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Sorbate Detoxification by Spoilage Yeasts Isolated from Marzipan Products

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Summary

Two yeast species were isolated from spoiled marzipan-based products, using enrichment procedures in Yeast Morphology Broth (YMB) supplemented with 330 g L^{-1} glucose. The isolates were identified as Zygosaccharomyces rouxii and Debaryomyces hansenii. They were tested in an experimental medium containing 600 g L^{-1} table sugar (sucrose) and 0.5 g L^{-1} potassium sorbate for reproduction of the alteration observed in the marzipan samples. Both yeasts reproduced the 'petroleum-like' off-odor, while only Z. rouxii produced gas. Using the same growth conditions, the volatile compound responsible for the characteristic off-odor was identified by gas chromatography coupled to mass spectrometry (GC/MS) as 1,3-pentadiene. It was concluded that this volatile compound is due to decarboxylation of sorbate to 1,3-pentadiene, a compound previously reported in Penicillium moulds and lactic acid bacteria as being a detoxification product of sorbate. To our knowledge this is the first report of this detoxification activity in spoilage yeasts associated with high sugar foods.

Key words: spoilage yeasts, marzipan, sorbate, 1,3-pentadiene

Introduction

Yeasts are the most common spoilage agents in high-sugar foods; these so-called osmotolerant yeasts are capable of growing in 800 g L^{-1} glucose or sucrose *(1)*, a concentration at which bacterial growth is inhibited. Preservatives such as sorbic acid and its salts, generally known as sorbates, are added to sugar-rich products in order to inhibit osmotolerant microorganisms like certain yeasts and moulds. Despite this prevention, foods may be spoiled by some mould and yeast species that are capable of resisting and even degrading certain amounts of sorbate. This kind of alteration has been observed in marzipan and related products *(2)*.

In Spain, marzipan mass is made from ground almonds mixed with approximately the same amount of sugar. An amount of up to 1 g L^{-1} of sorbic acid is added as a preservative, in addition to adequate concentrations of citric acid, according to Good Manufacturing Practice (GMP), to obtain a pH value of 5.0–5.5. In these conditions of high proportion of sugar and the presence of sorbate, yeasts are able to grow in the product and give rise to a peculiar alteration: a softening of the texture that is accompanied by an intense 'petroleum-like' off-odor and flavor, and swelling of some individual wrappings.

In a previous work *(2)*, we analyzed a number of spoiled marzipan samples, and several yeasts were isolated from them. These yeast strains were tested in an experimental sugar-rich medium both with and without sorbate, with the result that yeasts were only able to reproduce the characteristic off-odor in the presence of sorbate.

There are several reports describing presumbly the same off-odor in sorbate-treated foods like cheeses and beverages *(3–5)*. Some moulds of the genus *Penicillium* and lactic acid bacteria can metabolize sorbate, in a detoxification reaction, producing a volatile compound, 1,3-pentadiene *(3–6)*. This compound is reported to have a 'hydrocarbon-like', 'plastic paint' or 'kerosene' off-odor and flavor. It is believed that sorbate is degraded through a decarboxylation reaction:

$$CH_3\text{–}CH\text{=}CH\text{–}CH\text{=}CH\text{–}COOH \rightarrow CH_3\text{–}CH\text{=}CH\text{–}CH\text{=}CH_2 + CO_2$$

sorbic acid(2,4-hexadienoic acid) 1,3-pentadiene

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The aim of the present work has been to study the mentioned alteration in marzipan products by isolating the spoilage yeasts and determining if the volatile compound responsible for the 'petroleum' odor was 1,3--pentadiene. It would be, to our knowledge, the first report of this detoxification activity in spoilage yeasts.

Material and Methods

Samples studied

Three spoiled marzipan-based samples, rejected by the consumers, were studied:

– **M1**: 'Egg yolk cake', consisting of marzipan mass filled with confited egg yolk. The sample displayed softened texture, 'petroleum' odor and wrapping swelling.

– **M2**: 'Sweet potato cake', elaborated with marzipan mass filled with confited sweet potato. The alteration was the same as described for M1.

– **M3**: 'Sweet potato cake', which presented softened texture and 'petroleum' odor.

