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Identification of yeast strains isolated from a two-phase decanter system olive oil waste and investigation of their ability for its fermentation

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Abstract

A dynamic fed-batch microcosm system is described which permits assessment of the progressive growth of yeasts through olive oil waste. We report on its application to measure the effects of the growth of yeast strains upon the chemical composition of "alpeorujo", the waste of a two-phase decanter system used for the extraction of olive oil. Six phenotypically distinct groups of yeasts were isolated. Three selected isolates were identified as being most closely related to *Saccharomyces* sp., *Candida boidinii* and *Geotrichum candidum* using biochemical tests and partial 18S rDNA gene sequence analysis. This is the first report of yeast growth on "alpeorujo" by the use of a fed-batch microcosm system, resulting in the change of the initial chemical composition of "alpeorujo" and in the decrease of the toxic substances such as phenols.

Keywords: Fermentation; Microcosm systems; Olive oil waste treatment; Yeast diversity

1. Introduction

The management of wastes from olive oil extraction is an industrial activity submitted to three main problems: the generation of waste is seasonal, the amount of waste is enormous and there are various types of olive oil waste (Rozzi and Malpei, 1996). In Greece, with the exception of a few oil mills that use the two-phase system, over 90% of the existing olive oil mills operate according to the three-phase decanter system, while the rest use the traditional press cake system.

The most recent method, applied since 1992, in the extraction of olive oil waste is the so-called "ecological process" or in other words the two-phase decanter system. The decomposed pulp of this system is separated in a two-phase scroll centrifuge into oil and a liquid–solid mixture, called "alpeorujo" in Spain, where a significant percentage of olive oil mills are already using it. The resulting solid waste is about 800 kg per ton of processed olives. This "alpeorujo" still contains 2.5–3.5% residual oil and about 60% water. There are various advantages of the two-phase decanter system when compared to the three-phase system, such as reduction of the produced waste since water consumption decreases considerably, higher oil yield and less energy consumption. One disadvantage reported by Balis et al. (1996) is the fact that the high water content inhibits transportation and further waste treatment at olive oil refineries.

Considering the lack of experience and information on detailed composition and treatment of "alpeorujo", as well as the possibility of further application of the two-phase system in Greece (Pavlea, 1997), it has been thought meaningful to study the chemical composition of this new waste and to use it as substrate for microbial fermentation in order to have a more friendly product released in the environment. "Alpeorujo" could have a role as a fertilizer or as a food additive, providing that it can be detoxified through bioremediation by breaking down the toxic phenolic compounds. While adaptive responses of bacteria grown on "alpeorujo" have been already studied (Jones et al., 2000); up to date, there is no information describing yeast strains isolated from "alpeorujo", nor a system monitoring their growth and the subsequent changes in "alpeorujo" chemical profile.

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In this paper, we investigate the growth of indigenous yeast isolates on ''alpeorujo'' using as a tool the dynamic fed-batch microcosm system.

2. Methods

2.1. Isolation of yeasts and culture conditions

Yeast strains were isolated from Spanish ''alpeorujo'' (supplied by Dr S. Hrushka, Westfalia Co., Oelde, Germany). The ''alpeorujo'' samples were derived from two-phase decanters for olive oil production and had been stored according to local practice in the open air for approximately 3 months prior to sampling. Yeast isolations from ''alpeorujo'' samples were made initially as follows: Samples (100 g) were placed in sterile flasks, mixed with 900 ml sterile Ringers solution (0.25 strength) and shaken on an orbital shaker (Stuart Scientific Co. Ltd., Redhiil, Surrey, UK) at maximum speed (500 revmin^{-1}) for 30 min (Katsifas et al., 1999). Mixtures were allowed to settle before making serial dilutions of the supernatant fluids and plating on malt extract agar (MEA) and czapek dox agar (CzA) media (Atlas, 1993). Plates were incubated at 30 °C for 72 and 96 h. In order to promote growth of strains occurring in low frequencies, serial dilutions of the supernatant fluids were used to inoculate flasks containing malt extract broth and czapek dox broth media. The flasks were incubated at 30 °C for 36 h. Serial dilutions were plated on MEA and CzA.

