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Spoilage of oat bran by sporogenic microorganisms revived from soil buried 4000 years ago in Iranian archaeological site

Claudio Milanesi [a, *], Mauro Cresti [a], Lorenzo Costantini [e], Michele Gallo [b], Giuseppe Gallo [c], Silvia Crognale [a, d], Claudia Faleri [a], Alessandro Gradi [a], Baldi Franco [b]

[a] Archaeobotany Laboratory, Department Life Science, Via Mattioli 4, 53100 Siena University, Italy
[b] Dipartimento di Scienze Molecolari e Nanosistemi, University Cà Foscari Venice, Calle Larga Santa Marta 2137, 30127 Venice, Italy
[c] Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF), University of Palermo, Viale delle Scienze, Ed. 16, 90128 Palermo, Italy
[d] Department for Innovation of Biological, Agro-food and Forestry Systems, General and Applied Microbiology Lab., University of Tuscia, Viterbo, Italy
[e] ISMEO – International Association for the Study of the Mediterranean and the Orient, Via Merulana 248, 00185 Rome, Italy

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ABSTRACT

The Bronze Age archaeological site of Shahr-i Sokhta (30° 39′ N; 61° 24' E), located today in southeastern Iran, Sistan region, is a special archaeological deposit in which the exceptional preservation of human, plant and animal remains, due to the dry climate of the region, can provide detailed information on one of the first complex proto-urban societies. In recent years, there has been growing interest in changes in local climate and environment as major reasons why the settlement was abandoned about 4000 years ago. Food shortage has been regarded as a direct effect of these changes. No attention has been paid to the potential health hazards associated with ancient urban/domestic pollution, although large garbage deposits have been found in several parts of the site.

During excavations in 2007, four soil samples were taken under aseptic conditions at a depth of 1.5–2 m in a stratified deposit sealed by the floor of a small house, dated to the second half of the third millennium BC. Microbiological, palynological, carpological and microanalytical studies were performed on the four soil samples. Site C was identified as the most affected by human activity. Failure of conventional methods of detecting culturable and unculturable microbes in site C indicated the need for specific culture conditions suggested by palynological observations. Since oat seed residues were identified among the archaeobotanical material, oat bran was used as carbon and energy source to make a new medium to revive microbes. Coarsely ground oat bran was sterilized twice and soaked with minimal medium as sole carbon source. About 50 mg of buried soil from site C was added to the medium in cell culture flasks with aerobic and anaerobic stoppers and incubated at 28–30 °C and at 4 °C.

After incubation under aerobic and anaerobic conditions, five sporogenic microbes were identified by sequencing 16S rDNA and ITS rRNA regions: a sporogenic strain IRC3 identified as *Bacillus* sp. was the only isolate under anaerobic conditions, whereas under aerobic conditions four moulds were isolated: *Aspergillus flavus* IRC1, *Penicillium crysogenum* IRC2, *Cladosporium* sp. IRC4, and the psychrotroph *Aspergillus restrictus* IRC5. *Bacillus* sp., with 99.7% similarity to *Bacillus subtilis*, broke down oat bran, producing a gel, while *Cladosporium* sp., with 99.8% similarity to *Cladosporium sphaerospermium*, grew on oat bran by synthesizing intracellular lipids. All these microbes are known to spoil food and they are common where there is intense human activity.

1. Introduction

The archaeological site of Shahr-i Sokhta (*Burnt City* in Persian) is located in southeastern Iran, about 57 km south of Zabol, the capital of the Sistan district. The site lies on a plateau-like fluvial terrace and the ruins of the ancient settlement and associated

* Corresponding author.
E-mail address: milanesi@unisi.it (C. Milanesi).

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graveyard cover an area of about 151 ha (Biscione et al., 1977). Shahr-i Sokhta was the most ancient proto-urban settlement in Iranian Sistan and the excavations carried out by an Italian team in 1967–1978 (Tosi, 1968, 1983; Salvatori and Vidale, 1997) and since 1997 by the Iranian expedition of ICAR (Sajjadi et al., 2003) revealed four periods of occupation, covering a time span from 3200 to 1800 BC (Salvatori and Vidale, 1997; Salvatori and Tosi, 2005; Piperno, 2007). The settlement was situated on a third millennium BC branch of the Helmand river, close to a lake into which the river flowed.

The particularly hot and dry climate and the saline crust that formed over most of the site has ensured preservation of unburnt organic remains with only slight deterioration. It has therefore been possible to collect large quantities of wood fragments, grains of food plants and seeds of wild grasses, as well as considerable charcoal. Analysis and study of these materials has made it possible to reconstruct the agriculture and the ancient ecosystems of this part of Sistan in the 3rd millennium BC (Biscione et al., 1974; Tosi 1978; Costantini, 1977, 1979; Costantini and Costantini-Biasini, 1985; Sajjadi et al., 2003; Costantini et al., 2007; Sajjadi et al., 2008).