Strains isolation

To isolate the yeast strains, two portions of approximately 5 g of each sample were respectively incubated in Erlenmeyer flasks containing two enrichment media:

– Yeast Morphology Broth (YMB), composed as follows (g L^{-1}): glucose, 10.0; proteose-peptone no.3 (Difco), 5.0; malt extract (Difco), 3.0; yeast extract (Difco), 3.0.

– YMB supplemented with 330 g L^{-1} glucose, with an a_w of 0.93, sterilized by autoclaving at 0.7 atm (115 °C) for 10 min *(1)*.

Both media were incubated at 28 °C for 30 days. Isolations were made weekly by streaking on Yeast Morphology Agar (YMA) plates.

Yeast identification

Identification of the strains was performed according to morphological and physiological tests described by Lodder *(7)* and Kreger-van Rij *(8)*, and compiled by Barnett *et al. (9)*.

Reproduction of the alteration

An experimental liquid medium *(2)*, composed of Nutrient Broth no.2 (Oxoid), 600 g L^{-1} sucrose and 0.5 g L^{-1} potassium sorbate, was employed to reproduce the 'petroleum' odor and/or production of gas.

1 mL of a dense suspension in water (6 Mc Farland standard) of each strain was inoculated in 100 mL airtight flasks containing 50 mL of the medium and incubated at 28 °C up to 7 days. 'Petroleum' odor and gas production were tested daily.

GC/MS tests for 1,3-pentadiene

Yeast strains were inoculated as described above in airtight flasks closed with rubber caps, containing 20 mL of the experimental medium with sorbate. Flasks were incubated at 28 °C for three to four days. After this period, headspace gas was analyzed in search of 1,3--pentadiene, according to the method of Liewen and Marth *(4)*, modified.

Gas chromatography (GC) was carried out using an HP 5890 Series II chromatograph (Hewlett Packard) coupled to an HP 5971 A Mass Selective Detector (MS) equipped with a quadrupole. Instrument control and data reduction were accomplished by a 59970 HP Chem Station. A capillary column 25 m × 0.25 mm (internal diameter) of vitreous silica with stationary phase SE-54 and 0.31 μm film thickness was run isothermally at 40 °C with He_2 as carrier gas at 0.55 bar head pressure. The injector was at 250 °C and the ionization voltage was 70 eV.

Analysis of each sample was made with split injection (1:50) of 1 μL of each headspace gas with a 5 μL Hamilton gas-tight syringe. 1,3-pentadiene was identified with reference to the spectra of the program library and standard pentadiene (Aldrich Chemical).

Results

Table 1 shows the results of the isolation by enrichment procedures of yeasts from the spoiled marzipan samples, together with the reproduction of the alteration. No strains were isolated when using 10 g L^{-1} glucose in the enrichment liquid medium. Four yeast strains were isolated from the spoiled samples by enrichment in the presence of 330 g L^{-1} glucose. Strains L1 and L2, isolated from samples M1 and M2 respectively, were identified as *Zygosaccharomyces rouxii*, and reproduced, in laboratory conditions, the same alterations observed in the samples, *i.e.* gas and 'petroleum' odor. Two yeast strains, L3 and L4, were isolated from sample M3, and identified as *Debaryomyces hansenii*; these strains were able to reproduce the characteristic odor in the experimental medium, but did not produce gas.

Figs. 1, 2 and 3 show the chromatograms (a) and mass spectra (b) of standard pentadiene, and metabolites present in the headspace gas of cultures of strain L1 and L3, respectively. Fig. 1 shows the chromatogram of standard pentadiene (1a); the peak corresponds to the

Table 1. Description of the samples, results of the isolations and reproduction of the alteration by the yeasts

Sample	Main alteration	Isolation medium/isolated strains		Species isolated	Spoilage reproduction
		10 g L^{-1} glucose	330 g L^{-1} glucose		
M1	'Petroleum' odor, wrapping swelling	–	L1	*Zygosaccharomyces rouxii*	'Petroleum' odor, gas
M2	'Petroleum' odor, wrapping swelling	–	L2	*Zygosaccharomyces rouxii*	'Petroleum' odor, gas
M3	'Petroleum' odor	–	L3	*Debaryomyces hansenii*	'Petroleum' odor
		–	L4	*Debaryomyces hansenii*	'Petroleum' odor

