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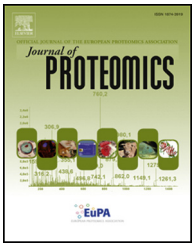

# Protein extraction method for the proteomic study of a Mexican traditional fermented starchy food☆

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ABSTRACT

Pozol is a traditional fermented maize dough prepared in southeastern Mexico. Wide varieties of microorganisms have already been isolated from this spontaneously fermented product; and include fungi, yeasts, and lactic- and non-lactic acid bacteria. Pozol presents physicochemical features different from that of other food fermentation products, such as a high starch content, in addition to a low protein content. It is these qualities that make it intractable for protein recovery and characterization. The aim of this study was to develop a methodology to optimize the recovery of proteins from the pozol dough following fermentation, by reducing the complexity of the mixture prior to 2D-PAGE analysis and sequencing, to allow the characterization of the metaproteome of the dough. The proteome of 15 day fermented maize dough was characterized; proteins were separated and analyzed by mass spectrometry (LC–MS/MS). Subsequent sequence homology database searching, identified numerous bacterial and fungi proteins; with a predominance of lactic acid bacterial proteins, mainly from the *Lactobacillus* genus. Fungi are mainly represented by *Aspergillus*. For dominant genera, the most prevalent proteins belong to carbohydrate metabolism and energy production, which suggest that at 15 days of fermentation not only fungi but also bacteria are metabolically active.

Biological significance
Several methodologies have been employed to study pozol, with a specific focus toward the identification of the microbiota of this fermented maize dough, using both traditional cultivation techniques and culture independent molecular techniques. However to date, the dynamics of this complex fermentation is not well understood. With the purpose to gain further insight into the nature of the fermentation, we used proteomic technologies to identify the origin of proteins and enzymes that facilitate substrate utilization and ultimately the development of the microbiota and fermentation. In this paper we overcome the first general challenge for such studies, obtaining a protein sample with adequate quality capable of representing the system.
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## 1. Introduction

Metaproteomics is a powerful tool to study microbial activity and interactions in complex samples such as fermented food and beverages. In contrast to DNA and RNA, most proteins have an intrinsic metabolic function and can thus be used to relate specific microbial activities to define organisms in multi-species communities. Therefore, the identification of the microbial proteins of a given habitat together with the analysis of their phylogenetic origin and their temporal distribution is expected to provide fundamentally new insights into the role of microbial diversity in processes [1]. This approach has been applied successfully to complex samples such as soil, ground water and fermented foods like cheese and wine [2,3].

Several techniques and kits have been designed to aid in the characterization of the proteome of many kinds of cells including animal tissues such as muscle, brain, liver and others [4–6]; microorganisms such as yeast, bacteria and fungi [7–9]; and plant tissues [10–13]. However, each one of these methods is generally optimized only for a specific type of sample. The chemical and physical characteristics of the sample are important factors to consider when selecting the best extraction method to optimize protein recovery, so usually, the first option is the use of methods reported for samples with similar characteristics. Nevertheless there are some samples with very particular characteristics such as the pozol.

Pozol is a traditional non-alcoholic beverage, made from the fermented dough of corn kernels. It is consumed traditionally by the inhabitants of Southeastern Mexico, in the Maya region, as an important component of their diet [14]. It has cultural importance not only as food, but also as traditional medicine and as a ceremonial element [15]. Cobs of white corn are husked and the kernels are cooked in the presence of lime (calcium hydroxide), a process called "nixtamalization" [16]. The grains are allowed to cool at room temperature in the cooking fluid, known as "nejayote", and then kernels are washed to remove most of the pericarp and subsequently coarsely ground. The resulting heterogeneous dough is shaped into balls, wrapped in banana leaves, and allowed to ferment spontaneously at an ambient temperature for 2 to 7 or more days [17,18]. The resulting fermented dough is suspended in water and consumed daily as a refreshing beverage [19]. More than 40 different species of bacteria, fungi and yeast have been reported in this fermented dough [14,18,20–22]. However, the fermentation mechanism remains unclear.

Corn kernels, like most seeds, accumulate important quantities of stored compounds as reserves for early growth of seedlings, including proteins, lipids, and carbohydrates. Carbohydrates in the form of starch are the most abundant constituent (approximately 75%), with protein making up between 6 and 12% of the stored compounds [23]. It is evident that the main problem for corn protein extraction is the interference due to the presence of high amounts of carbohydrates. Moreover, the nixtamalization process modifies the composition of corn kernels, and the starch undergoes many structural changes including partial gelatinization, melting, pasting, retrogradation [24,25], and starch cross-linking [26,27]. These changes increase the complexity of the extraction because the partially gelatinized starch tends to form networks that trap proteins, as it does with DNA or RNA [28].

Furthermore, corn kernels have important quantities of store proteins such as zeins, which represent the major component of corn storage proteins (50% of the total protein in mature seeds [29]). These highly-abundant proteins may mask low-abundant proteins, dominating the protein profiles. Furthermore, due to their particular characteristics, zein proteins gel easily [23]. Thus, the challenge was to obtain the proteins in the dough trapped within the gelatinized starch and zein network, including those proteins of the fermenting microbiota. Here we describe the procedure developed for the efficient extraction of pozol proteins aimed to obtain a high protein yield and an extensive meta-proteome for the characterization of the fermentation.

## 2. Materials and methods

### 2.1. Pozol samples

Two samples of pozol were obtained from the central market of the city of Villahermosa, Tabasco State, Mexico. Freshly ground nixtamal dough was wrapped in banana leaves. The resulting ball was fermented at 37 °C for 15 days. Following the respective fermentation period, the dough balls were ground in an industrial blender with solid $CO_2$ until a powder was obtained. Samples were stored at −70 °C until use.

### 2.2. Selection of homogenization method

Because of the nature of the sample, before weighing, the sample was subjected to a second grinding in a mortar with solid $CO_2$ to obtain a fine powder. Sample (0.1 g) was mixed with 1.0 mL of 40 mM Tris–HCl buffer pH 8.5 with protease inhibitor (Sigma Protease Inhibitor cocktail for general use) and six different homogenization methods, commonly used in proteomic protocols, were evaluated (Table 1). Resulting samples were centrifuged twice (13,200 ×*g* for 10 min) at 4 °C to obtain a clarified supernatant. Each extraction was performed in triplicate.

### 2.3. Solubility and extraction of proteins

After the selection of the most efficient sample homogenization method, by comparing protein yield; six different extraction/solubilization buffers were assessed. One mL of each different buffer (Table 2) was added to 0.1 g of macerated sample and vortexed with glass beads (Sigma 710–1180 μm). The samples were kept at room temperature for one hour with 5 min of vigorous vortexing each 20 min. Samples were centrifuged twice

**Table 1 – Methods used for sample homogenization.**

| Method | Description |
|---|---|
| I | French press (20,000 psi) |
| II | Sonication (5 pulses of 10 s, 130 W, 20 kHz) |
| III | Grinding mortar with solid $CO_2$ |
| IV | Vortexing with glass beads (710–1180 μm) |
| V | III + IV Grinding mortar with solid $CO_2$ plus vortexing with glass beads |
| VI | Freeze-thaw cycling (3) |

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