In recent years, there has been growing interest in changes in local climate and environment as major reasons why the settlement was abandoned (Biscione, 2010). Food shortage has been regarded as a direct effect of these changes. No attention has, however, been paid to potential health hazards associated with ancient urban/domestic pollution, although large garbage deposits have been found in several parts of the site. Palynology and carpology studies could provide useful information on agriculture and food practices and for reconstructing climate and the dietary habits of the inhabitants (Milanesi et al., 2006a,b; Riehl, 2009), while preliminary palaeobotanical analysis of soil and vessel contents show abundant cereals, cucurbits and grapes (Costantini et al., 2007).

Light microscopy (Suyama et al., 1996) Transmission electron microscopy (Milanesi et al., 2006a,b) and Scanning electron microscopy with energy dispersive X-ray has been used to study microorganism ultrastructure and soils prehistoric fertilization strategies buried for millennia (Nielsen and Kristiansen, 2014). This instrument is excellent for identifying elements indicative of metallurgical activities and metals used in prehistoric handicrafts (Wilson et al., 2008).

During excavations in 2007, soil was sampled to search for and identify fungal and bacterial spores. The aim of the study was to combine cultural, microscopy and genetic techniques to isolate and identify microorganisms that survived 4000 years of burial under a salty crust at a depth of more than one meter, in order to obtain additional information about food spoilage at Shahr-i Sokhta. The presence of specific microbial species in soil suggests that sudden abandonment of the city may not only have been influenced by diversion of the river but also by food deterioration.

2. Materials and methods

2.1. Study area

The archaeological site of Shahr-i Sokhta, Sistan, Iran (30°39′N; 61°24′E) lies on a Plio-Pleistocene plateau near the now dry branch of the Helmand River, which formed a terminal delta. Today it lies about 55 Km SWof Zabol in SE Iran (Fig. 1). At the beginning of the third millennium BC, the city did not exceed 20 ha. At the height of its prosperity around 2700–2600 BC, it covered an area of 151 ha (360 acres). The archaeological site includes buildings and structures occupying 75 ha, about two thirds of the area, flanked by a large necropolis of 20 ha (Biscione et al., 1977). The region was fertile and there was an abundance of all kinds of grains, as well as dates and grapes. Human settlement relied on the water of the above-mentioned river branch. At some stage, the course of the branch deviated and the region was hit by drought. The city was rapidly abandoned 4000 years ago (Costantini and Costantini-Biasini, 1985).

2.2. Sampling

Three samples from a buried house (30°39′00.63″N; 61°23′59.90″E) and one of a superficial reference soil outside the dwelling were selected for palynological and microbiological study (Fig. 1). Soil samples were obtained with sterile archaeology probes (Fig. 1 arrows) from three test trenches excavated at the archaeological site: sample A from trench 5, square 5, level 1 (depth 2 m below current surface) near the mud-brick wall of the house perimeter; sample B from trench 5, square 5, level 1 (depth 2 m) 1 m outside house perimeter; sample C from trench 5, square 5, level 0 (depth 1.85 m) on the floor of the house; contemporary sample D from the ground surface 3 m from the sample B. Samples were stored aseptically in sealed sterile test tubes at 4 °C.

2.3. Determination of major elements in soil

The overall composition of major elements was determined in soil samples. The latter were glued on standard vacuum-clean stubs and coated with graphite (Edwards, carbon scancoat, S150A). A scanning electron microscope (SEM) equipped with energy dispersive X-ray analyser (EDX, Philips XL20) and EDAX DX4 probe was used at an acceleration voltage of 20 kV for element analysis. Mean concentrations and standard deviations of each element were calculated from five random determinations on different spots of each sample. Standard deviation was 1%. The X-ray beam was 4 μm wide and penetrated to a depth of 2 μm.

2.4. Determination of seeds and palynomorphs

To identify seeds, all four archaeological soil samples were first observed by stereoscopic light microscope (Zeiss CL 1500 Eco). To separate pollen and spores from inorganic matter, 30 g of sample was washed with 17% HCl solution, centrifuged at 3000 rpm for 5 min in a microcentrifuge (mod. Eppendorf), washed with 37% HF solution and spun down again. The soil was washed in water to remove acid residues and then immersed in a high density liquid (iodide) to separate organic matter by flotation (Gorury and Beaulieu, 1979). The purified material was mounted on glass slides and the specimens were observed by light microscope (Zeiss Axiophot 400). Palynomorphs were identified by visual comparison with a reference guide and by analytical key that considered the number, location and type of openings in the sporoderm of individual pollen grains.

2.5. Isolation of aerobic and anaerobic microorganisms

Soil samples collected in aseptic conditions for microbiological study were sealed in sterile Eppendorf vials and stored at 4 °C until analysis. The palynological and carpological investigations suggested that sample C was the most promising for resuscitating microorganisms, because the soil showed the highest anthropogenic impact. A new oat-bran medium (OBM) was prepared using 0.5 g of coarsely ground oat bran (1–4 mm), as carbon source. The material was double sterilized and soaked in 2 ml mineral medium. The latter contained (per litre): 1.5 g NH_4Cl, 1.5 g $MgSO_4.7H_2O$, 0.6 g Na_2HPO_4, 0.1 g KCl. The OBM was prepared directly in cell culture flasks (50 ml) sealed with stoppers with aerated or unaerated plug seals. All flasks with OBM were inoculated with 50 mg of soil sample “C” and then incubated at 30 °C and/or at 4 °C. Before