– = absence of isolates

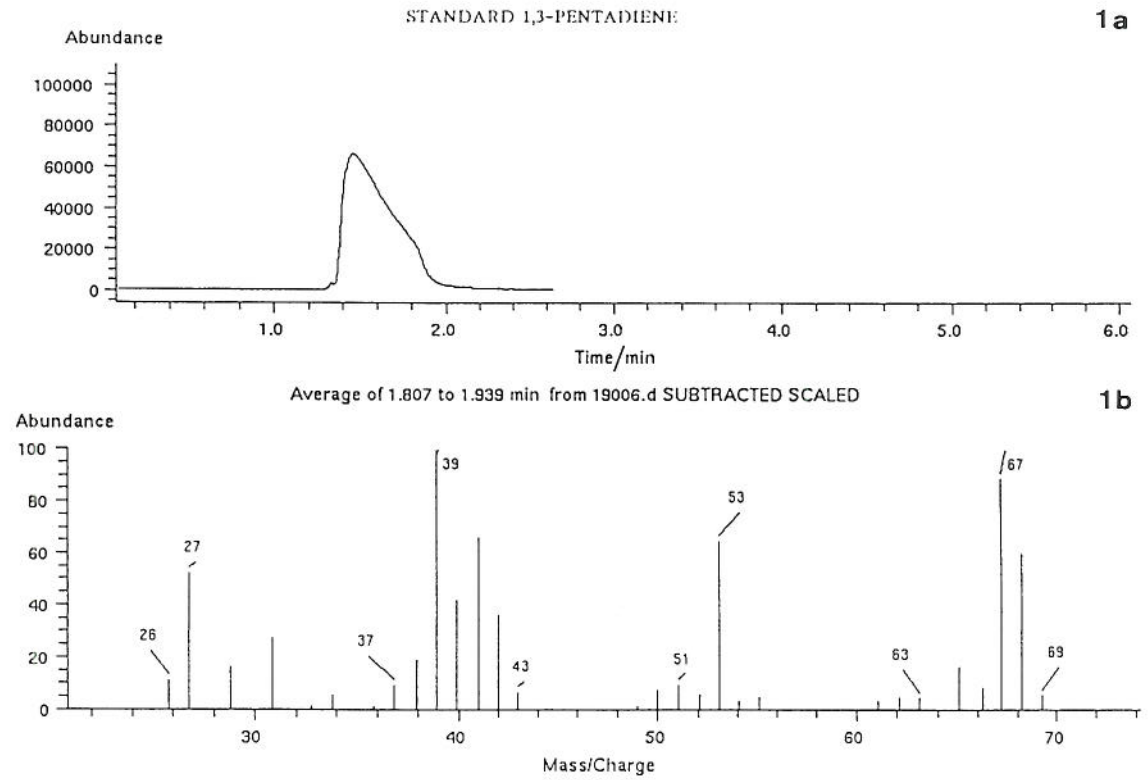

Fig. 1. Chromatogram (1a) and mass spectrum (1b) from the injection of 1 μL of standard pentadiene

