J. Dairy Sci. 100:1–10
https://doi.org/10.3168/jds.2016-11450

Identification of the major yeasts isolated from high moisture corn and corn silages in the United States using genetic and biochemical methods

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ABSTRACT

The objective of this study was to identify species of yeasts in samples of high moisture corn (HMC) and corn silage (CS) collected from farms throughout the United States. Samples were plated and colonies were isolated for identification using DNA analysis. Randomly selected colonies were also identified by fatty acid methyl esters (FAME) and by physiological substrate profiling (ID 32C). For CS, *Candida ethanolica*, *Saccharomyces bulderi*, *Pichia anomala*, *Kazachstania unispora*, and *Saccharomyces cerevisiae* were the predominant yeasts. *Pichia anomala*, *Issatchenkia orientalis*, *S. cerevisiae*, and *Pichia fermentans* were the prevalent species in HMC. The 3 identification methods were in agreement at the species level for 16.6% of the isolates and showed no agreement for 25.7%. Agreement in species identification between ID 32C and DNA analysis, FAME and ID 32C, and FAME and DNA analysis was 41.1, 14.4, and 2.2%, respectively. *Pichia anomala* and *I. orientalis* were able to grow on lactic acid, whereas *S. cerevisiae* metabolized sugars (galactose, sucrose, and glucose) but failed to use lactic acid. The yeast diversity in CS and HMC varied due to type of feed and location. Differences in species assignments were seen among methods, but identification using substrate profiling generally corresponded with that based on DNA analysis. These findings provide information about the species that may be expected in silages, and this knowledge may lead to interventions that control unwanted yeasts.
Key words: fermentation, *Pichia anomala*, spoilage

INTRODUCTION

Anaerobic fermentation and aerobic spoilage by yeasts are undesirable in silage. Yeasts tend to grow best aerobically but can ferment sugars anaerobically and produce ethanol and CO_2 when trace amounts of oxygen are present, resulting in large losses of DM (McDonald et al., 1991). The degree of anaerobiosis during storage and the concentration of organic acids are important factors that affect the survival of yeasts. Jonsson and Pahlow (1984) reported that the presence of oxygen enhanced survival and growth of yeasts, whereas a high level of acetic acid reduced their viability during storage.

When silage is exposed to air, during feed-out in the silo or in the feed bunk, aerobic microorganisms can become active. It is generally accepted that yeasts play a major role in initiating aerobic spoilage of silage (Jonsson and Pahlow, 1984). Under aerobic conditions, spoilage yeasts are able to oxidize sugars and lactic acid, resulting in the production of CO_2, H_2O, and heat, and thus causing spoilage (Woolford, 1990) and loss of important nutritional components (Kung et al., 1998). The degradation of lactic acid by yeasts causes a rise in silage pH to a level that allows opportunistic bacteria (e.g., bacilli) and molds (e.g., *Aspergillus*, *Fusarium*, and *Penicillium*) to grow and further reduce silage quality (McDonald et al., 1991).

The population of yeasts associated with aerobic spoilage of silages can be divided into 2 physiological groups. The first group is composed of yeasts with a high ability to ferment sugars but varying ability to assimilate lactic acid. These organisms include *Saccharomyces cerevisiae* and species of *Torulopsis*. The second group is composed of yeasts that have a high respiratory affinity for lactic acid. These organisms include species of *Candida*, *Issatchenkia*, *Pichia*, *Hansenula*, and *Endomycopsis* (Moon and Ely, 1979; Jonsson and Pahlow, 1984; McDonald et al., 1991). Pahlow et al. (2003) reported that yeasts of the genera *Candida* and *Hansenula* (*Pichia*) are particularly prolific during the aerobic phase of silage spoilage because of their lactate-assimilating ability and their strong affinity for glucose.

