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(71) Demandeurs/Applicants:
UNIVERSIDADE DO MINHO, PT;
STAB-TRATAMENTO DE AGUAS E BIOTECNOLOGIA, LDA., PT

(72) Inventeurs/Inventors:
LEAO, CECILIA, PT;
CORTE-REAL, MANUELA, PT;
SCHULLER, DORIT, PT

(74) Agent: FETHERSTONHAUGH & CO.

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(54) Title: CULTURE MEDIUM FOR THE DETECTION OF ZYGOSACCHAROMYCES

(57) Abrégé/Abstract:
The present invention refers to a differential and selective culture medium, for the detection of yeasts of the species Zygosaccharomyces bailii and Zygosaccharomyces bisporus, allowing a drastic reduction in the time and work usually involved in the conventional detection of these species. According to the present invention, the detection of Zygosaccharomyces bailii and Zygosaccharomyces bisporus is accomplished with one single test that only requires the preparation, and inoculation of one liquid or solid culture medium. This culture medium is comprised by a base mineral medium supplemented with oligoelements and vitamins, by glucose and formic acid as the only energy and carbon sources, and by an acid-base indicator. The acid-base indicator, particularly bromocresol green, provides the medium with a green coloring that is converted into blue through the action of the above mentioned yeasts. Additionally, the blue color presented by the coloniesis a specific characteristic of these species and can be observed in the medium after 48 to 96 hours of incubation, depending upon the inoculation methodology used. The invention can be used either with previously isolated and purified yeast strains or with cell suspensions of mixed yeast populations containing other yeasts different from Zygosaccharomyces bailii and Zygosaccharomyces bisporus, for the detection of these species in the food industry, namely in wines and other beverages. The medium can also be included in galleries of yeast identification tests.

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(71) Applicants *(for all designated States except US)*: **UNIVERSIDADE DO MINHO** [PT/PT]; Largo do Paço, P-4700-320 Braga (PT). **STAB-TRATAMENTO DE ÁGUAS E BIOTECNOLOGIA, LDA.** [PT/PT]; Quinta de Stª. Teresa, P-2825 Charneca da Caparica (PT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEÃO, Cecília** [PT/PT]; Urbanização do Salgueiral, 32, P-4800 Guimarães (PT). **CÔRTE-REAL, Manuela** [PT/PT]; Rua Senhora do Porto, 798 R/C Dir, P-4200 Porto (PT). **SCHULLER, Dorit** [PT/PT]; Rua Monsenhor Ferreira, 118 1º Esq., P-4700 Braga (PT).

(74) Agents: **FERREIRA MAGNO, Fernando, António,** et al.; Rua das Flores, 74 - 4º andar, P-1200-195 Lisboa (PT).

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(54) Title: CULTURE MEDIUM FOR THE DETECTION OF ZYGOSACCHAROMYCES

(57) Abstract: The present invention refers to a differential and selective culture medium, for the detection of yeasts of the species *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, allowing a drastic reduction in the time and work usually involved in the conventional detection of these species. According to the present invention, the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* is accomplished with one single test that only requires the preparation, and inoculation of one liquid or solid culture medium. This culture medium is comprised by a base mineral medium supplemented with oligoelements and vitamins, by glucose and formic acid as the only energy and carbon sources, and by an acid-base indicator. The acid-base indicator, particularly bromocresol green, provides the medium with a green coloring that is converted into blue through the action of the above mentioned yeasts. Additionally, the blue color presented by the coloniesis a specific characteristic of these species and can be observed in the medium after 48 to 96 hours of incubation, depending upon the inoculation methodology used. The invention can be used either with previously isolated and purified yeast strains or with cell suspensions of mixed yeast populations containing other yeasts different from *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, for the detection of these species in the food industry, namely in wines and other beverages. The medium can also be included in galleries of yeast identification tests.

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In mixed cultures of *S. cerevisiae* and *Z. bailii*, a few colonies (*ca.* 2-3%) with a light blue coloring and with a morphology similar to that of *S. cerevisiae* can be present, and that were judged as belonging to this species. The light coloring is due to the affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*.

In mixed cultures of *P. membranaefaciens* and *Z. bailii,* an intense blue coloring of the colonies formed by *P. membranaefaciens* was observed. This characteristic is equally due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. However, the discrimination between those colonies is clear as can be seen in the appended Figure 5.

Culture medium for ~~the~~ detection of *Zygosaccharomyces* ~~*bailii and Zygosaccharomyces bisporus Yeasts*~~

Object of the Invention

The present invention refers to a differential and selective culture medium containing glucose, formic acid and an acid-base indicator, for the detection in a sample, after 48 hours, of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, two of the most dangerous species when considering food deterioration, and to a process for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts using the referred culture medium. It is ~~It is~~ a further object of the present invention the use of the referred culture medium in a gallery of yeasts identification tests.

State of the prior art

Yeasts are a growing problem in the food industry. The use of milder preservation processes in order to maintain the organoleptic properties of the product, of packages with modified atmospheres, and of new formulations, designed to avoid bacterial contamination are, nevertheless, favorable to yeast contamination. Although some pathogenic yeast species have been detected in food and the opportunistic strains may be dangerous to a fraction of the population, the fundamental risk of contamination that arises is not one of sanitary nature, but it consists in the spoilage effects that certain yeasts, such as *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* have in food products, with the consequent economic losses involved.