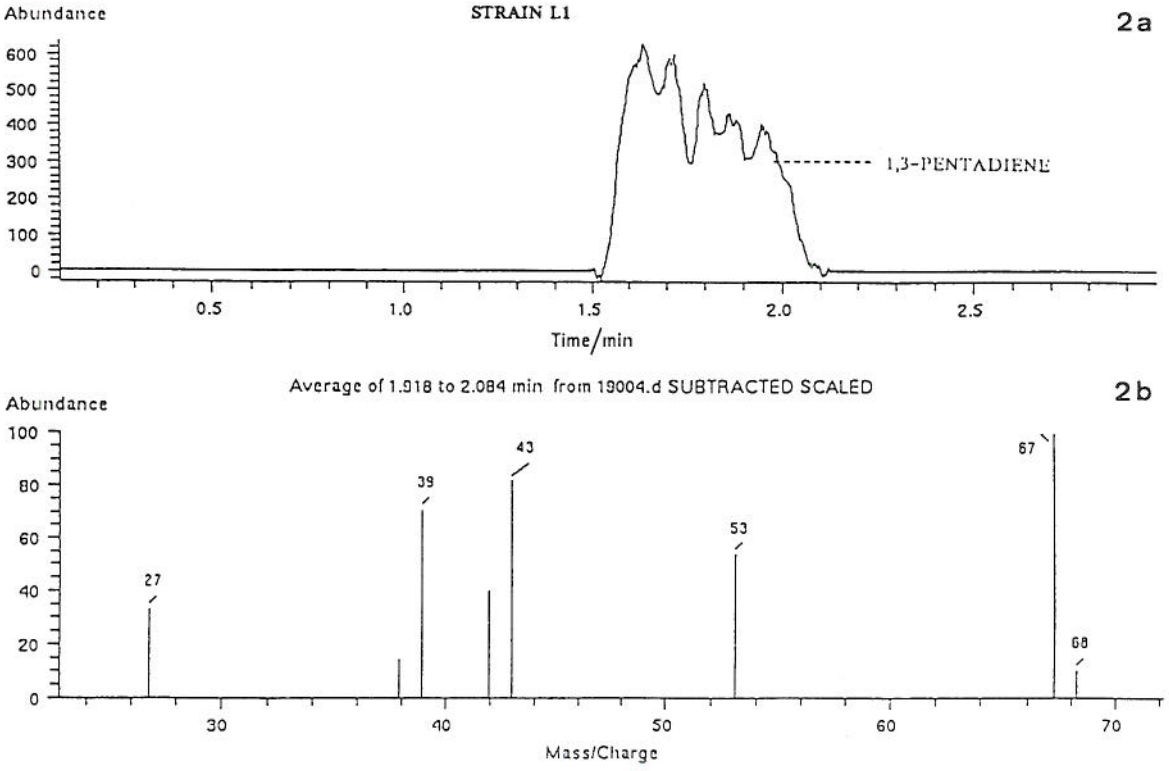

Fig. 2. Chromatogram (2a) and mass spectrum (2b) from the injection of 1 μL of headspace gas from the culture of yeast strain L1 (*Z. rouxii*), showing the scan for metabolites with MS base peak of 67 (ion 67 is selected from standard pentadiene spectrum)

