Grasas y Aceites
Vol. 51. Fasc. 4 (2000), 225-229

Characterization of yeast strains isolated from bloaters of fermented green table olives during storage

By **A. Asehraou**[1], **C. Peres**[2], **D. Brito**[3], **M. Faid**[4*] and **M. Serhrouchni**[1]

[1] Department of Biology, Faculty of Sciences, Mohamed I[st] University, BP 524, Oujda, Morocco.
[2] Instituto de Tecnologica Química e Biológica, Apt 127,2780 Oeiras, Portugal.
[3] Instituto Nacional de Investigacao Agraria, 2780 Oeiras, Portugal.
[4] Food Microbiology and Biotechnology Department, IAV Hassan II, BP:6202, Rabat, Morocco. Fax: 212 7 77 81 35. E-mail: mfaid@iav.org.ma.

RESUMEN

Caracterización de cepas de levaduras aisladas de las cavidades de aceitunas verdes durante la conservación.

Con el fin de obtener frutos afectados de «alambrado» se tomaron muestras de aceitunas verdes conservadas a granel (recipientes de plástico de 200 litros) de dos fábricas de Marruecos. El aislamiento de las especies de microorganismos de las cavidades se realizó tomando con el asa una porción de fruto de la zona afectada y realizando la siembra en medios idóneos para levaduras, bacterias Gram-negativas y bacterias lácticas. Los resultados mostraron que en las mismas únicamente se encontraban colonias de levaduras. Se obtuvieron ciento cuatro cepas, a las que se caracterizó y se les investigó la actividad «killer» frente a cepas predeterminadas. Los resultados indicaron que las cepas aisladas pertenecían a las cuatro especies siguientes: *Saccharomyces cerevisiae, Pichia anomala, Candida etchellsii, Candida versatilis y Rhodotorula glutinis.* Algunas de las cepas aisladas de cada especie mostraron actividad «killer» frente a las especies de referencia. Las cepas más activas pertenecieron a *P. anomala y C. etchellsii,* seguidas de las de *S. cerevisiae.*

PALABRAS-CLAVE: Aceituna verde de mesa - Actividad «killer» - Alambrado - Conservación - Levadura.

SUMMARY

Characterization of yeast strains isolated from bloaters of fermented green table olives during storage.

Fermented green olives stored in bulks (200 litres plastic containers) were sampled from two factories in Morocco to collect the attacked fruits (bloaters). The microbial species present in the bloaters were isolated by taking a loop from the attacked regions of the fruits and streaked onto appropriate media for the determination of yeasts, Gram negative bacteria and lactic acid bacteria. Results showed that only yeast colonies appeared and no growth of other microorganisms was detected. One handered and four isolates of yeasts were collected for the characterization and research of the killer activity on selected target strains. Results showed that the isolates fit into four species: *Saccharomyces cerevisiae, Pichia anomala, Candida etchellsii, Candida versatilis,* and *Rhodotorula glutinis.* Some of the studied isolates from each species showed killer activity on the target strains. Strains of *P. anomala* and *C. etchelsii* were the most active followed by the strains belonging to *S. cerevisiae.*

KEY-WORDS: Bloater - Green table olive - Killer activity - Storage - Yeast.

1. INTRODUCTION

Gaseous deteriorations or bloaters formation in fermented green olives are one of the most known problem in the field of table olive fermentation. These are more and more occuring during the storage of fermented olives in bulk. In some cases these deteriorations are more severe in non controlled conditions storage and may consequently induce large losses of the production.

The gaseous attack is called in Morocco «roppy spoilage» and it would correspond to the «alambrado» in Spain or to the «fish eye» in USA. This attack leads to the floating of the fruits at the upper layer of the brine and would help in the onset of other attacks such as the softening and browning of the fruits.

Various microorganisms could be involved in the gaseous attack of olive fruits such as Gram negative bacteria (Borbolla y Alcala *et al.* 1960), yeasts (Vaughn *et al.* 1972) and heterofermentative lactic acid bacteria (Etchells *et al.* 1968). Some authors (Durán Quintana *et al.* 1979) reported that the attack can be due to non microbial effects. The control of these attacks were deeply studied in black olives (Garrido Fernández *et al.* 1979; Fernandez Díez *et al.* 1985). However, little had been done on the microorganisms involved in the bloater spoilage of fermented green olives during storage.

In the present study the microorganisms that could be associated with the gaseous attack of fermented green olives during storage were determined as well as their properties.

2. MATERIAL AND METHODS

Samples collection

Samples of fermented green olives from three different fermentations were collected in sterile glass

screw capped flasks from two factories and transported to the laboratory. They were analysed immediately.

pH

The pH of the brine was measured using a pH-meter apparatus (type Orion research).