2.2. Physiological growth tests

Strains were grown on MEA and CzA in order to study the colony colour and texture. For the examination of the micromorphological characteristics, cells taken from a young pure culture were examined microscopically (magnification $\times 1000$) for the presence of budding yeast cells and filaments (mycelium or pseudomycelium). The examination of sporulation and the examination of the formation of ascospores were performed as described by Phaff et al. (1978).

2.3. Assimilation of carbon compounds

In order to study the pattern of carbon compound assimilation, which in many cases is species-specific, the yeast identification system ID 32 C system (BioMerieux Sa, France) was used. For inoculation the manufacturer's instructions were followed. The test strips were inspected for growth daily, up to 7 days.

2.4. Identification using rDNA gene sequence

Chromosomal DNA was isolated by the method of Reader and Broda (1985). Then, it was subjected to PCR amplification using the primers P108, P1190 and M2130 as described by James et al. (1994). The PCR product was purified using a Qiaquick purification kit (Qiagen, Germany) according to the manufacturer's instructions. Direct sequencing of the purified PCR products was performed by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) and an Applied Biosystems model 373A automatic DNA sequencer (James et al., 1997).

2.5. Analysis of sequence data

Strains were characterised by partial sequence analysis of the 18S rDNA gene. The sequences were aligned, by using the BLAST program, with complete or nearly complete 18S rDNA gene sequences retrieved from the EMBL nucleotide sequence data libraries.

2.6. Growth of isolates on ''alpeorujo''

The growth of isolates in different concentrations of ''alpeorujo'' was tested by plating on 1–10% (w/v) ''alpeorujo''. The effect of ''alpeorujo'' on growth was assessed using the concentration gradient plate technique described by Pawsey (1994).

2.7. Dynamic fed-batch microcosm system handling

In the present study, a dynamic fed-batch microcosm system was used for the investigation of long-term growth and activity of indigenous yeast strains under controlled conditions (Vionis et al., 1998). Hundred grams of dry sterile ''alpeorujo'' were placed in sterile polyethylene pots (growth chambers) composing the microcosm. Nutrients were not added to the waste microcosm system before or after the sterilisation. About 10^5 cfu of each strain per gram of dry ''alpeorujo'' were added in the sterile water used for rewetting the ''alpeorujo'' to the desired water content (60%), which was kept constant throughout the experiment. Incubation temperature was 30 °C. Microcosms were sampled at 1, 2, 3, 5, 12, 13, 14, 16 days after inoculation, each sample being exactly 10% of the total ''waste'' volume. In order to achieve repeated cycles of growth in ''alpeorujo'', every 12 days 50% of the microcosm volume was removed and replaced as described by Katsifas et al. (2000).

2.8. Extraction and enumeration of colony forming units and determination of metabolic activity

The extraction and enumeration of cells from ''alpeorujo'' samples was performed as described by Wellington et al. (1992). Determination of respiration rates of microorganisms in ''alpeorujo'' was based on the method described by Katsifas et al. (2000).

2.9. Analytical methods

Sugar and phenol extraction was performed as described by Lambraki et al. (1994). Determination of total sugars was made according to the method of Dubois et al. (1956), while total phenols were determined with the Folin–Ciocalteau's method (Makkar et al., 1988). Total nitrogen (TN) was estimated by the Kjeldhal method (Silva et al., 1995). Protein nitrogen was estimated by the Kjeldhal method, after the extraction of soluble nitrogen by adding Cl_3COOH. True protein was determined by multiplying the protein nitrogen with the factor 6.25 (Silva et al., 1995). Total lipids were determined by the method of Bligh and Dyer (1959), while pH was measured as described by Ohlinger (1996).

2.10. Statistical analysis

All the points on graphs and tables are the means of three replicate samples. Statistical analyses were performed using MINITAB statistical package (Minitab Statistical Software, State College, PA) minimum significant differences (MSD) were calculated from analysis of variance using the Tukey–Kramer method (Fry, 1989).

3. Results and discussion

3.1. Chemical characterisation of "alpeorujo"

The chemical composition of fresh "alpeorujo" that was used in the microcosm system experiments is shown at Table 1. This type of "alpeorujo" was selected among various types of olive mill waste, not only because it had the highest concentration of sugars, which could be used as a carbon source for the growth of microorganisms, but also because of its relatively high percentage of proteins and nitrogen.