Heretofore, the study of the yeast microflora present in the most diverse habitats (*e.g.* food, nature), comprises a first strain isolation stage, using the general selective yeast culture media, and a second identification stage of the isolated strains, through the use of conventional and/or molecular biology based methods. The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a

large range of physiologic and biochemical tests. It is a demanding work that only produces results after at least one to two weeks, and requires a great deal of experience for the correct interpretation of the results. The molecular biology based methods are, generally, faster than the classical ones, but they also require a good amount of operator experience and involve expensive equipment and reactants.

There are some culture media commercially available for the detection of yeasts in wines, namely the Wallerstein Laboratory Nutrient Medium, WLN, used for detecting fermenting yeasts, and the Wallerstein Laboratory Differential Medium, WLD, which allow the detection of lactic and acetic bacteria as well as of yeasts belonging to the non-fermenting flora (both from Difco). However, these prior art media are not capable to differentiate the yeasts, particularly the *Zygosaccharomyces bailii* species.

There is therefore the necessity for a culture medium and a process for the detection and identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, rapid and efficient, and which is thus an alternative means to the conventional techniques for the rapid detection of these species.

Description of the invention

It was surprisingly found that *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, when grown in a medium containing glucose, formic acid and an appropriated acid-base indicator, lead to a rapid change in the medium color and to the formation of colored colonies after at least 48 hours, these changes being characteristic and exclusive of the referred yeasts in the referred culture medium.

It was also found that the medium according to the invention is differential for the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts,

through the inclusion of an appropriate acid-base indicator, and can be selective for the growth of the referred yeasts, depending on the formic acid concentration present in the medium.

Thus, according to the present invention, a new differential and selective culture medium was developed, which permits the identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, assuring results after 48 hours of incubation, and which is therefore an alternative means to the conventional techniques for the rapid detection of these species, allowing a drastic reduction in the time and work involved in their identification.

The culture medium according to the present invention comprises a base mineral medium, supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator, namely one having a pK_i between 4.5 and 4.8, particularly bromocresol green, and optionally agar and an antibiotic inhibitor of bacterial growth, such as cloramphenicol.

According to the present invention, the formic acid is present in the culture medium in a concentration from 0.1% to 0.5% (v/v), the concentration being selected depending if the culture medium is to be selective or only differential.

When the concentration of formic acid is increased in the culture medium according to the present invention, the selectivity of the medium for *Z. bailii* and *Z. bisporus* yeasts increases, although at expenses of some differentiability, as shown in examples 6 and 7 below.

According to the present invention, the glucose is present in a concentration from 0.05% and 0.1% (p/v), preferably 0,1% (p/v).

The culture medium according to the present invention further allows, through the choice of appropriated conditions, in particular the inoculation methodology, the enumeration of *Z. bailii* and *Z. bisporus* yeasts in a sample, regardless the presence of other yeasts, since it is selective as shown in examples 4 and 8.

In an embodiment of the present invention, the acid-base indicator is bromocresol green which provides the medium with a green color, that is converted to blue by the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts. Additionally, the colonies of the referred yeasts present, in the medium of the invention, a blue coloring.

In another embodiment, the culture medium according to the invention may contain additionally an inhibitor of bacterial growth, being particularly useful for application in samples of mixed populations including bacteria.

The culture medium object of the invention is prepared by autoclave sterilization of the base mineral medium in deionized water. The medium is then allowed to cool, and before solidifying, the glucose, formic acid, oligoelements and vitamins, prepared as adequate solutions and previously sterilized, are added under aseptic conditions. The whole medium is homogenized and aseptically dispensed into Petri dishes.

The present invention also refers to a process of detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts present in a sample, using a culture medium according to the present invention, as characterized above.

According to this feature of the present invention, a process was developed which comprises: (i) preparing a medium according to the present invention; (ii) inoculating it, by spreading, streaking or by deposition of a drop of cell

suspension, with a sample to be analyzed for *Zygosaccharomyces bailii* and/or *Zygosaccharomyces bisporus* yeasts; (iii) incubating it in a incubator at a suitable temperature and for a time enough for the yeast development (minimum 48 hours); and (iv) observing the color changing in the medium and the colonies formation, to conclude the presence of the referred yeasts when occurs a changing of the medium color and formation of colored colonies in agreement with the acid-base indicator used.

The present invention can be used with previously isolated and purified strains, there being no kind of limitation concerning the type of inoculation that is used. However, the time needed to observe the turning of the indicator depends on the cell concentration of the inoculum and on the method of inoculation.

The present invention can also be used with cell suspensions of mixed yeast populations, containing yeasts other than *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, providing information about the presence of these species, every time that blue colonies are detected in conjunction with a change in the medium color.

One of the objects of the present invention is to provide the food industry, particularly the wine and beverages industry, with a procedure for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts. The procedure is simple and easily reproducible by any microbiological analysis laboratory. Additionally, the production of the culture medium doesn't require new technologies. Once prepared, the culture medium finds immediate use in any industrial facility or quality control laboratory, since there is no need for highly skilled personnel other than the one in charge of the routine microbiological analyses.

