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A Differential Medium for the Isolation of *Kluyveromyces marxianus* and *Kluyveromyces lactis* from Dairy Products

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ABSTRACT

A selective and differential solid medium, called *Kluyveromyces* Differential Medium (KDM), is described for the isolation of *Kluyveromyces marxianus* and *K. lactis* from dairy products. Its discriminative potential is based on the detection of the enzyme β-galactosidase, in the absence of lactose. Of the more than 95 strains tested, including yeasts, bacteria, and filamentous fungus, only the strains of *K. marxianus* and *K. lactis* produced blue colonies on the medium due to the presence of X-Gal/IPTG. The bacterial strains were not able to grow in KDM. On this basis, the medium was very satisfactory when testing naturally or experimentally contaminated dairy food products. When quality assessment tests were performed, optimal values of productivity (growth and color) and selectivity were obtained for *K. marxianus* and *K. lactis.*

At present, microbial quality control in food industries is mainly focused on detecting pathogenic bacteria, while only total numbers of yeasts, together with filamentous fungi, are considered. Nevertheless, in recent years, economic losses due to spoilage by yeasts have increased in European companies because of use of lower concentrations of preservatives, less severe preservation procedures, packaging in modified atmospheres, or new formulations that on occasion allow the growth of yeasts.

When investigation of specific spoilage yeasts is required, traditional identification methods are used after isolation of the yeasts, but these methods are laborious and time-consuming.

A number of selective culture media are currently available for quantitative recovery of yeasts from foods. In general, they are acidified or contain antimicrobial agents to inhibit bacterial growth. Certain substances, such as dichloran or rose bengal, are also added to inhibit radial growth of microfungi *(9, 19).*

Kluyveromyces marxianus and *K. lactis* are two of the most frequent spoilage yeasts of milk and dairy products because of their capacity to assimilate or ferment lactose, as well as to hydrolyze fats or proteins present in milk *(9, 10).* In a previous work *(8),* we studied the enzymatic profiles of a number of spoilage yeasts in search of discriminative activities, and we found that *K. marxianus* was the only species able to show β-galactosidase activity in the experimental conditions. Several chromogenic and fluorogenic substrates were also screened for the detection of this enzyme on solid media. On this basis, the aim of the present work has been to develop a selective and differential culture medium for the isolation and rapid detection of *Kluyveromyces* in milk and dairy products.

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MATERIALS AND METHODS

Strains. A total of 87 yeast strains have been used in this work (Table 1). They had been isolated in our laboratory from spoiled food products or obtained from culture collections. Seven strains of bacteria and one filamentous fungus were also included.

Media. The new culture medium, called *Kluyveromyces* Differential Medium (KDM), consisted of a basal medium containing the following: yeast extract (Difco Laboratories, Detroit, Mich.), 5 g/liter; proteose-peptone no. 3 (Difco), 3 g/liter; malt extract (Difco), 3 g/liter; glucose, 10 g/liter; and agar, 20 g/liter. After autoclaving and cooling to 45 to 50°C, adequate volumes of filter-sterilized solutions of the following substances were added: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) (Sigma-Aldrich Quimica S.A., Barcelona, Spain), 0.08 mg/liter; isopropil-β-D-thiogalactopyranoside (IPTG) (Sigma), 0.1 mg/liter; and chloramphenicol (Sigma), 0.5 mg/liter. This medium was poured into Petri dishes (15 ml/plate) and stored at 4°C. The final pH of the medium was 7.0 ± 0.2.

As reference media, yeast morphology agar (YMA; Difco) was used for yeast and mold strains, and Trypticase soy agar (TSA; Panreac, Panreac Quimica S.A. Barcelona, Spain) for bacteria.

Inoculation. The media were inoculated with 10 μl of a suspension in water (approximately 6 MacFarland) of an actively growing culture of the strains. The drops were left to absorb on the surface, and the plates were incubated at 28°C (yeasts and mold) or 37°C (bacterial strains) for 7 days. Growth and color were checked daily.

Influence of incubation temperature. The strains of *K. marxianus* and *K. lactis* were inoculated on KDM plates as previously described, and plates were incubated at 40 and 42°C for 7 days. Growth and color were recorded daily. As a growth control, the strains were inoculated in a similar manner on YMA plates, those being incubated in parallel at the same temperatures.