3.2. Yeast diversity in "alpeorujo"

As already mentioned by Middelhoven (2002), who has isolated yeast species from alpechin, these habitats are unique because of their chemical composition. In our effort to study the yeast diversity of "alporujo", 12 yeast strains were isolated from Spanish "alpeorujo" and were classified into six morphological distinct groups. Of these, six strains (ALP 3, 4, 7, 8, 9, 10) formed pseudohyphae, one (ALP 11) formed septate hyphae and the other five did not present any form of mycelium. Only one (ALP 12) had a yellowish colony appearance, while all the others were white cream. Six of them (ALP 1, 2, 3, 4, 5, 6) had butyrous colony texture, five (ALP 7, 8, 9, 10, 11) membranous and only one had mucoid colony texture (ALP 12). Budding was observed in all isolates except ALP 11 which formed arthroconidia. In five strains (ALP 1, 2, 5, 6, 7), round or oval ascospores were observed, while no sexual spores were formed in the other seven strains under the conditions of examination.

All isolated strains were able to assimilate glucose for their growth (Table 2). Group 1 seemed to degrade saccharides (mono-, di- and tri-) while Group 2 degraded aminosugars and polyols. Groups 3 and 4 showed a limited ability to assimilate organic compounds degrading only some polyols and two aminosugars, respectively. ALP 11 (Group 5) strain as well as ALP 12 (Group 6) showed a unique degradation profile.

Although it was difficult to know if the isolated yeasts were representative of the native "alpeorujo" populations, they were, nonetheless, capable of growing on this type of waste. The strains that grew better were ALP 1 (Group 1), ALP 3 (Group 2) and ALP 11 (Group 5), all three belonging to Groups capable of utilising various carbon sources. Strains belonging to Groups 3 and 4 showed limited ability to grow on "alpeorujo" plates. This would be expected, since they did not show any variety in the carbon sources they could assimilate. While the assimilation ability of each strain could be indicative of its ability to grow on "alpeorujo", it was noticed that this was not the case, in all strains tested. ALP 12 (Group 6) showed pure growth on "alpeorujo" medium, despite its ability to degrade various saccharides, aminosugars, polyols and organic acids. Since the two-phase system is a new technique, data on the biotoxicity of the waste is rather limited. Balis and Antonakou (2000) during the IMPROLIVE (CT 96 1420) project, showed that a strain of *Pleurotus* sp., previously

Table 1
Chemical analysis on the fermented samples of "alpeorujo" and comparison with the unfermented

Chemical Analysis	Unfermented alpeorujo sample	Fermented alpeorujo sample		
		Candida boidinii	*Saccharomyces* sp.	*Geotrichum candidum*
Total sugars (w/w%)	2.31 ± 0.33	0.036 ± 0.008	0.055 ± 0.012	0.021 ± 0.006
Phenols (w/w%)	2.70 ± 0.31	1.14 ± 0.03	1.05 ± 0.11	1.16 ± 0.03
Total lipids (w/w%)	4.34 ± 0.58	6.63 ± 0.95	5.70 ± 0.90	6.42 ± 0.76
pH	5.30 ± 0.20	7.15 ± 0.80	7.50 ± 0.85	5.40 ± 0.83
Proteins (w/w%)	13.56 ± 1.8	10.30 ± 1.21	9.93 ± 1.34	6.81 ± 1.25

Standard errors were calculated by common numerical analysis.