Titratable acidity

The titratable acidity was determined by titrating 10 ml of the brine with a N/9 sodium hydroxyde solution in the presence of phenolphtalein. Results are expressed as% (v/v) of lactic acid.

Sodium chloride

Ten ml of the brine were diluted to 1/100 with distilled water and titrated with 0.01 N silver nitrate solution in the presence of potassium chromate.

Microbiological determinations

The attacked fruits were opened aseptically and a loop was taken from the infected area and streaked directly onto the respective culture media. Different culture media for determining the microorganisms existing in the bloaters were used: Deoxycholate Agar (Merck, Germany) for Gram negative bacteria, MRS (DeMan, Rogosa and Sharpe) (Merck, Germany) agar for lactic acid bacteria and PDA (Potato Dextrose Agar) (Biokar, France) acidified to pH 3.5 with sterile lactic acid, for yeasts. The plates were incubated at 30°C for 24 to 48 hours for Gram negative bacteria and lactic acid bacteria, and at 30°C for 3-4 days for the yeasts.

Identification

Yeast isolates were picked up at random from the plates, purified and identified according to the method described by Barnett *et al.* (1990).

Characterization

Salt resistance, cellulolytic activity and utilization of organic acids as the sole source of carbon were studied according to the methods described by Faid *et al.* (1994).

Killer activity

a) *Test on the target strains*

Thirty isolates of yeasts belonging to the species *Saccharomyces cerevisiae, Pichia anomala, Candida etchellsii, Rhodotorula glutinis,* and *Candida versatilis,* were tested for their killer activity on target strains. These were kindly supplied by the «Instituto de Tecnologia Quimica y Biologica, Oeiras, Portugal). Five strains namely *Pichia membranaefaciens* IGC 4619, *Saccharomyces cerevisiae* IGC 4620, *Candida boidinii* IGC 3430 *Kloeckera lactis IGC* 4358 and *Saccharomyces bayanus* IGC 4565 were used in this test. Cultures of the different strains were diluted in sterile saline water to make a suspension of 10^5 cells/ml. One ml of the suspension was placed in a sterile Petri dish and the melted medium YMAM (Marquina *et al.,* 1992) supplied with agar (10 g/l) and methylen blue (0.03 g/l), and buffered to pH 4 with citrate buffer (0.2M) was poured on and well mixed to make a homogeneous inoculation. This medium seeded was spot inoculated by the strains to be tested. The plates were incubated at 25°C for 10 days. The killer activity was shown by the clear zones around the spotted cultures.

b) *Test on the selected strains*

The yeast strains that had shown a killer activity were tested for their sensitivity or resistance to the killer activity of those strains isolated from the bloaters. The method used was the same as the one described above. The selected strains were *P. anomala* (S18) and C. *etchellsii* (S92 and S95). The strain *C. versatilis* (S105) which showed the highest sensitivity was used in this test as control.

3. RESULTS AND DISCUSSION

The pH values varied slightly from 3.8 to 4.05 in all the samples (Table I). This low pH value would stop the growth of the Gram negative bacteria in the brines. González Cancho *et al.* (1970) reported that Gram negative bacteria are inhibited when the pH reach values below 4.5. However, a low pH, may encourage growth of yeasts and some lactic acid bacteria. So to prevent this growth, addition of antifungals (sorbic and benzoic) is encouraged. These compounds are more active at low pHs.

Chloride concentrations in the brine varied from 6.88 to 7.26 % (Table I). These concentrations are inhibitory to the Gram negative bacteria and may not inhibit the lactic acid bacteria (Balatsouras, 1985). High salt concentrations would also improve the organoleptic quality of olives and may have an effect on the killer activity of yeasts in the brine (Llorente *et al.* 1997).

Yeasts isolation and identification

Since the Gram negative and lactic acid bacteria could not have been isolated from the attacked fruits

Table I
Physico-chemical characteristics of stored fermented green olive brines

Samples (%)	pH	NaCl
1	3.92	7.26
2	4.05	7.52
3	3.8	6.88

(bloaters), and the only isolation of yeasts would suggest that the main cause of roppy attacks are most probably due to yeasts. All the isolated microorganisms were confirmed by the culture on PDA and the microscopic examination of the grown colonies on the medium and furtherly by the physiological and biochemical tests indicated by Barnett *et al.* (1990).

The isolated strains of yeasts fit into five species (Table II). Most of the isolates were sporogenous and mainly represented by *P. anomala* (46.15%) and *S. cerevisiae* (38.46%). The other species were considered as secondary contaminating agents and were represented by *C. etchellsii* (7.69%) *C. versatilis* (4.8%) and *R. glutinis* (2.9%).

Vaughn *el al.* (1972) isolated *P. anomala* as the main species from brines of gaseous deteriorated olive fruits. Marquina *et al.* (1992) and Asehraou *et al.* (1997) had isolated other species such as *C. etchelsii, C. versatilis* and *R. glutinis* from the brine of fermented green olives. The later indicated that these species were the most frequent yeasts in the brine of natural fermented olives.