Quality assessment of the culture medium. Twenty three strains of *K. marxianus* and 9 strains of *K. lactis,* listed in Table

TABLE 1. *Growth of the studied strains on KDM*

Organisms	No. of strains	Growth[b]	Color[c]
Candida albicans[a]	2	+	−
C. guillermondii	1	+	−
C. lipolytica	1	+	−
C. parapsilosis[a]	2	+	−
C. rugosa	1	+	−
Debaryomyces hansenii[a]	7	+	−
Issatchenkia orientalis[a]	4	+	−
Kluyveromyces lactis[a]	8	+	+
	1	+	−
K. marxianus[a]	22	+	+
	1	+	−
K. thermotolerans	1	+	−
K. veronae[a]	1	+	−
Pichia anomala[a]	1	+	
P. membranaefaciens[a]	1	+	−
Rhodotorula glutinis	1	+	−
R. minuta[a]	2	+	−
R. mucilaginosa[a]	3	+	−
Saccharomyces cerevisiae[a]	6	+	−
Soporidiobolus salmonicolor	1	+	−
Torulaspora delbrueckii	8	+	−
Zygosaccharomyces bailii[a]	4	+	−
Z. bisporus	1	+	−
Z. rouxii[a]	7	+	−
Eurotium amstelodamii	1	+	−
Bacillus cereus	1	−	−
Staphylococcus aureus	1	−	−
Escherichia coli	1	−	−
Enterococcus faecalis	1	−	−
Lactococcus lactis var. *lactis*	1	−	−
Pseudomonas aeruginosa	1	−	−
P. fluorescens	1	−	−

[a] Type strain included.
[b] +, growth; −, absence of growth.
[c] +, blue color, −, absence of blue color.

3, together with reference strains *Zygosaccharomyces rouxii* NCYC 1152, *Saccharomyces cerevisiae* ATCC 7754, *Eurotium amstelodamii* ATCC 16018, and *Bacillus cereus* CECT 193 were used. Productivity and selectivity were tested by both the ecometric streaking procedure and the modified Miles-Misra colony counting method *(6, 14, 16),* following the recommendations of the International Committee for Food Microbiology and Hygiene Working Party for Culture Media (ICFMH-WPCM) *(6).* Productivity was expressed using two parameters: the relative growth index (RGi) (ecometric method) and productivity rate (PR) (modified Miles-Misra method).

Shelf life. In order to assess the shelf life of the medium, plates were stored at 4°C for 1 month after preparation. They were checked weekly for performance (growth and color) using two selected *Kluyveromyces* strains. Inoculation and incubation were performed as previously described.

Sample analysis. The performance of KDM as a differential medium for dairy products was analyzed on a total of 15 natural samples (Table 4). Five microliters of each liquid sample were streaked on TSA, YMA, and KDM media. For solid samples, 5 g were homogenized in 45 ml of sterile distilled water and 5 μl

TABLE 2. *Influence of high incubation temperatures on the growth and color production of* K. marxianus *and* K. lactis *on KDM*

Strains (no.)	Temperature (°C)			
	40		42	
	Growth	Color	Growth	Color
K. marxianus (23)	15[a]	15	6	6
K. lactis (9)	2	2	0	0

[a] Results are expressed as number of strains that grew or produced blue color at the tested temperatures.

TABLE 3. *Productivity and selectivity of KDM assessed by the ecometric method and the modified Miles-Misra method*[a]

Strains		RGi	PR	Selectivity
K. lactis	CYC 1386^T	0.95	ND	1
	CYC 1009	0.93	ND	1
	CYC 1010	1	ND	1
	CYC 1361	1	ND	1
	CECT 1362	0.81	1	1
	CECT 10.361	0.86	ND	1
	CECT 10.366	1.18	ND	1
	CECT 10.356	1.06	ND	1
	CECT 10.669	1[b]	ND	1
K. marxianus	CYC 1162^T	1	1.4	1.1
	CYC 1059	1.25	0.6	1
	CYC 1165	1	1.1	1
	CYC 1167	0.85	1	0.9
	CYC 1182	1	0.9	1
	CYC 1355	1	1	1
	CYC 1366	1	0.95	1
	CYC 1392	1	1.2	1
	CYC 1363	1	0.8	1
	CYC 1364	1	0.9	1.1
	CYC 1365	0.76	0.8	0.9
	CYC 1394	1	1.4	1
	CECT 10.315	1	1.4	1
	CECT 10.257	1	1	1
	CECT 10.367	1.12	0.95	1
	CECT 10.368	1	1	1
	CECT 10.369	1	1	1
	CECT 10.370	1	1.2	1
	CECT 10.371	1	1.1	1
	CECT 10.374	1	1.4	1
	CECT 10.379	1	1	1
	CECT 10.668	1	0.9	1
	CECT 10.649	1[b]	0.92[b]	1
S. cerevisiae	ATCC 7754	1.1[b]	1[b]	1
Z. rouxii	NCYC 1522	0.9[b]	0.8[b]	1
B. cereus	CECT 193	0	0	0

[a] CYC, Complutensis Yeasts Collection; CECT, Colección Española de Cultivos Tipo; ATCC, American Type Culture Collection; NCYC, National Collection of Yeast Cultures; T, Type strain; ND, not determined.
[b] The data corresponded to growth productivity. Color productivity equals 0, as no blue color was observed.