Table 2
Assimilation of carbon compounds by yeast isolates

		Group 1		Group 2		Group 3			Group 4			Group 5	Group 6
		ALP	ALP	ALP	ALP	ALP	ALP	ALP	ALP	ALP	ALP	ALP	ALP
		1	2	3	4	5	6	7	8	9	10	11	12
1	D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
2	D-Galactose	+	+	–	–	–	–	–	–	–	–	–	+
3	L-Sorbose	–	–	–	–	–	–	–	–	–	–	+	+
4	D-Ribose	–	–	+	+	–	–	–	–	–	–	–	–
5	D-Xylose	–	–	+	+	–	–	–	–	–	+	+	+
6	L-Arabinose	–	–	–	–	–	–	–	–	–	–	–	+
7	Rhamnose	–	–	–	–	–	–	–	–	–	–	–	+
8	a-Methylglucoside	–	–	–	–	–	–	–	–	–	–	–	+
9	Sucrose	+	+	–	–	–	–	–	–	–	–	–	+
10	Maltose	+	+	–	–	–	–	–	–	–	–	–	+
11	Trehalose	+	–	–	–	–	–	–	–	–	–	–	+
12	Cellobiose	–	–	–	–	–	–	–	–	–	–	–	+
13	Melibiose	+	+	–	–	–	–	–	–	–	–	–	–
14	Lactose	–	–	–	–	–	–	–	–	–	–	–	+
15	Raffinose	–	+	–	–	–	–	–	–	–	–	–	+
16	Melezitose	–	–	–	–	–	–	–	–	–	–	–	+
17	D-Glucosamine	–	–	+	+	–	–	+	+	+	+	–	–
18	Acetyl-D-glucosamine	–	–	+	+	–	–	+	+	–	–	–	–
19	Glycerol	–	–	+	+	–	–	–	–	–	–	+	–
20	Erythritol	–	–	+	+	–	–	–	–	–	–	–	–
21	Glucitol	–	–	+	+	+	+	–	–	–	–	+	+
22	Mannitol	–	–	+	+	+	+	–	–	–	–	+	+
23	Inositol	–	–	–	–	–	–	–	–	–	–	–	+
24	DL-Lactate	–	+	–	+	–	–	–	–	+	+	–	–
25	D-Gluconate	–	–	–	–	–	–	–	–	–	–	–	+
26	D-Glucuronate	–	–	–	–	–	–	–	–	–	–	–	+
27	2-Keto-D-gluconate	–	–			–	–	–	–	–	–	–	+
28	Cycloheximide	–	–	+	+	–	–	–	–	–	–	+	–

capable of growing on the extracted press cake of the three-phase decanters, was unable to grow on "alpeorujo", due to is high concentration of phenolics.

Sequences of the selected ALP 1 (Group 1), ALP 3 (Group 2) and ALP 11 (Group 5) strains have been deposited in EMBL/GenBank/DDBJ with the following accession numbers: ALP 1 strain (Sequence accession no. AY181094), ALP 3 strain (Sequence accession no. AY181096) and ALP 11 strain (Sequence accession no. AY181095).

Taking into consideration the colonial morphology, the formation of filaments, the presence and type of sexual sporulation as well as the results of the comparison of the 18S rDNA gene sequence of each microorganism with complete or nearly complete 18S rDNA gene sequences retrieved from the EMBL/GenBank/DDBJ nucleotide sequence data libraries, ALP 1 is closely related to *Saccharomyces* sp. (99.7%), ALP 3 to *Candida boidinii* (99%) and ALP 11 to *Geotrichum candidum* (100%), the anamorph of *Endomyces geotrichum*.

As far as yeast classification is concerned, the presence of sexual reproduction is considered critical. Therefore, 18S rDNA gene sequence comparison may not be able to discriminate the anamorph from the teleomorph state. This has been noticed in the comparison of ALP 11 with known sequences: the strain is closely related to *Galactomyces geotrichum*, *Endomyces geotrichum* and *G. candidum* species. Since no formation of ascospores was observed, it should be closely affiliated to *G. candidum.* In conclusion, only the combination of morphological, biochemical and genetic properties can lead to a safe and reliable classification of the isolated microorganism.

3.3. Survival and growth of yeast strains in "alpeorujo" microcosms

Yeasts could grow on "alpeorujo" microcosm systems and their population increased by 4-fold (Fig. 1). Data obtained suggested that the population was metabolically active at the beginning of the fermentation and during the turnover period of the system. Total sugars of the fermented products were decreased just after each growth cycle of the inoculum (Table 1). This means that the three yeast species use sugars as carbon sources for their growth. The parallel phenol decrease that was observed, indicates that they all display an ability to degrade polyphenols. The biodegradation of polyphenols by yeasts has been previously reported by Ramos-Cormenzana (1986) and Saiz-Jimenez and