Further, the culture medium according to the present invention can be used to integrate galleries of identification of yeasts.

Brief Description of the Figures

Figure 1 is a photograph showing the response of several yeasts (*Z. bailii* ISA 1265 and *Z. bailii* IGC ~~3806~~ 4806: positive response; *T. delbrueckii* ISA 1229 and *I. orientalis* IGC 3806: negative response) in a solid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% v/v) at the end of 96 hours of incubation at 30°C. The *Z. bailii* yeasts shown a positive response revealed by a blue coloring of the culture medium in the dish, while the negative responses are shown as a green coloring which did not change during the incubation.

Figure 2 is a photograph showing the response of several yeasts in a liquid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% w/v) at the end of 48 hours of incubation at 30°C. All the *Z. bailii* strains induced the medium to change color to blue, while all the others maintained the green color.

Figure 3 shows the morphology of *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.3% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The colonies can be observed well defined with a blue color.

Figure 4 shows the morphology of *S. cerevisiae* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies shown a blue coloring, perfectly distinct from the creme coloring of the other colonies.

Figure 5 shows the morphology of *P. membranaefaciens* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies are totally distinguishable by its morphology and blue color.

Preferred embodiments of the invention

In a preferred embodiment of the present invention the differential and selective culture medium, for identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts in a sample, after at least 48 hours of incubation, comprises a base mineral medium, including bromocresol green as the acid-base indicator, supplemented with oligoelements and vitamins, 0.05% to 0.1% (w/v) of glucose and 0.1% to 0.5% (v/v) of formic acid as the only energy and carbon sources, and optionally agar and an inhibitor of bacterial growth.

In this embodiment of the invention, the bromocresol green provides the medium with a green coloring that will be converted into blue through the action of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts during incubation under appropriate conditions. Additionally, the colonies of these yeasts will also present a blue color. The change of color of the culture medium is characteristic of these yeast species, as illustrated in examples 1 and 2, thus allowing the detection of the presence thereof in a sample only by the color changing.

The process according to the present invention will now, be illustrated by means of the non limitative examples below:

Examples

Example 1

This example illustrates the preparation of a solid culture medium according to the present invention and shows that it is effective in the identification of *Z. bailii* and *Z. bisporus* yeasts.

A culture medium is prepared comprising the following ingredients:

Table 1 **Culture medium composition for the detection of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts**

Compound			Concentration (%)	
Base Medium	Ammonium sulphate	$(NH_4)_2SO_4$	0.5	(w/v)
	Potassium dihydrogen ~~sulphate~~ phosphate	KH_2PO_4	0.5	(w/v)
	Magnesium sulphate ~~hepta~~ hydrate	$MgSO_4 \cdot 7H_2O$	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	0.4	(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 ~~hexa~~ tetra hydrate	$MnSO_4 \cdot 4H_2O$	0.8	(w/v)
	Zinc ~~Tin~~ sulphate ~~hexa~~ hepta hydrate	$ZnSO_4 \cdot 7H_2O$	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 sterilization is accomplished in 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 ~~annealed~~ mixed at 50±5°C before being mixed together. The whole medium is homogenized and dispensed into Petri dishes.

The yeast strains to be identified, previously purified and inoculated in agar slants with a generic yeast culture medium (yeast extract medium, peptone, and glucose), are incubated for 48 hours at 28°C. An loopful is transferred to the culture medium with glucose and formic acid, prepared above. The inoculation is made by streaking and the plates are incubated at 30°C, for a minimum time of 48 hours. Alternatively, the inoculation may be done with a cotton smear containing an equivalent biomass amount.

The results obtained are presented in Table 3. Typical Responses of the below mentioned yeasts are shown in Figure 1.

Table 3 **Inoculation by streaking - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~rouxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 24-48 hours

A change in the culture medium color from green to blue was observed in all of the tested *Z. bailii* strains. However, it was observed that 3 of the 8 tested *Z. bisporus* strains converted the medium color only after an additional incubation period of 24 to 48 hours. For all the strains of the other species tested a negative result was observed, since the medium color did not change.

The results that were obtained show that the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* directly inoculated from cultures in solid medium after a minimum incubation period of 48 hours.

(*) Zygosaccharomyces rouxii 6 green

Example 2

The same procedure as Example 1 was used, differing only in that the inoculation was made with single strain cell suspensions instead of cells originated in solid medium. The cells are also originated from agar slants as disclosed in Example 1. The cell suspensions are prepared in deionized water in such a way that the optical density (OD_{640}) lies within the range of 0.7 to 1.0. 10 µl drops of these suspensions are placed on the surface of Petri dishes containing the medium disclosed in Example1. The plates were incubated at 30°C for 48 hours.

The results obtained are presented in Table 4. These results are ~~similar~~ identical to the ones presented for Example 1.

Table 4 **Application of cell suspensions on the surface of the solid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~rouxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 72-96 hours

(see page 10)

The culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions after a minimum incubation period of 48 hours.