The subsequent presence of non-fermentative species, such as *R. glutinis* and *C. etchellsii,* could be due to the contamination of the bloaters. In fact, the *Rhodotorula* species could be involved in the softening of olives from which they were isolated. This phenomenon is due to the pectinolytic activity present in these species (Vaughn *et al.* 1969).

Both *S. cerevisiae* and *P. anomala* can ferment sucrose and release large amounts of carbone dioxide in the fruits causing the gaseous deterioration (Table II). The presence of the two species in the brines of fermenting olives should be avoided to stop the occurrence of various deteriorations and spoilage of the fermented olives during storage. In fact, the highly fermentative properties of the species *S. cerevisiae* and *P. anomala* which are the most frequent, may induce the bloater defects because of the large amounts of CO_2 formation in the olive flesh. Durán Quintana *et al.* (1979) demonstrated that the occurence of the roppy attack or the gaseous deteriorations in the olive flesh can be facilitated by an alkali treatment, mechanical perforation and UV treatment of the olive fruits. The yeasts growth in the flesh could be related to the gas production leading to bloaters formation in the fruits.

Characterization

Most of the yeast species could grow in high chloride concentrations (Table III) *P. anomala* was not inhibited by 10 and 12% NaCl. This may show that these salt concentrations and pH values, which are the main factors monitored during the storage of fermented olives, are not sufficient to avoid yeast growth.

About half of the isolates, mainly those of *P. anomala* showed cellulolytic activity (Table III). The

Table II
Physiological and biochemical characteristics of yeast strains isolated from gaseous deteriorations of fermented green olives in storage

Species									Growth														Fermentation					
	Nb	Sp	My	Ps	Ur	NO_3	37	F	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6
S. cerevisiae	40	1-4*	-	-	-	-	+	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+
P. anomala	48	1-4	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	-	+
C. etchellsii	8	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rh. glutinis	3	-	-	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-
C. versatilis	5	-	-	+	-	±	+	+	+	+	-	-	-	±	+	-	-	+	-	-	+	-	+	+	-	-	-	-

Legends: Nb: number of isolates, Sp: spore formation, My: mycelium, Ps: pseudomycelium, Ur: urea, NO_3: nitrate utilization, 37: growth, 37°C, F: Film formation, 1: D(+)-glucose, 2: D(+)-maltose, 3: D(+)-galactose, 4: D(+)-raffinose, 5: lactose, 6: D(+)-saccharose, 7: trehalose, 8: D-xylose, 9: sorbitol, 10: D(-) mannitol, 11: myo-inositol, 12: L(+)-rhamnose, 13: cellobiose, 14: starch. *: Spores number.

Table III

Characterization of the yeast strains isolated from gaseous deterioration of fermented green olives

	NaCl (% w/v)					
	10	12	15	A.A.	L.A.	Cel.
S. cerevisiae	50*	0,05	0	0	0	20
P. anomala	33,33	10,42	0	100	97,92	54,17
C. etchellsii	50	0	0	75	62,5	75
Rh. glutinis	100	66,67	0	100	33,33	100
C. versatilis	0	0	0	60	60	100

Legends: A.: acetic acid, L.A.: lactic acid, Cel: Cellulose.
*: Figures are % of strains growing on the medium

Table IV

Yeasts species distribution in the different types of bloaters

Sample	Type of attack	Species
1F1	dry bloater	*S. cerevisiae*
1F2	dry bloater	*S. cerevisiae* and *P. anomala*
1F3	crack	*S. cerevisiae*
1F4	crack	*S. cerevisiae* and *P. anomala*
1F5	crack	*S. cerevisiae*
1F6	crack	*S. cerevisiae* and *P. anomala*
1F7	dry bloater	*P. anomala*
2F1	soft bloater	*S. cerevisiae*
2F2	dry bloater	*S. cerevisiae* and *P. anomala*
2F3	dry bloater	*S. cerevisiae* and *P. anomala*
2F4	dry bloater	*S. cerevisiae* and *P. anomala*
2F5	dry bloater	*P. anomala* and *C. etchellsii*
2F6	soft bloater	*S. cerevisiae, P. anomala, C. versatilis, Rh. glutinis*
2F7	dry bloater	*P. anomala*
2F8	dry bloater	*S. cerevisiae* and *P. anomala*
3F1	dry bloater	*P. anomala*
3F2	crack	*S. cerevisiae*
3F3	bloater	*S. cerevisiae*
3F4	dry bloater	*P. anomala*
3F5	soft bloater	*C. etchellsii* and *P. anomala*
3F6	crack	*C. etchellsii, P. anomala, S. cerevisiae*
3F7	crack	*C. versatilis*

Legends: Bloater: a pocket inside the flesh of the fruits; Dry bloater: a non soft pocket in the flesh of olives; Soft bloater: a soft zone inside the fruit; Crack: a fissure in the flesh of the olives.

yeasts would not only induce gaseous problems in the olive fruits, but they could be also involved in the degradation of the fruits texture by an active enzymatic hydrolysis of the cellulolytic material.

