Research: Syrup production

Chemical and microbial characterization of ropy maple sap and syrup

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Ropiness of maple syrup is a phenomenon that can occur several times in the season. The alteration known as "ropiness" is characterized by a viscous, thick, slimy/jelly-like texture which, although not noticeably altering the taste, renders the product unpleasant in terms of mouthfeel. Ropy maple syrup is unsaleable according to Quebec's current regulation, causing it to be discarded and leading to a substantial loss for the industry (Quebec, M-35.1, r. 18, a.17). Year after year, this type of defective syrup is produced to varying extent. A syrup is graded ropy when the length of the string is equal or above 10 cm (http://www.centreacer.qc.ca/Service/document-formulaire). It is automatically graded as improper and must be destroyed.

Ropy maple syrup is generally caused by fermentation of bacteria present in sap (Fabian and Buskirk, 1935). These bacteria possess the ability to produce exopolysaccharides (EPS) in maple sap resulting in a stringy maple syrup after concentration. Several bacteria were found to contribute to the development of stringiness in concentrated maple sap such as *Aerobacter aerogenes*, *Bacillus ,aceris* or *Enterobacter agglomerans* (Fabian and Buskirk, 1935; Edson and Jones, 1912; Britten and Morin, 1995). These bacteria are usually found in the environment of sugarbushes and can develop in improperly handled or stored maple sap (Morin et al., 1993). Polysaccharides (PS) such as dextrans, arabinogalactans and rhamnogalacturonans (Sun et al., 2016; Storz, Darvill and Albersheim, 1986; Adams & Bishop, 1960) were previously reported in maple syrup. Arabinogalactans and rhamnogalacturonans were suspected to mainly originate from cell walls of plants, while dextran was presumed to result from bacterial contamination of sap (Storz, Darvill and Albersheim, 1986).

The aim of this study was to estimate the economic impact of production of ropy maple syrup in the region of Quebec, to more deeply identify and characterize bacteria associated to this type of quality defect, and to study the composition of PS found in stringy maple syrup.

MATERIAL AND METHODS

Sampling

A total of 25 samples were obtained in 2011, including 15 ropy maple syrups, six concentrates and four saps, from several producers in different regions of Québec. It should be noted that sampling sap corresponding to ropy maple syrup was not always possible since ropiness cannot always be predicted from sap or concentrate. Samples were then stored at -20°C until further analysis.

Physico-chemical analysis

Each sample was analyzed for its

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soluble solids (°Brix), pH and viscosity. Light transmittance (at 560 nm) was measured in syrup samples. Ropiness was measured in each syrup sample by measuring the string's length by dipping a spatula into the ropy syrup.

Microbial counts and culture isolation

Dilution and plating of sap and concentrate samples were performed to provide total bacterial mesophilic counts in aerobic and anaerobic conditions, and total bacterial psychrophilic aerobic counts. Specific growth media were prepared to obtain counts of microorganisms of the genus *Pseudomonas* and total yeast and mold counts. The viable cell counts were expressed in terms of log of colony forming unit per millimeter (log CFU/ml). Eighteen different colonies with distinctive morphologies were purified with three subcultures in their corresponding growth medium before storage at -80°C in tryptic soy broth (Difco, NJ, USA) until DNA extraction.

Total DNA extraction

The DNA of bacterial isolates was extracted with the Nucleospin® Tissue extraction kit (Marcherey-Nagel, Düren, Germany). Concentrations of purified DNA were measured using ND-1000 spectrophotometer (Nanodrop Technologies, USA).

PCR Amplification

Amplified 16S rRNA gene was obtained from each isolates by PCR, by using the universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-GGYTACCTTGTTACGACTT-3') (Invitrogen, Carlsbad, CA). PCR amplification was achieved with Taq PCR Core kit (QIAgen, Hilden, Germany) and carried out in a TGradient PCR thermocycler (Biometra, Goettingen, Germany) according to a published protocol (Lagacé et al., 2004).

Cloning and plasmidic DNA extraction

Amplified PCR products were ligated into the pCR2.1-TOPO plasmid, inserted in Escherichia coli and cultured on LB Miller agar for 24 hours at 37°C. Prior to sequencing, plasmids were extracted according to the method described by Holmes and Quigley (1981) and digested with EcoRI enzyme. DNA fragments were then sequenced and partial sequences were obtained. Consensus sequences of 1500 bp were retrieved with InfoQuestTMFP software and identified by comparison to database of DNA sequences with BLASTn (NCBI).