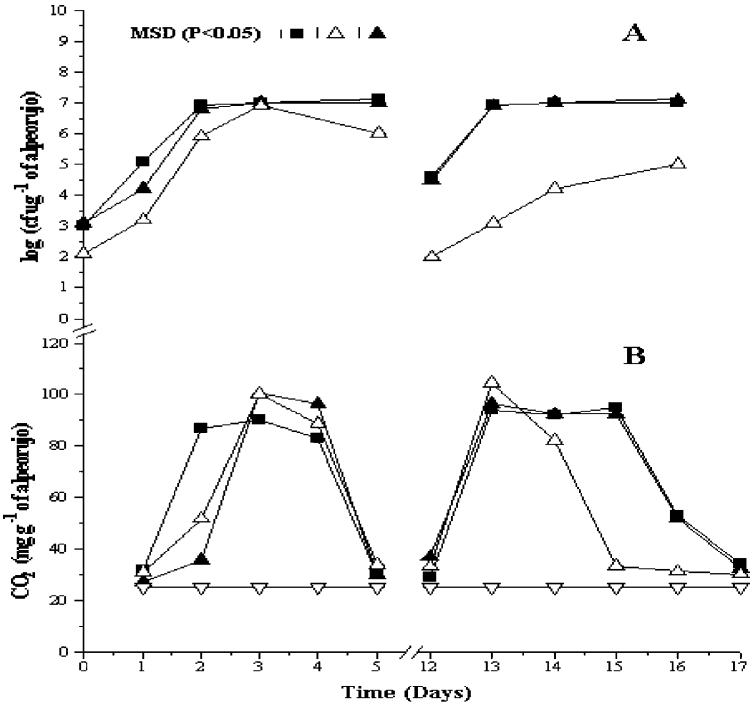

Fig. 1. Survival (A) and metabolic activity (B) of ALP 1 (■), ALP 11 (△) and ALP 3 (▲) in a dynamic fed-batch microcosm system of "alpeorujo" (control respiration ▽).

Gomez Alarcon (1986). An interesting work on this field has been performed by Fiestas and Borja (1996), where a strain of *G. candidum* has been used to pretreat olive mill wastewater in order to reduce the phenolic content before anaerobic digestion and methane production. The results showed that the reduction in phenolic compounds resulted in lower biotoxicity levels of the treated waste. Consequently, this result enhances the suggestion that the two phase olive oil waste, which is released at the environment after its fermentation with the three fungal strains, is much more friendly and less toxic than the untreated waste and can be used as a substrate for the growth of other fungal species, such as the *Pleurotus* strain already reported, which was incapable of growth on the untreated waste.

Total lipid content increased after the fermentation in all cases. Despite the fact that yeasts usually exhibit lipolytic activity, an increase in the total lipids after fermentation has already been reported from other workers on other substrates. Samelis et al. (1993) have noticed an increase on the fat and free fatty acids content of the Greek dry sausage after its fermentation and ripening by lipolytic microorganisms. It is believed that the fatty acids that are exclusively esterified with glycerol molecules to form triglycerides or that participate in the structure of the polar lipids of the biological membranes, are released by a specific or non-specific lipase and exposed to the action of various enzymes and oxidation factors. The final products of the biochemical reactions are, usually, carbonyl compounds of small molecular weight which are the main ingredients of the aroma of the product. The production of aroma was also noticed by the yeast strains since there was a significant change in the initial smell of the waste after the fermentation. It is obvious that lipid enrichment and aroma production are two factors that improved the organoleptic properties of a product and can increase its potential as an animal feed. The increase in the pH can be indicative of the deamination of the aminoacids by the yeasts and the subsequent ammonia production (Welthagen and Viljoen, 1999). The small decrease in the protein content at the fermented products can be attributed to the probable proteolytic activity of the microorganisms. *Geotrichum* sp., in the microcosm of which the highest decrease of protein content after fermentation has been observed, is a well known yeastlike fungus with both proteolytic and lipolytic activity as described by several workers (Daigle et al., 1999).

4. Conclusion

Our results suggest that "alpeorujo" is a suitable substrate for yeast growth and probably a promising fermented product could be obtained from its fermentation. This product could be used as a feed additive, as a fertilizer in crops or as a substrate for the growth of edible mushrooms. Further experiments will follow for the evaluation of the fermented product, in order to select the most appropriate microorganism for fermentation.

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