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Fungi in Ontario maple syrup & some factors that determine the presence of mold damage

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ABSTRACT

Maple syrup is a high value artisanal product produced mainly in Canada and a number of States primarily in the northeast USA. Mold growth (*Wallemia sebi*) on commercial product was first reported in syrup in 1908. Since then, few data have been published. We conducted a systematic examination for fungi in maple syrup from 68 producers from all of the syrup-producing areas of Ontario, Canada. The mean pH of the samples was pH 6.82, sugar content averaged 68.0 ± 0.89 °Brix and a_w averaged 0.841 ± 0.011. Some 23 species of fungi were isolated based on morphology and molecular techniques. The most common fungus in the maple syrup samples was *Eurotium herbariorum*, followed by *Penicillium chrysogenum*, *Aspergillus penicillioides*, *Aspergillus restrictus*, *Aspergillus versicolor* and two species of *Wallemia*. *Cladosporium cladosporioides* was also common but only recovered when fungi known from high sugar substrates were also present in the mold damaged sample. The rarely reported yeast *Citeromyces matrinsis* was found in samples from three producers. There appear to be three potential causes for mold damage observed. High a_w was associated with about one third of the mold damage. Independently, cold packing (bottling at ~25 °C) was a risk factor. However, syrup of good quality and quite low a_w values was contaminated. We hypothesize that sanitation in the bottling line and other aspects of the bottling process may be partial explanations. Clarifying this requires further study.

1. Introduction

The production of maple products, including maple syrup, sugar and butter in Canada represents approximately $360 million to the Canadian economy (Anon., 2013). Of this total, approximately 10% is from rural Ontario. Almost all of the production can be described as artisanal, i.e., small producers on their sugar maple woodlots. It has long been known that some fungi can grow on packaged maple syrup that has not been sufficiently heated before sealing (Heald and Pool, 1908; Whalen and Morselli, 1984) or stored for a long time after opening. The common wisdom has been that moldy maple syrup "unless severely damaged" can be filtered, re-heated and blended and packaged (Perkins and van den Berg, 2009). While this advice reflects common sense, there is little information on the consequences of the mold growth. Whalen and Morselli (1984) recommended that further studies be conducted on "the possible damage to pure maple syrup by fungi". Consumer complaints of mold growth in maple syrup are quite common throughout the maple production areas in the US and Canada (Drake and James, 1992), particularly in product purchased from roadside stands.

Maple syrup is made by collecting and concentrating maple sap by boiling. Maple syrup making was common to all the tribes in Colonial America and Canada and quickly adapted by European settlers (Chamberlain, 1891). The dominant sugar of maple syrup is sucrose with small amounts of glucose and fructose as well as trace concentrations of polysaccharides and oligosaccharides (Perkins and van den Berg, 2009; Stuckel and Low, 1996). The pH of maple syrup varies from 4.73 to 8.26; the central value reported is pH 6.7 (Perkins and van den Berg, 2009; Stuckel and Low, 1996; Robinson et al., 1988; Takano, 2005). Syrup pH varies primarily due to soil mineral content (Greenough et al., 2010; Robinson et al., 1988) and the bacterial and yeast populations in the lines collecting the syrup (Filteau et al., 2012).

In the sugar bush, sugar content in maple syrup is typically determined by the measurement of degrees Brix. Two methods are typically used. The first involves the measurement of specific gravity with a hydrometer calibrated to solutions of pure sucrose. This assumes that the solutions being measured have the same specific gravity as pure sucrose. It has been long known that the measurement of °Brix for sugar solutions other than sucrose had the modest accuracy (Deerr, 1921). Reasons for this included the variable presence of very fine crystals (Shorey, 1896), mineral salts from the soil (Fort and McKaig, 1936) and ash (King, 1931). Thus, even in the hands of the same analyst, the analyses of samples of high sugar solutions from different plants/locations are not directly comparable. The second common method is the use of refractometers. Misclassification between the estimated sugar content and the weight of sugar solids and

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inter-sample variation (samples from different sources) were greater than for maple syrup compared to other high sugar syrups (Bryan, 1908, 1909).

Probably the first report of fungal growth on maple syrup was made by Heald and Pool (1908). They found that a mold which they called *Torula saccharina* grew quite well in media with up to 75% sucrose as well as good quality maple syrup. This species was reduced to synonymy with *Wallemia sebi* by Ciferri and Redaelli (1943). *W. sebi* also spoils cakes, dates, jams, dried fruits, salty meat, peanuts, and/or dry cereals, bread and rice (Samson et al., 2010). Well conducted studies done on spoilage of various kinds of sugars including maple sugar were done over the first half of the 20th century. This is because raw sugar would spoil because of the presence of invertase produced by the associated fungi. The dominant fungi in sugars were some yeasts, *Eurotium* species and *Aspergillus penicillioides*. In maple products, these fungi, plus *W. sebi* are listed as common in spoiled maple sugars and syrup (Browne, 1918; Hucker and Pederson, 1942). Whalen and Morselli (1984) reported a laboratory study of mold on maple syrup. They took one lot, heated it to 82 °C and hot packed the syrup with an automatic filling machine. The containers were stored for up to one year at 4 °C, 24 °C and 30 °C. Samples were taken and plated on Rose Bengal Agar and Sabouraud Dextrose Agar and allowed to incubate. Approximately 7% of the samples were contaminated by visible mold with incubation temperatures of 24 °C and 30 °C. Whalen and Morselli (1984) reported mostly *Penicillium chrysogenum*, *Aspergillus niger* and *Aspergillus ochraceus* and some *Eurotium repens*, along with some yeasts.