Identification of microorganisms causing stringy maple syrup

Each of the 18 bacterial isolates were inoculated in an 8°Brix maple sap concentrate previously filtered through a membrane (0.22 μm) to remove microbial biomass. Identification of microorganisms responsible for stringy maple syrup was performed with inoculation of each isolate at 106 CFU/ml in sterile 8°Brix concentrate, incubated at 15°C for two days followed by 4°C for four days. Resulting fermented media were evaluated for ropy properties and corresponding bacterial isolates were selected for growth conditions and associated syrup characteristics evaluation.

Fermentation of concentrated maple sap by slimy bacterial isolates and syrup production

Fermentations were done by inoculating selected bacterial isolates at 106 CFU/ml in an 8°Brix maple sap con-

centrate previously filtered through a membrane (0.22 μm). Three incubation conditions were selected: 4°C, 15°C and alternating incubations of 23°C for eight hours followed by 4°C during 16 hours for three days. Syrups were subsequently produced and physicochemical analysis were performed.

Polysaccharides purification and characterization

Three out of the 15 ropy syrup samples were selected for polysaccharides characterization based on the difference between total soluble solids measured by the refractometer and total sugar (sucrose, glucose and fructose) content quantified by high-performance liquid chromatography (HPLC). The greater the difference, the more polysaccharides were suspected to be present in syrup.

Polysaccharides of each syrup samples were purified using HPLC (Waters, Milford, MA, USA). Molecular weights of polysaccharides were estimated by HPLC with a TSK-GEL 4000PWXL column (Waters, Milford, MA, USA) heated at 40°C and using ultrapure water as the eluent at a flow rate of 0.5 ml/min.

Polysaccharides were then hydrolyzed by adding trifluoroacetic acid (TFA) 2N and monomeric saccharides were identified by GC Agilent 6890 equipped with an MS 5973 as detector. Reference solutions of glucose, fructose, mannose, rhamnose, arabinose and galactose were as well used to confirm the peak identification. Results were presented as area percentage out of total areas of identified peaks to demonstrate relative monosaccharide proportions. Peaks were identified using the NIST database (2008) as well as reference solutions of monosaccharides.

RESULTS AND DISCUSSION

Economic impact

Ropy maple syrup volumes may vary from year to year. For the last ten years, a maximum was reached in 2014 with 358,607 lbs. of bulk ropy syrups produced (Table 1). It is important to mention that the amount presented is underestimated since producers tend to destroy ropy maple syrups themselves when they are detected after evaporation. However, in 2014, more than $1 million (CAD) was lost without including those barrels discarded by producers. In total, an estimated more than $5.5 million was lost in production of ropy maple syrup since 2008.

Over the last ten years, the highest proportion of ropy syrups has been classified as dark color syrup, representing 39.77% of total ropy maple syrups (Results not shown). This is followed by amber and medium syrups representing 25.20% and 17.57% respectively, while extra-light and light ropy syrups represented less than 10%. Therefore, the darker the syrup, the higher the probability of ropiness occurrence. Ropy syrup production is an important issue that needs to be addressed.

Microbial counts

Sap and concentrate samples retrieved from sugarbushes producing ropy syrups were diluted and plated to characterize the microbial profile of each sap and concentrate samples (Results not shown). Aerobic plate count ranged from 6.12 to 8.49 log CFU/ml. For most samples, psychrotrophic counts were higher than *Pseudomonas* counts and varied between 6.14-8.41 log CFU/ml and 5.77-7.63 log CFU/ml

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respectively. Other psychrotrophic species are therefore suspected to be present in sap/concentrate samples. Anaerobic plate counts were lower or equal to aerobic plate counts and ranged from 5.05 to 8.56 log CFU/ml. Yeast and mold counts ranged from 2.22 to 5.05 log CFU/ml.

Globally, samples showed a high total bacterial load, with concentrates containing higher aerobic plate counts than saps.

Phylogenetic tree of bacterial isolates

Analysis of 16s rDNA sequences of the 18 isolates through BLASTn revealed that 15 bacterial isolates were Gammaproteobacteria with nine isolates belonging to the genus *Pseudomonas* and six isolates belonging to the family of *Enterobacteriaceae* (Figure 1). Pseudomonas are psychrophilic microorganisms and are usually present in sap throughout the season. *Pseudomonas* genus is generally associated with good quality sap or concentrate (Lagacé et al., 2004, Filteau et al., 2012). Bacterial isolates A and 2 were identified as *Leuconostoc mesenteroides*.