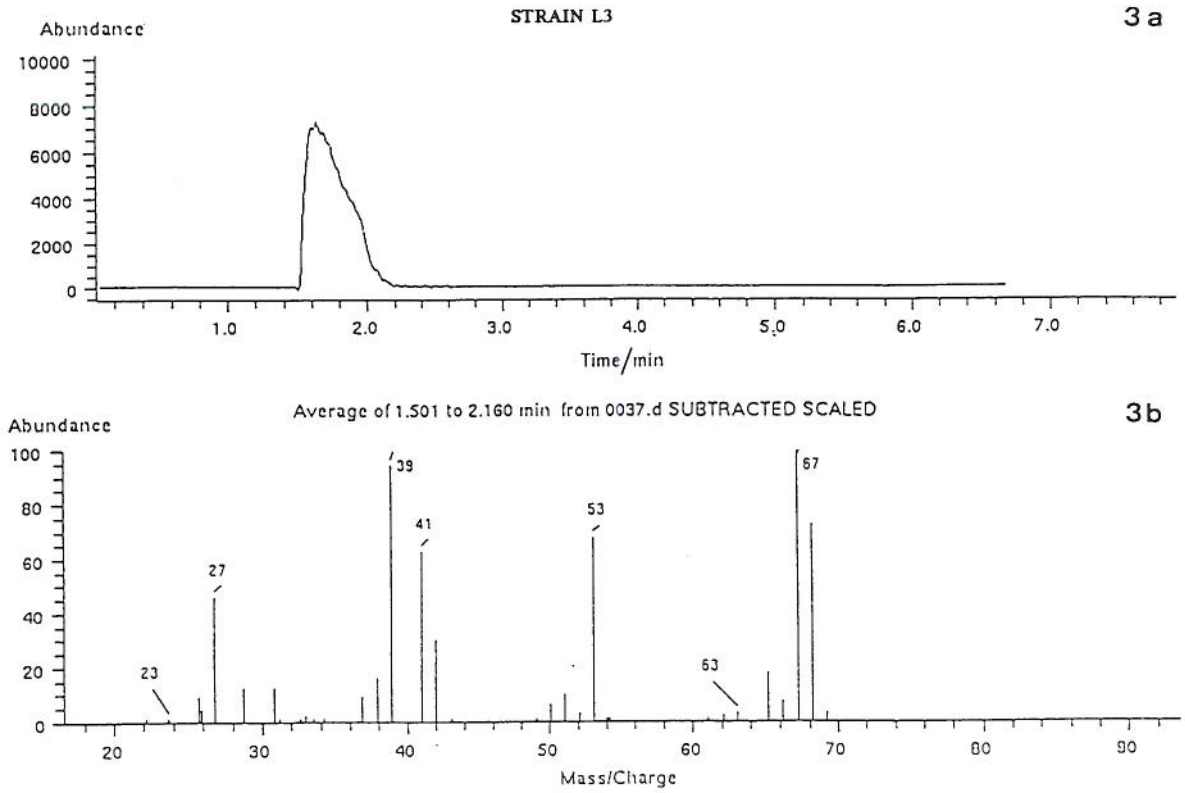

Fig. 3. Chromatogram (3a) and mass spectrum (3b) from the injection of 1 μL of headspace gas from the culture of yeast strain L3 (*D. hansenii)*, showing the scan for metabolites with MS base peak of 67 (ion 67 is selected from standard pentadiene spectrum)

injection of 1 μL of the chemical, whose mass spectrum appears below (1b). Figs. 2 and 3 show chromatograms and mass spectra of metabolites present in the headspace gas of the cultures of the yeast strains L1 and L3, respectively. It may be observed that these two latter spectra (Figs. 2b and 3b) resembled that of standard pentadiene (1b). Likewise, the retention time of peaks corresponding to 1,3-pentadiene produced by *Z. rouxii* (Fig. 2a) and *D. hansenii* (Fig. 3a) were 1.91–2.08 and 1.80–1.93 min respectively, which were similar to the retention time of standard pentadiene, 1.5–2.1 min (Fig. 1a). It is also worth noting that interfering peaks appeared on chromatogram 2a, which corresponded to sugar fermentation compounds produced by *Z. rouxii*, such as CO_2 and ethanol. Otherwise, chromatogram of less-fermentative *D. hansenii* only showed the characteristic peak of 1,3-pentadiene. As mentioned, chromatograms and spectra corresponding to *D. hansenii* and *Z. rouxii* isolates resembled those of the standard chemical. This allowed us to identify the compound produced by the spoilage strains in the presence of sorbate, and responsible for the 'petroleum-like' odor, as 1,3-pentadiene.

Discussion

A peculiar alteration of marzipan, consisting in an intense 'petroleum-like' off-odor, sometimes the presence of gas, and a softening of the texture, has been studied in this work. Four yeast strains, presumably responsible for the observed alteration, were isolated from three different samples.

The first remarkable aspect to note is the medium from which these yeasts were isolated. It has been reported *(1)* that the detection of osmophilic yeasts in high-sugar products can be improved by using media formulated to resemble more closely the natural environment of the cells. The results of this work support the utility of these glucose-supplemented media. As Table 1 shows, yeasts could be recovered from the samples incubated in YMB plus 330 g L^{-1} glucose, but not from the same medium (YMB) containing only 10 g L^{-1} glucose. Therefore, this sugar-enriched culture medium is recommended to recover low numbers and/or sublethally injured osmotolerant yeasts, as well as dangerous yeasts present in such low numbers that they have not yet caused evident alteration of the sample (data not published).