Example 3

The same procedure as Example 2 was used, but using the culture medium in its liquid form. 25 µl of the cell suspension are transferred to 225 µl of the medium disclosed in Example 1 but without the agar (contained in the wells of a microplate), and in a concentration such that after the 25 µl addition of the cell suspension the final medium components concentration equals the ones disclosed in Example 1. The incubation conditions are similar to those described in Example 2. In addition the culture is homogenized by mechanical mixing at 160 rpm.

The results obtained are presented in Table 5. These results are similar to the ones obtained in the above examples.

Table 5 **Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~fouxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

The culture medium according to the present invention, in the liquid form, is equally suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions after a minimum incubation period of 48 hours.

Example 4

This Example shows that the culture medium according to the present invention is selective for yeasts of the *Z. bailii* and *Z. bisporus* species in samples of mixed yeasts populations.

A similar procedure as in Example 3 is used, differing only in that the cell suspensions used are pure or mixed (in equal ratios) yeast cell suspensions, and in that the method of membrane filtration is used. The cell suspension is prepared as in Example 2. The mixed cultures are prepared from pure culture

(*) (See page 10)

suspensions. In this case, the inoculations are accomplished using an aliquot of the suitably diluted suspension that is filtered under vacuum through a sterilized filtration membrane (pores of 0.45 μm), the filters are then placed Petri dishes, and the dishes containing the filters on the surface of the medium disclosed in Example 1, are incubated at 30°C for 96 hours. As a reference culture medium (corresponding to a recovery ratio of 100%) a generic yeast culture medium is used (yeast extract ~~medium~~, peptone, and glucose). medium containing

The results obtained are presented in Table 6. The recovery ratio of *Z. bailii* cells in the medium disclosed in Example 1 is about 60 to 70 %, regardless of the presence of other yeast species. The culture medium was shown to be highly selective since the recovery ratio of *S. cerevisiae, P. membranaefaciens* and *D. anomala* was significantly reduced, lower the 0.01%.

S. cerevisiae, P. membranaefaciens and *D. anomala* being representative examples of contaminant species in wines, the culture medium according to the invention will be useful and appropriate for the identification of *Z. bailii* in contaminated wines samples.

Table 6 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio
Zygosaccharomyces bailii	65
Zygosaccharomyces bailii	57
Saccharomyces cerevisiae	n.d
Zygosaccharomyces bailii	67
Pichia membranaefaciens	n.d.
Dekkera anomala	n.d
Saccharomyces cerevisiae	< 0,002
Pichia membranaefaciens	0,011
Dekkera anomala	<0,004

n.d. not determined

The Figures 3, 4, and 5 show the colony morphology of different species in pure and mixed cultures, being remarkable the easy discrimination between the 3 species using only the colonies color and morphology.

~~In some cases non typical colonies (*ca.* 2-3%) with a light blue coloring or with an intense blue coloring can be present. The first of these (Fig. 4), with a morphology similar to that of *S. cerevisiae*, were judged as belonging to this species. The light coloring of these colonies is due to the incorporation of the indicator after the color change induced by the presence of *Z. bailii*. The second kind of colonies (Fig. 5), with a similar morphology to that of *P. membranaefaciens* were judged as belonging to this species, the intense coloring being due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. This characteristic was equally observed for the pure cultures of *P. membranaefaciens* that showed a very intense green coloring in contrast with those of *S. cerevisiae*, that under these conditions, showed a green cream coloring. However, the discrimination between these colonies is clear as can be seen in the appended Figures 4 and 5.~~ (insert page 15a)

Example 5

This example shows the differential ability of the culture medium according to the present invention and the enumeration of *Z. bailii* cells in wine samples.

The enumeration of *Z. bailii* cells in wine samples is made using membrane filtration (according to the method disclosed in Example 4). For the determination of the number of colony forming units (CFU)/ml of wine is done after 96 hours of incubation at the temperature of 30°C. Other commercial culture media presently used for the detection of yeasts in wines (Wallerstein Laboratory Differential Medium, WLD, and Wallerstein Laboratory Nutrient Medium, WLN, both marketed by Difco) are tested in parallel. The WLN medium is used for the detection of fermenting yeasts, while the WLD

medium allows the detection of lactic and acetic bacteria as well as yeasts belonging to the non-fermenting flora.

The results are summarized in Table 7.

Table 7 **Number of CFU/ml in 2 contaminated wines, obtained by the method of membrane filtration after 96 hours of incubation in culture medium containing glucose and formic acid (0.4% v/v) at 30°C**

Culture medium	Wine 1	Wine 2
Medium disclosed in Example 1	75 [1]	90 [1] + 170 [2]
WLN	685	620
WLD	10	200

[1] cream-yellowish colored colonies
[2] blue colored colonies, typical of *Z. bailii*

The identification of blue colored colonies and white-yellowish colonies as belonging or not to the *Z. bailii* species was confirmed by molecular methods.

The culture medium described in Example 1 is an ideal culture medium for the isolation of yeasts of the *Zygosaccharomyces bailii* species, allowing the discrimination between this yeast and other yeasts species, just by the color. The WLN and WLD media do not show this differentiation ability that is a characteristic of the medium of the present invention. This property makes this medium superior to those presently commercially available.

Example 6

This example shows the effect of formic acid concentration in the solid culture medium according to the present invention.