Plating silage extracts on solid agar medium yields the number of culturable colony-forming units per gram of fresh material that appear on a particular me-

Received May 12, 2016.
Accepted October 2, 2016.
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dium at a given temperature. The colonies can then be studied with different methods to determine the species of yeast (Querol and Ramòn, 1996). Classical or traditional microbiological procedures are based on microbial culturing, and macroscopic and microscopic analysis of colonies and cells or phenotypic traits such as fermentation products and enzyme activity. In recent years, rapid identification methods have been developed to overcome the complexity of traditional yeast identification methods. These methods are based on substrate assimilation and are characterized by the development of growth of an organism in the presence of chemically pure substrates. The analytical profile index (**API**) 20C system (bioMérieux, Marcy-l'Etoile, France; Buesching et al., 1979) is considered a reliable and proven system (Willemsen et al., 1997; Heelan et al., 1998) and has been a commonly used system for identification of yeasts. A similar identification system is the ID 32C (bioMérieux). The advantage of the ID 32C for the identification of yeasts in silage samples is the presence of lactic acid as a substrate for growth. The ID 32C has been used as a reference system due to its extensive database and accuracy (Gutierrez et al., 1994; Fricker-Hidalgo et al., 1995, 1996). Fatty acid methyl ester profiling of the cell wall is another method that can be used for the identification of microorganisms, including yeasts (Botha and Kock, 1993).

In the last decade, molecular methods have been developed for the identification of yeast (Chang et al., 2001; Luo and Mitchell, 2002). For species differentiation and to establish phylogenetic relationships, the most common method is sequencing of ribosomal DNA (Kurtzmann, 1992; Yamada et al., 1994; Cai et al., 1996). The approach consists of amplification of yeast rDNA sequences or the internal transcribed spacer (**ITS**) region by PCR, followed by sequencing of the resulting DNA products. Application of molecular approaches has had a considerable effect on species assignments and the shift to a genomic basis for species delineation caused the merger of physiological or morphological variants into larger and more diverse species (Lachance, 2006).

The type of yeasts and their metabolism in silage has not been well studied in corn silage (**CS**) or high moisture corn (**HMC**) in the United States. Thus, the aims of the present study were to identify and characterize the major species of yeasts associated with samples of CS and HMC collected from dairy farms throughout the United States. Yeast identification was conducted by sequencing of the ITS region of the DNA, FAME analysis of the cell wall, and physiological substrate profiling, and the results obtained by these methods were compared.

MATERIALS AND METHODS

Sample Collection and Preparation

Ten CS and 11 HMC samples from dairy farms located in 9 states (New York, Idaho, Ohio, Washington, West Virginia, Michigan, Iowa, Minnesota, and Wisconsin) were used for the isolation and characterization of yeasts associated with silages. Silages were stored in a variety of silo types between 7 and 11 mo and sampled directly from the silos. The samples were characterized as having "high" populations of yeasts as described by a nutritionist or technical service representative servicing the farm. Samples were immediately shipped on ice via expedited delivery services to the University of Delaware. Upon arrival, water extracts of the samples were prepared by blending 25 g of silage with 225 mL of sterile quarter-strength Ringers solution (Oxoid BR52, Unipath, Basingstoke, UK) for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). Silage pH was measured and a portion of the water extract was filtered through Whatman 54 filter paper (Whatman, Florham, NJ), acidified with 50% H_2SO_4, and frozen before analysis of their lactic acid, acetic acid, and ethanol contents, using HPLC (Muck and Dickerson, 1988). The HPLC system consisted of a Shimadzu system controller (CBM-20A), pump (LC-20AT), and refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan) with a Bio-Rad Aminex HPX-87H ion exchange column (300 mm × 7.8 mm i.d., Bio-Rad Laboratories, Hercules, CA) heated to 35°C and with a mobile phase composed of 7.5 m*M* H_2SO_4 plus 0.25 m*M* EDTA at 0.6 mL/min. A portion of each sample was dried in a forced-draft oven at 60°C for 48 h for the determination of DM content.

Enumeration and Isolation of Yeasts

A portion of the silage water extract was filtered through a double layer of sterile cheesecloth and 1 mL was 10-fold serially diluted for enumeration of yeasts and molds on pour-plates containing malt extract agar (Oxoid CM59, Unipath) acidified with 85% lactic acid at a rate of 0.5% vol/vol. After incubation at 32°C for 48 to 72 h, colonies were enumerated and all microbial counts were transformed to $\log_{10}$ cfu/g and expressed on a fresh weight basis. Between 15 to 20% of the yeast colonies were randomly picked from the Petri dish at the highest dilution and containing >30 colonies. The colonies were streaked onto Sabouraud dextrose agar (Becton Dickinson, Sparks, MD), grown at 28°C for 24 to 48 h, and a single colony-forming unit was recovered. The colony purification process was repeated 2 more