The strains isolated from spoiled samples are described in Table 1. Strains L1 and L2 corresponded to *Z. rouxii*, strains L3 and L4 to *D. hansenii*. *Z. rouxii* is one of the most dangerous yeasts contaminating and spoiling high-sugar products, due to its tolerance to very low water activities (a_w), its fermentative capability and its resistance to preservatives commonly used in food industries. *D. hansenii* is described as a highly osmophilic yeast, able to tolerate high concentrations of both sugars and salts *(10,11)*. *Z. rouxii* isolates produced 'petroleum' odor and a large quantity of gas in the experimental medium containing sorbate and 600 g L^{-1} sucrose, so consequently they must be responsible for the package swel-

ling, due to sugar fermentation, of samples M1 and M2. In contrast, yeast strains isolated from the non-swollen sample (M3), and identified as *D. hansenii*, only reproduced the characteristic odor, without gas formation.

The volatile metabolite exhibiting the characteristic 'petroleum' odor has been identified as 1,3-pentadiene, but a number of derivatives of sorbic acid different from pentadiene have been identified as detoxification products from sorbate. These products, yielded by some moulds and/or lactic acid bacteria, are a result of certain esterification, reduction and ether formation reactions affecting the carboxyl group of sorbic acid. Therefore, it is suggested that the free carboxyl group is necessary for antimicrobial activity, and so the microorganisms, which have developed a detoxification system, do so by chemically suppressing the charged carboxyl group *(6)*.

Among yeasts, there are sorbate resistant strains *(3,12,13)*; sorbate resistance implies an inducible energy--requiring mechanism which 'pumps' the preservative out of the yeast cell *(5,12,13)*. Yeasts built up this ability after repeated exposures to the preservative *(5,13)*. Certain strains are capable of degrading it and others even assimilate it as sole carbon and energy source *(13,14)*. As sorbic acid is a fatty acid, it may be degraded as in animal cells, through a β-oxidation, like its saturated counterpart, *i.e.* caproic acid, yielding acetyl coenzyme A *(5,6,14)*.

To our knowledge, no detoxification reactions, as described for other microorganisms, have been reported in yeasts. *Z. rouxii* and *D. hansenii* isolated in this work were able to develop in a substrate with high sugar content and where sorbic acid is present, *i.e.* marzipan. Thus a selection may have taken place that allows only the growth of those yeasts that, in addition to being osmotolerant, are sorbate resistant or, as the strains reported here, are even capable of detoxifying the preservative, so producing inactive volatile end products like pentadiene.

Very little is known about the conversion of sorbate into 1,3-pentadiene, dealing with the metabolic pathway and implicated enzymes. It also remains unclear if the detoxification mechanism in these pentadiene-producing yeasts is the same as in penicillia and lactic acid bacteria. Further investigation is needed to clarify if the ability to degrade sorbate has any genetic determination which allows only certain strains to do so, or if this ability is exclusively due to continuous exposure to the preservative.

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Detoksikacija sorbata s kvascima kvarenja izoliranim iz proizvoda od marcipana

Sažetak

Iz pokvarenih proizvoda od marcipana izolirane su dvije vrste kvasaca, koristeći postupak pojačanog rasta u kvaščevoj morfološkoj podlozi (Yeast Morphology Broth - YMB) uz dodatak 330 g L^{-1} glukoze. Izolati su identificirani kao Zygosaccharomyces rouxii i Debaryomyces hansenii. Ispitivane su promjene njihove reprodukcije u uzorcima marcipana u eksperimentalnoj podlozi, koja je sadržavala 600 g L^{-1} šećera (saharoze) i 0,5 g L^{-1} kalijeva sorbata. Oba kvasca proizvela su neugodan miris sličan petroleju, dok je samo Z. rouxii proizveo plin. Koristeći iste uvjete rasta, kao hlapljivi je spoj, što je uzrokovao neugodan miris, utvrđen je 1,3-pentadien plinskom kromatografijom povezanom s masenom spektrometrijom (GC/MS). Zaključeno je da je taj hlapljivi spoj nastao dekarboksilacijom sorbata do 1,3-pentadiena. Navedeni je spoj ranije ustanovljen u plijesnima Penicillium i bakterijama mliječne kiseline kao detoksikacijski produkt sorbata. Prema našim spoznajama to je prvi izvještaj o detoksikacijskoj aktivnosti kvasaca kvarenja u namirnici s velikom koncentracijom šećera.