A culture medium was prepared as in Example 1, but using different concentrations of formic acid, and inoculation was done with various yeast strains following the same procedure as described in Example 2, as presented in Table 8.

The results obtained are presented in Table 8. For the lower formic acid concentration the basification of the solid culture medium is observed for all the strains belonging to the species *Z. bailii* and *Z. bisporus*. The increase in concentration resulted for 3 *Z. bailii* strains in a slower change in the culture medium color. All the strains of the other tested species induced no color change in the culture medium.

Table 8 **Application of cell suspension drops on the surface of solid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d.
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia membranaefaciens	13	green	green
Debaryomyces hansenii	2	green	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined

The present culture medium is therefor suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions, applied as a drop on the surface of the solid medium, for all the tested concentrations of formic acid, after a minimum incubation period of 48 hours. The results obtained show that for 0,3% acid formic concentration, the culture medium according to the invention is appropriate and efficient for the detection of *Z. bailii* and *Z. bisporus* in pure culture suspensions, inoculated in liquid culture medium, after a minimum incubation period of 48 hours. The same is valid for the detection of *Z. bailii* in a medium with 0,5% formic acid concentration. Both concentrations are suitable to guarantee a negative response from the other tested species. However, the medium with 0.5% (v/v) of formic acid is not

the best suited one for the detection of *Z. bailii* strains that show lower tolerance to acid conditions.

Example 7

This Example shows the effect of formic acid concentration in a culture medium according to the present invention.

A culture medium was prepared as in Example 3, but using different concentrations of acid formic and inoculation was done with various yeast strains following the procedure of Example 3, as presented in Table 9.

These results obtained are similar to the ones in Example 6 and are presented in Table 9. In Figure 2 are shown typical responses of yeast strains belonging and not belonging to the *Z. bailii* species.

Table 9 Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d.
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia membranaefaciens	13	green	green
Debaryomyces hansenii	2	green	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined

As in Example 6 the results obtained show that, for 0,3% acid formic concentration, the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions, inoculated in liquid media after a minimum incubation

period of 48 hours. The same is valid for the detection of Z. bailii in a medium with 0,5% acid formic concentration.

Example 8

This example shows the effect of formic acid concentration in the culture medium according to the present invention on the medium selectivity.
The procedure of Example 4 was used, but using different concentrations of formic acid in the culture medium.

The results obtained are presented in Table 10. These results and the ones from Example 4 show that the recovery ratio of *Z. bailii* cells in the medium decreases with the increasing of the acid formic concentration, being independent of the presence of other yeast species such those that can be found in contaminated wines. For 2 of these other 3 tested species the recovery ratio also decreases with the increase in the formic acid concentration.

Table 10 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio		
	formic acid 0.2%	formic acid 0.3%	formic acid 0.5%
Zygosaccharomyces bailii	82	78	42
Zygosaccharomyces bailii	82	81	35
Saccharomyces cerevisiae	n.d.	n.d.	n.d.
Zygosaccharomyces bailii	99	94	34
Pichia membranaefaciens	n.d.	n.d.	n.d.
Dekkera anomala	n.d.	n.d.	n.d.
Saccharomyces cerevisiae	30	4	<0.002
Pichia membranaefaciens	55	5.9	<0.004
Dekkera anomala	<0.004	<0.004	<0.004

n.d. not determined

Thus, it was shown that the culture medium according to the present invention has characteristics of a selective and differential culture medium appropriated and highly effective for the detection, identification and

enumeration of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts species in samples either containing previously isolated strains of these yeasts or containing mixed yeasts populations. These characteristics of differentiability and selectivity can be optimized. Lower formic acid concentrations provides the medium with remarkable differentiation ability although with lower selectivity. On the other hand, for higher formic acid concentrations the medium is highly selective.

The culture medium can also be supplemented with an inhibitor of bacterial growth, which makes it useful for using with mixed populations samples including also bacteria, as for example food and beverages.

Although the present invention is described based on its preferred embodiments, it should be apparent to any person skilled in the art that variations and modifications within the spirit and scope of the appended claims are possible.

Claims

1. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. Culture medium according to claim 1 characterized in that glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. Culture medium according to claim 1 characterized in that formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v).

4. Culture medium according to claim 3 characterized in that the formic acid concentration is preferably 0.4% (v/v).

5. Culture medium according to claim 1 characterized in that the base mineral medium comprises ammonium sulphate (0.5% (w/v)), potassium dihydrogenphosphate (0.5% (w/v)), magnesium sulphate heptahydrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid (1.0% (w/v)), potassium iodide (0.2% (w/v)) and sodium molibdate dihydrate (0.4% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahydrate (0.08% (w/v)), iron chloride hexahydrate (0.4% (w/v)), manganese sulphate tetrahydrate (0.8% (w/v)), zinc sulphate heptahydrate (0.8% (w/v)) and hydrochloric acid (HCl 10^{-3}N, 0.8% (v/v)); and the vitamin solution (0.05% (v/v)) comprises biotin (0.001% (w/v)), calcium panthotenate (0.08% (w/v)),

mioinositol (4.0% (w/v)), niacin (0.16% (w/v)), pyridoxine hydrochloride (0.16% (w/v)) and thiamin hydrochloride (0.16% (w/v)).