The purpose of this study was to conduct a systematic survey of the fungi in maple syrup from the 2014 production season in Ontario, determining °Brix by specific gravity and refractometer, water activity (a_w) and pH and make a preliminary assessment of the risk factors for post-market mold damage.

2. Materials and methods

2.1. Sampling

Coordinated through the Ontario Maple Syrup Producers Association, 68 maple syrup producers sent samples proportional to the volume of production in each of the 11 maple-syrup producing districts in Ontario which span an area of ~250,000 km^2. The samples from each producer comprised 6 250 ml containers of syrup (48 medium grade, 9 amber, 8 light and 3 ungraded). Samples were blind-coded for producer location with information on factors thought to predispose samples to mold contamination (e.g., cold packing, container type, reverse osmosis, wood versus oil heat, preheating containers). The samples were received at Carleton University over a three week period from the annual production in the spring of 2014 shipped via Canada Post by the producers. Samples were generally received within two days and were stored at room temperature in their original boxes until processed.

2.2. Physical measurements

The measurements recorded below apply to three of the six samples received from each producer except for those which had fungal damage when opened. A total of 21 out of 204 bottles could not be tested due to mold growth (two samples of three came with visible mold from 5 producers; one sample of three came with visible mold from 11 producers).

2.2.1. pH

The pH meter (Mettler Toledo, Mississauga, ON) was used to directly measure the pH of the maple syrup samples, in duplicate. The electrode was washed in deionized water between each reading. The pH meter was calibrated with standard buffers of 4.0, 7.0 and 9.0 (BDH Chemicals, Mississauga, ON).

2.2.2. Water activity

The a_w of the syrup was measured in duplicate with an AquaLab Series 3 water activity meter (Decagon Devices, Pullman WA) according to the manufacturer's instructions. Certified water activity standards with a_w values of 0.760 ± 0.003 (6.00 mol/kg NaCl) and 1.000 ± 0.003 were obtained from the manufacturer.

The estimated sugar content as °Brix was determined by two techniques used by maple syrup producers. Except for the cases where there were mold-damaged samples, the readings were taken in all three remaining samples.

2.2.3. Specific gravity

For each reading, a °Brix hydrometer (Dominion & Grimm, Anjou, QC) and the 250 ml graduated cylinder were washed in hot running water and dried with clean paper towel. For each sample tested, 240 ml was poured into the graduated cylinder. The hydrometer was carefully lowered into the syrup taking care not to touch the side of the graduated cylinder. The reading was taken after no air bubbles were present in the syrup. The reading was taken at eye level according to the manufacturer's instructions.

2.2.4. Optical refractometer

An aliquot of syrup was enclosed in the measurement chamber of a handheld optical refractometer (Baker Instrument 76018T; Dominion & Grimm, Anjou, QC). The instrument was held up to the eye level towards a source of light. The density was recorded when a clear and straight boundary was observed through the lens. Measurements were taken at 20 °C.

2.3. Mycological analysis

From each producer sample, three bottles were randomly selected for mycological analysis. Aliquots of 300 μl were drawn from the surface of the syrup from each bottle and transferred onto Petri dishes containing either yeast extract sucrose agar (YES) or Dichloran Glycerol agar (DG18) in triplicate (Samson et al., 2010). Plates were incubated at 25 °C in the dark and inspected every 2–3 days for three weeks. Colonies were enumerated and, as necessary, transferred to appropriate media for identification. From sample receipt to plating was completed in less than two weeks. Distinct colonies of representative isolates were transferred to 2% Malt Extract agar for DNA extraction and on Malt Extract and Czapek Yeast Extract agars for identification (Pitt, 2000; Samson et al., 2010).

Separately, DNA was extracted from 50 mg of mycelium from each fungal culture with an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop ND1000 UV–Vis Spectrophotometer (Thermo Scientific, Wilmington, DE). The complete ITS region of the extracted DNA was amplified by polymerase chain reaction (PCR) using primers ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTATTGATATGC (White et al., 1990) synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). PCR amplification was performed in a 20 μl volume using 12.2 μl ddH_2O, 0.2 μl of Taq DNA polymerase (Invitrogen, Carlsbad, CA), 2.0 μl of PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 0.4 μl dNTPs (Invitrogen, Carlsbad, CA), 1.0 μl of each primer and 2 μl of template DNA. PCR was conducted using a TECHNE TC-3000 thermocycler (Bibbly Scientific Ltd.) using the following parameters: initial denaturation at 94 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 53 °C for 30 s, and extension at 72 °C for 90 s. Final extension was at 72 °C for 10 min. The full length amplification product was sequenced in both directions by the Génome Québec Innovation Centre (Montreal, Canada). All sequences were trimmed to begin with the 'CATTA' motif