Results concerning the nature and state of the deteriorations in the olives reported in table IV would not show clearly a correlation between the species involved in the deterioration and the nature of this deterioration. That is, each species can cause alone or associated with another species the same attack.

Table V

Killer activity of the yeast strains isolated from gaseous deteriorations of fermented green olives

	Sensitive strains								
	P.m 4619	S.c. 4620	C.b. 3430	K.l. 4358	S.b. 4565	C.v. 105	C.e 92	C.e 95	P.a. 18
S. cerevisiae (S10)	-	+	-	-	-	+	-	-	-
S. cerevisiae (S13)	-	+	-	-	-	+	-	-	-
S. cerevisiae (S22)	-	+	-	-	+	+	-	-	-
S. cerevisiae (S28)	-	+	-	-	-	+	-	-	-
S. cerevisiae (S39)	-	+	-	-	-	+	-	-	-
S. cerevisiae (S42)	+	+	-	-	+	+	-	-	-
S. cerevisiae (S54)	+	+	-	-	-	-	-	-	-
S. cerevisiae (S78)	+	-	-	-	-	+	-	-	-
S. cerevisiae (S79)	+	-	-	-	-	+	-	-	-
S. cerevisiae (S84)	-	-	-	-	-	-	-	-	-
S. cerevisiae (S99)	-	-	-	-	-	-	-	-	-
S. cerevisiae (S103)	+	-	-	-	-	-	-	-	-
P. anomala (S18)	+	+	+	+	+	+	-	-	-
P. anomala (S34)	+	+	-	-	-	+	-	-	-
P. anomala (S48)	+	+	-	-	-	+	-	-	-
P. anomala (S57)	+	+	-	-	-	+	-	-	-
P. anomala (S66)	+	-	-	-	-	+	-	-	-
P. anomala (S70)	+	+	-	-	-	+	-	-	-
P. anomala (S89)	+	+	-	-	-	+	-	-	-
P. anomala (S90)	+	+	-	-	+	+	-	-	-
P. anomala (S91)	+	+	-	-	+	+	-	-	-
P. anomala (S98)	+	+	-	-	+	+	-	-	-
C. etchellsii (S55)	+	-	-	-	-	+	-	-	-
C. etchellsii (S92)	+	+	-	-	+	+	-	-	-
C. etchellsii (S95)	+	+	-	+	+	+	-	-	-
C. etchellsii (S100)	-	-	-	-	-	-	-	-	-
Rh. glutinis (S63)	+	-	-	-	-	-	-	-	-
Rh. glutinis (S69)	+	-	-	-	-	-	-	-	-
C. versatilis (S105)	-	-	-	-	-	-	-	-	-
C. versatilis (S108)	-	-	-	-	-	-	-	-	-

Legends: +: killer activity, P.m. 4619: *Pichia membranaefaciens* IGC4619, S.c. 4620: *Saccharomyces cerevisiae* IGC4620, C.b. 3430: *Candida boidinii* 3430, K.l. 4358: *Kloeckera lactis* IGC4358 et S.b. 4565: *Saccharomyces bayanus* IGC4565. C.v. (S105): *C. versatilis*, C.e. (S92 et S95): *Candida etchellsii*, P.a. (S18): *P. anomala*.

The killer activities of the yeast strains are reported in Table V. These may show high proportions of strains belonging to *P. anomala* relatively to those of *S. cerevisiae, C. etchellsii* and *R. glutinis.* Strains S18 of *P. anomala* and S95 of *C. etchelsii* showed the highest activity. These strains were also resistant to the other killer yeasts. Our results agree with those found by several authors about the activity of strains of *P. anomala* on yeasts (Palpacelli *et al.* 1991; Marquina *et al.* 1992; Almeida and Pais, *1996)* and pathogenic bacteria (Izgu and Altinbay, 1997). Meanwhile, compared on the basis of the diameter of inhibition our strains would have higher activities than that reported in the last work. In fact, the killer activity would be improved by salt as it was stated by Llorente *et al.* (1997).

Among the target strains, *P. membranaefaciens* showed the highest sensitivity to the killer strains. This would be very important to eliminate the oxidative yeasts by these killer yeasts if they were used, but, on the other hand, the presence of the killer yeasts could cause gaseous defects in the olives.

ACKNOWLEDGMENTS

The authors are grateful to the Ministry of Sciences and Technology (JNICT), Portugal, for funding this research.

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Recibido: Noviembre 1998
Aceptado: Julio 1999