6. Culture medium according to claim 1 characterized in that the acid-base indicator is one having a pK_i between 4.5 and 4.8, preferably bromocresol green.

7. Culture medium according to claim 6 characterized in that the pH is adjusted to 4.3-4.8, preferably 4.5.

8. Culture medium according to claim 1 characterized in that it further contains an antibiotic inhibitor of bacterial growth, in the usually used concentrations for this purpose, for use with mixed population samples containing bacteria.

9. A culture medium according to any previously claim characterized in that it contains all the ingredients except agar, that is in its liquid form.

10. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it is composed of

Glucose	0.1% (w/v)
Formic acid	0.4% (v/v)
Base Medium:	
Ammonium sulphate	0.5% (w/v)
Potassium dihydrogen ~~sulphate~~ phosphate	0.5% (w/v)
Magnesium sulphate ~~hetpa~~ hepta hydrate	0.05% (w/v)
Calcium chloride dihydrate	0.013% (w/v)
Bromocresol green	0.005% (w/v)
Agar	2.0% (w/v)
Oligoelements Solution A	0.05% (v/v)
Boric acid	1.0% (w/v)

Potassium Iodide	0.2% (w/v)	
Sodium molibdate dihydrate	0.4% (w/v)	
Oligoelements Solution B		0.05% (v/v)
Copper sulphate pentahydrate	0.08% (w/v)	
Iron chloride hexahydrate	0.4% (w/v)	
Manganese sulphate tetrahydrate	0.8% (w/v)	
Zinc ~~Tin~~ sulphate heptahydrate	0.8% (w/v)	
Hydrochloric acid, HCl 10^{-3}N,	0.8% (v/v)	
Vitamin Solution		0.05% (v/v)
Biotin	0.001% (w/v)	
Calcium panthotenate	0.08% (w/v)	
Mioinositol	4.0% (w/v)	
Niacin	0.16% (w/v)	
Pyridoxine hydrochloride	0.16% (w/v)	
Thiamin hydrochloride	0.16% (w/v)	

the pH being adjusted to pH 4.~~6~~ 5 with HCl 1M.

11. Culture medium according to any previously claim characterized in that the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, annealing this solution and the base medium at about 50 ± 5°C, before mixing the same and to adjust the final pH value to the desired value.

12. Process for the detection of *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts characterized by the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucose and

formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

13. Process according to claim 12, characterized in that the acid-base indicator is bromocresol green and in that, after inoculating the referred culture medium with a sample containing *Zygosaccharomyces bailii* and/or *Zygosaccharomyces bisporus* yeasts and incubating in conditions appropriated for the growth of the referred yeasts, it is possible to conclude for the presence of said yeasts species, by means of a medium color change from green to blue after about 48 hours, and if desired to number said yeasts species, by development of blue colored colonies after about 96 hours.

14. Process according to claims 12 and 13 characterized in that it is applied to the detection and numbering yeasts of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* species in wines, as well as in other beverages or food containing or not mixed yeast populations.

15. Use of a culture medium according to claims 1 to 11, to be included in yeast identification galleries.

16. Use of a culture medium according to claims 1 to 11 in an industry, particularly in the quality and process control in the food and beverage industry.

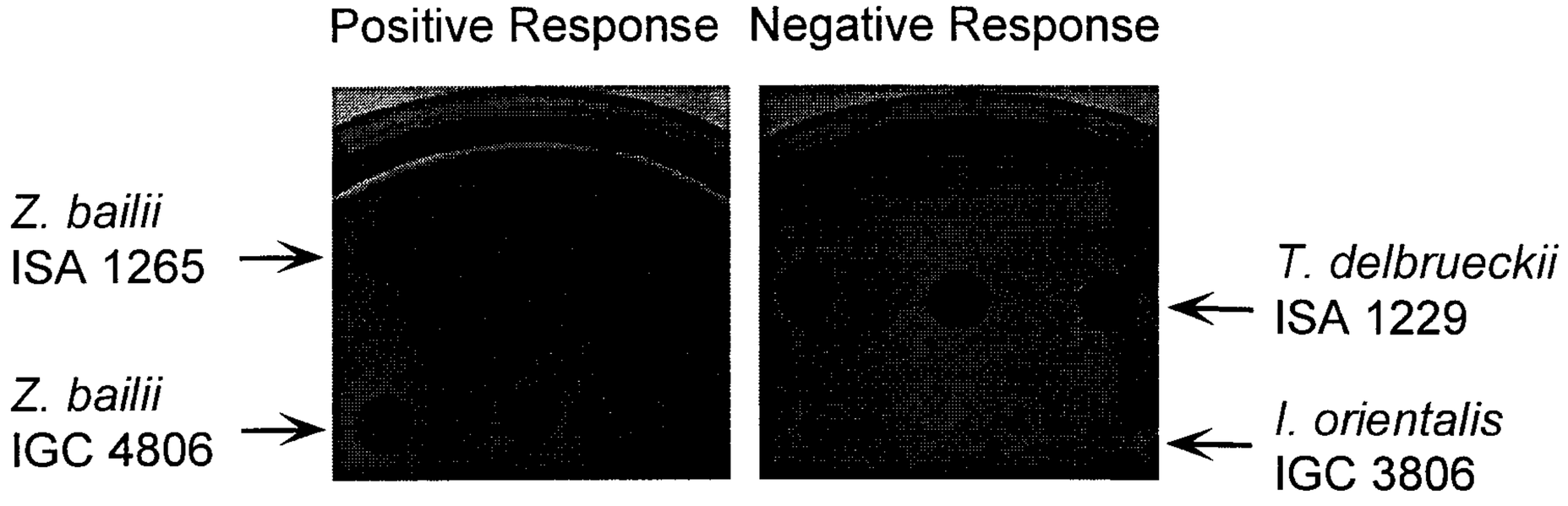
Positive Response
Negative Response
Z. bailii
ISA 1265
Z. bailii
IGC 4806
T. delbrueckii
ISA 1229
I. orientalis
IGC 3806