TABLE 4. *Analysis of naturally contaminated dairy food samples*[a]

Sample	TSA	YMA	KDM		Yeasts identification
			Growth	Color	
Butter	M	M	M	Greenish[b]	
	Y	Y	Y	White	*D. hansenii*
Infant milk powder	B	B	—	—	
	Y	Y	Y	Pink	*R. mucilaginosa*
Cheese	Y	Y	Y	Pink	*S. salmonicolor*
Milk (pasteurized)	B	B	—	—	
Milk (UHT) 1	B	B	B+/−[c]	—	
Milk (UHT) 2	B	B	—	—	
Milk (raw)	B	B	B+/−[c]	—	
Fruit yogurt 1	Y	Y	Y	White	*I. orientalis*
Fruit yogurt 2	Y	Y	Y	White	*I. orientalis*
Fruit yogurt 3	Y	Y	Y	White	*I. orientalis*
Liquid yogurt	Y	Y	Y	Blue	*K. marxianus*
Plain yogurt 1	Y	Y	Y	Pink	*R. mucilaginosa*
Plain yogurt 2	Y	Y	Y	White	*T. delbrueckii*
Plain yogurt 3	Y	Y	Y	White	*T. delbrueckii*
Muesli yogurt	Y	Y	Y	White	*T. delbrueckii*

[a] B, bacteria; M, mold; Y, yeasts; −, absence of growth.
[b] Filamentous appearance.
[c] Only a few minute colonies.

were inoculated on the above mentioned media. Plates were incubated at 28°C for 7 days and examined daily.

The recovery of *Kluyveromyces* from experimentally inoculated dairy products was tested on seven samples, including yogurt, infant milk powder, pasteurized and UHT milk, and raw milk. Approximately 10^5 CFU of *K. marxianus* type strain (CYC 1162) were seeded into 10 ml of milk or 10 g of yogurt and homogenized. Five microliters were streaked on KDM medium. The plates were incubated at 28°C for 7 days and examined daily.

Identification of yeasts. The yeast isolates were identified according to traditional morphological and physiological criteria *(2, 12).*

RESULTS

Colonies of *K. marxianus* and *K. lactis* on KDM were blue due to the expression of β-galactosidase activity in the presence of the chromogenic substrate XGal and its inductor IPTG. All the remaining yeast species assayed did not produce the characteristic blue color, and they showed their typical white, cream, or pink (*Rhodotorula* and *Sporidiobolus* strains) color on solid media (Table 1). The filamentous fungus *Eurotium amstelodamii* also grew on KDM plates with its normal filamentous appearance. None of the bacterial strains studied grew on the designed medium.

Table 2 shows the growth and color of *K. marxianus* and *K. lactis* on KDM at 40 and 42°C. As seen, it was not possible to differentiate *K. marxianus* and *K. lactis* on the basis of their growth at high temperatures.

The results of the quality assessment studies are shown in Table 3. The selectivity of the medium was optimal, because it allowed the growth of all the yeast strains assayed (selectivity, 0.9 to 1.1) but completely inhibited the development of *B. cereus,* the bacterial strain used as a reference (selectivity, 0). The productivity has been recorded as growth and production of blue color. Both RGi (ecometric method) and PR (modified Miles-Misra method) had high values, close to 1, for *K. marxianus* and *K. lactis,* except for two strains (*K. marxianus* CECT 20649 and *K. lactis* CECT 10669), which grew on the medium but did not produce blue colonies. The reference yeast strains *S. cerevisiae* ATCC 7754 and *Z. rouxii* NCYC 1522 grew optimally on KDM plates, but did not produce blue color (productivity for color: RGi and PR, 0). Both selectivity and productivity were 0 for the reference bacterial strain *B. cereus* CECT 193.

The plates of KDM could be stored at 4°C for 30 days after preparation, without alteration in the performance of its selectivity and differential properties.