Fig. 1

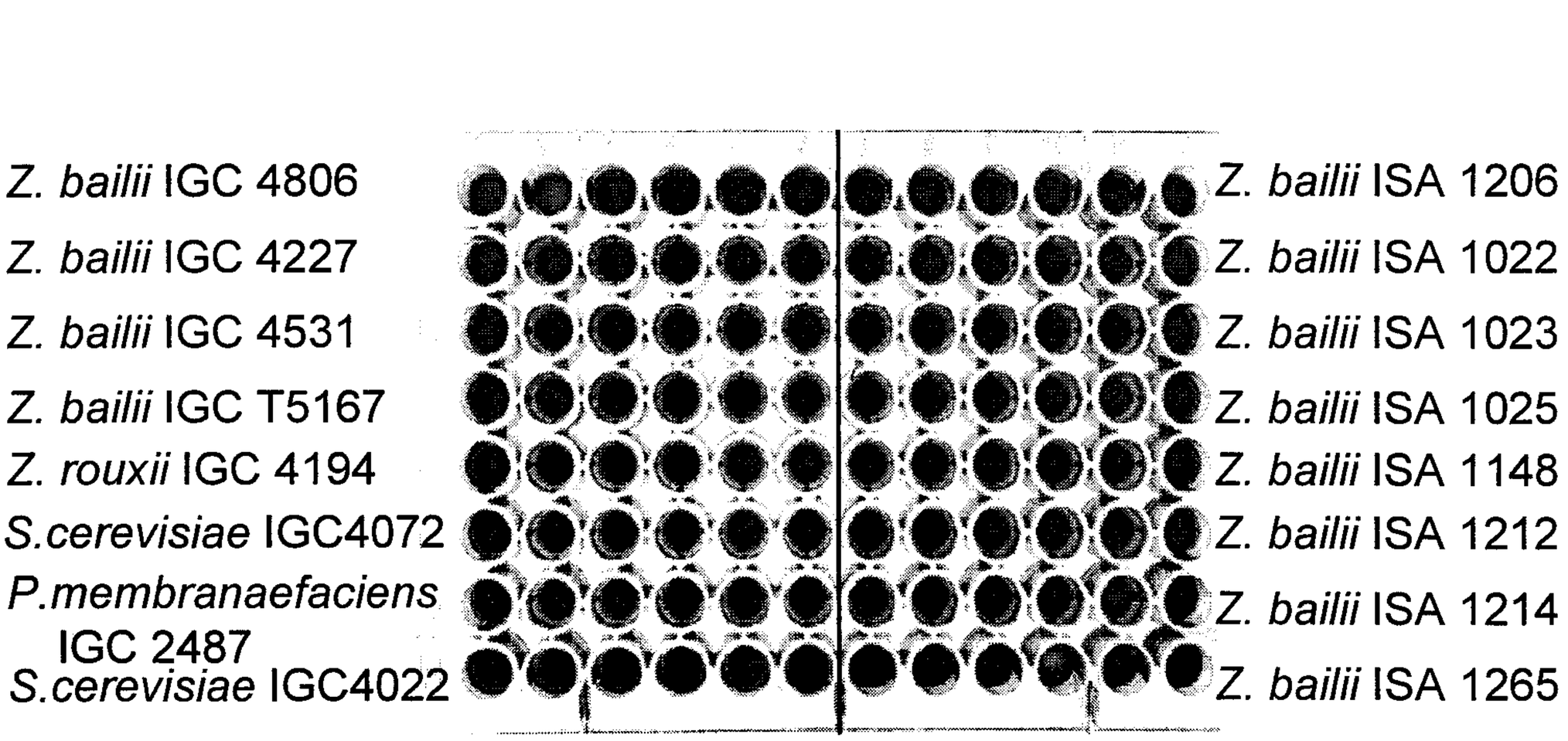
Z. bailii IGC 4806
Z. bailii IGC 4227
Z. bailii IGC 4531
Z. bailii IGC T5167
Z. rouxii IGC 4194
S.cerevisiae IGC4072
P.membranaefaciens IGC 2487
S.cerevisiae IGC4022
Z. bailii ISA 1206
Z. bailii ISA 1022
Z. bailii ISA 1023
Z. bailii ISA 1025
Z. bailii ISA 1148
Z. bailii ISA 1212
Z. bailii ISA 1214
Z. bailii ISA 1265

Fig. 2

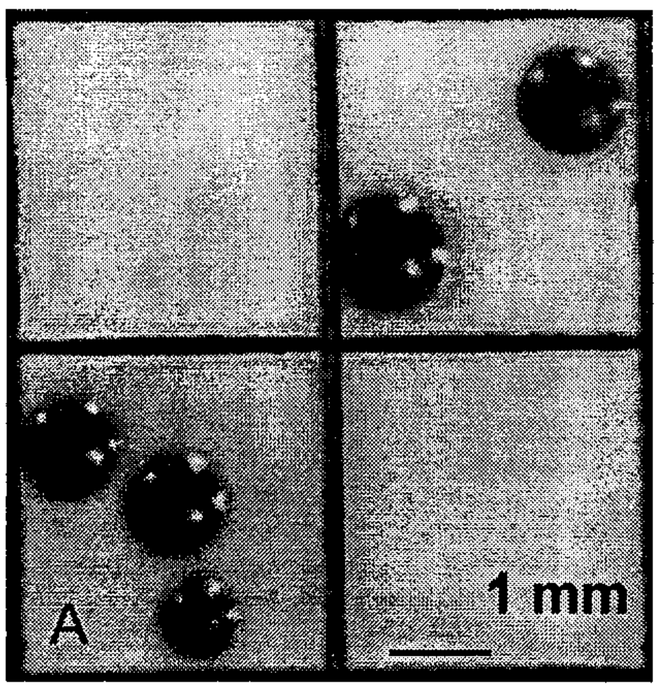

Fig. 3

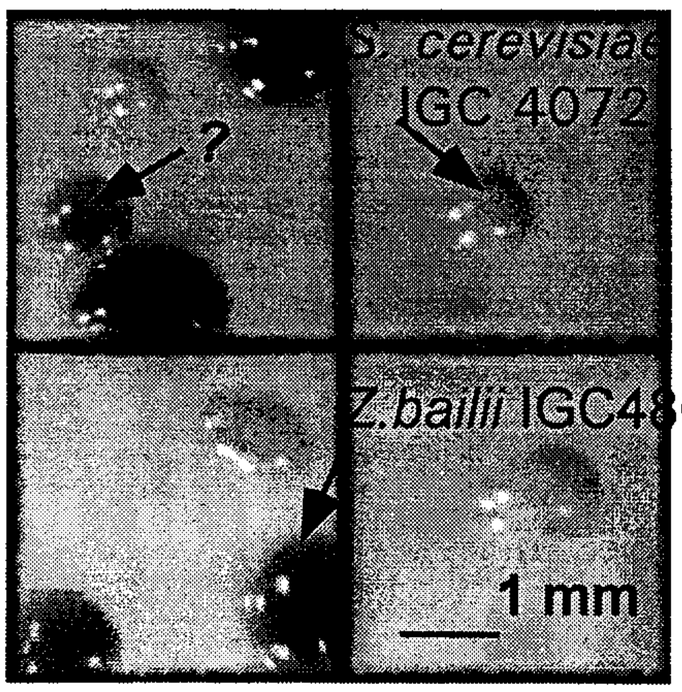

Fig. 4

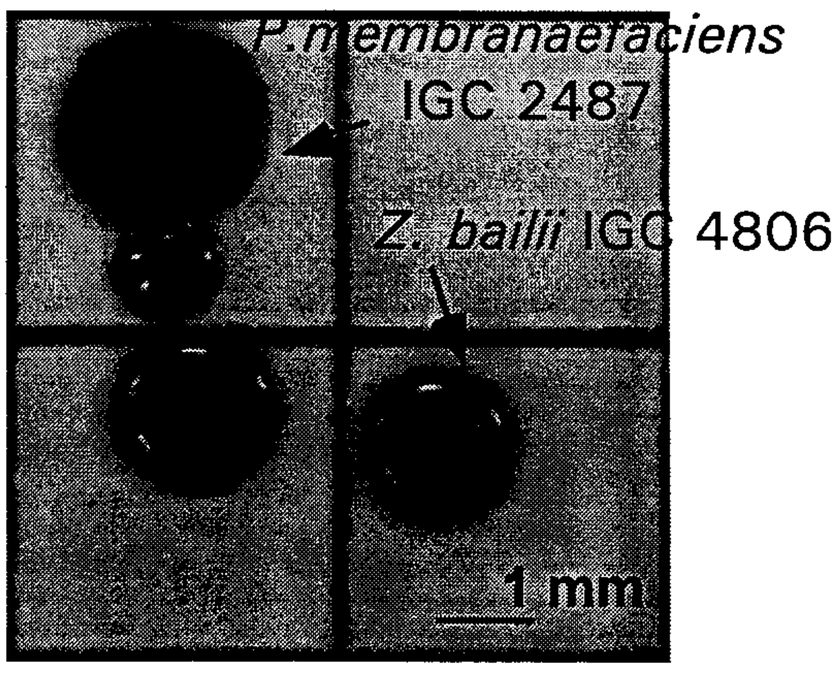

Fig. 5