Table 4 shows the results of the analysis of 15 dairy food products. Yeasts could be isolated from 10 samples. Blue colonies on KDM were only obtained from spoiled liquid yogurt, and they were subsequently identified as *K. marxianus.* All the remaining yeast strains isolated producing nonblue colonies were identified as *Debaryomyces hansenii, Issatchenkia orientalis, Torulaspora delbrueckii* (white in color), or *Rhodotorula mucilaginosa* and *Sporodiobolus salmonicolor* (pink colonies). Bacterial colonies were obtained on TSA and YMA from all the studied milk samples, but their growth was completely or highly inhibited on KDM.

K. marxianus was recovered from experimentally inoculated samples. Blue colonies were obtained from all the seeded milk and yogurt samples. They were all subsequently verified as *K. marxianus,* by using conventional identification methods.

DISCUSSION

During recent years, the use of conjugated substrates cleaved by specific enzymes has proved to be a powerful

tool for rapid and sensitive detection of certain microorganisms. Based on this approach, several culture media have been designed for differential isolation of medically important yeasts such as *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, and related species *(3, 18, 20).* To our knowledge, these type of procedures have not yet been used for detecting food spoilage yeasts.

This work describes a selective and differential solid medium for the isolation of *K. marxianus* and *K. lactis* from dairy food products. It is based on the detection of the enzyme β-galactosidase, which cleaves the chromogenic compound XGal. Colonies of positive strains turn blue due to the liberated aglycone. IPTG stimulates the synthesis and increases the activity of β-galactosidase.

Eighty seven yeast strains, belonging to different species commonly isolated from milk and dairy products, were assayed. Only the strains of *K. marxianus* and *K. lactis* produced blue colonies, thus allowing their differential detection in those foods (Table 1). No false-positive results were recorded. Nevertheless, 2 false-negative results were obtained, since two strains failed to produce blue colonies on KDM, thus accounting for 2.2% of the total of yeast strains assayed. As an initial screening for β-galactosidase activities, we perform in our laboratory a conventional enzymatic UV test with ONPG (*o*-nitrophenyl-β-D-galactopyranoside). In those conditions, the enzyme is detected in all the strains of *K. marxianus* and *K. lactis*, but not in the rest of the yeasts studied. We suggest that this simple test could be used as a confirmatory one for testing the nonblue yeast colonies after isolation KDM.

β-Galactosidase production by *K. marxianus* or *K. lactis* is well documented *(4, 5, 11).* Nevertheless, it is not an exclusive character, as other yeast species are able to do so *(2, 9, 19).* The goal of KDM is to provide the possibility of detecting β-galactosidase activity in the absence of lactose and of differentially discriminating *K. marxianus* and *K. lactis*, even though some of the other yeast strains used in this work were able to assimilate lactose *(13).*

Miller described a selective culture medium for *K. marxianus* (*K. fragilis*) based on its ability to utilize lactose and to grow at 45°C *(15).* Barnett et al. *(2)* also recorded growth at 40°C as a difference between *K. marxianus* and *K. lactis.* To improve the performance of KDM medium, we tested two supraoptimal incubation temperatures, 40 and 42°C. Our results showed (Table 2) that growth at elevated temperature was a strain character rather than a species one. As a consequence, incubation at 40 or 42°C could not be used as an additional selective or differential factor to discriminate between *K. marxianus* and *K. lactis*, both originating blue colonies on KDM. Temperature did not affect color production as an expression of β-galactosidase activity but did affect growth.

The ICFMH-WPCM proposed a standardized scheme for the quality control of microbiological media *(1)*, which has been also applied in the development of new formulations *(17).* The method standardizes each step of the procedure, from the preparation of the media and inocula, to the quantification techniques *(7).* When the recommended tests were applied to KDM, the results showed that the medium possessed a good productivity and selectivity for *K. marxianus* and *K. lactis* (Table 3). All but two strains grew well on KDM and produced blue colonies.

Since KDM had been demonstrated to be a promising diagnostic medium, it was used to examine spoiled or severely contaminated dairy food products. The commodities (15 in total) included yogurts, cheese, butter, raw or pasteurized milk, and infant milk powder. Additionally, seven samples were experimentally contaminated with *Kluyveromyces* in the laboratory. The medium was very satisfactory. Blue colonies were obtained from one of the naturally contaminated foods (Table 4) and from all the seeded samples. All were subsequently confirmed as *K. marxianus.* White to cream or pink yeast colonies were also isolated and identified as belonging to several yeast species other than *K. marxianus* or *K. lactis.* Bacteria present in natural products were completely or highly inhibited in KDM. Occasionally, a few minute bacterial colonies grew on the medium, but they could be easily confirmed by microscopic examination (Table 4). On the other hand, none of the pure cultures of bacterial strains could grow on the medium as previously shown in Table 1.

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