Identification of exopolysaccharides producing isolates

The ability of the isolates to produce ropy slime was tested by inoculation at 106 CFU/ml in filtered 8°B concentrate (0.22 μm) and incubation at 15°C for two days followed by 4°C for four days. The viscosity of resulting concentrates was monitored. Three isolates (A, 2 and N) were able to enhance the viscosity of the concentrate. Isolates A and 2 were previously identified as *L. mesenteroides* and isolate N belonged to *Enterobacteriaceae* family. They already have been reported for EPS production and enhancing the viscosity of the medium in which they were inoculated (Beech & Carr, 1977; Korkeala, Suortti and Mäkelä, 1988; Anderson and Rogers, 1963).

Influence of exopolysaccharides producing bacteria inoculated in maple sap concentrate

To test growth conditions of the selected bacterial isolates in maple syrup concentrate and properties of corresponding syrup, isolates A, 2 and N were inoculated at 106 CFU/ml in 8°Brix sap concentrate and incubated at 4°C, 15°C and an alternation of 23°C for

Year	Total sales of syrups in Quebec (millions lbs)[a]	Ropy syrups (lbs)[b]	% of ropy syrup	Weighted price ($/lbs)[a]	Economic loss ($)[c]
2008	58.772	146,125	25%	$2.20	$321,476
2009	109.373	101,300	9%	$2.74	$277,561
2010	88.078	142,243	16%	$2.74	$389,745
2011	101.869	117,536	12%	$2.78	$326,749
2012	96.138	208,952	22%	$2.80	$585,066
2013	120.324	121,065	10%	$2.89	$349,879
2014	113.722	358,607	32%	$2.84	$1,018,445
2015	107.168	314,134	29%	$2.86	$898,424
2016	148.177	221,659	15%	$2.94	$651,677
2017	152.250	240,013	16%	$2.92	$700,837

[a] Data from economic file, FPAQ (2017)

[b] Estimated by converting the number of ropy maple syrup's barrels (32 gal.us per barrel) produced to lbs.

[c] Estimation based on ropy syrup (lbs) and weighted price ($/lbs) for each year.

Table 1: Estimated economic impacts of ropy maple syrups production, Quebec (2008-2017)

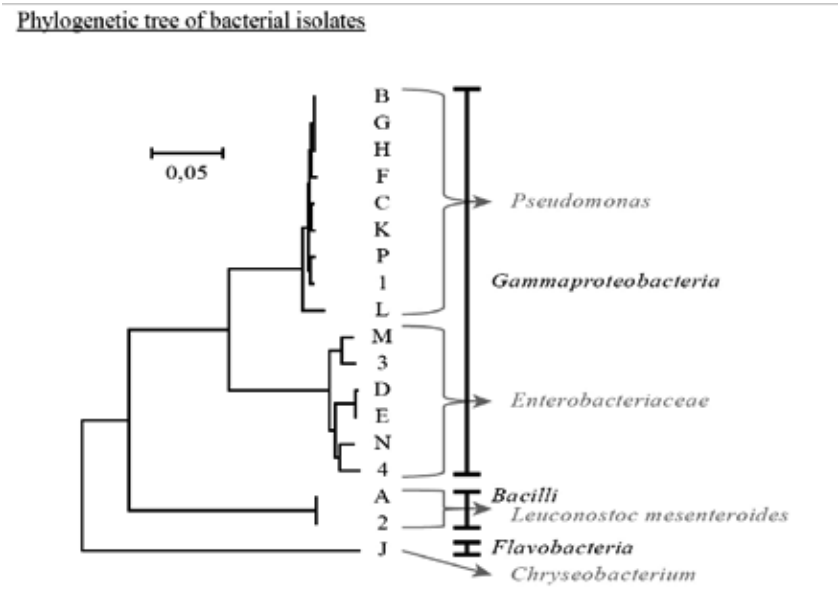

Figure 1: Neighbor-joining phylogenetic tree based on partial 16S rRNA gene of 18 bacterial isolates retrieved from sap and concentrate samples of producers having ropy maple syrup issues.

eight hours and 4°C for 16 hours, over three days for unaerated static fermentations (Figure 2).

Higher viscosity was noticed at 15°C for *Leuconostoc* (isolates A and 2) compared to higher temperature (23°C) after three days of incubation. At 15°C and 23°C, the pH of the fermenting concentrate dropped rapidly to about 5.7 by three days of fermentation (except for isolate 2 at 15°C) whereas at 4°C, the pH did not vary. The pH decrease is suspected to result from metabolic microbial activity and corresponding organic acids production. This sharp decrease is also correlated with bacterial proliferation during fermentation. Indeed, all bacterial isolates counts increased from 6 to about 8 log CFU/ml at all growth conditions except for *Leuconostoc* 2 at 4°C. Control showed a microbial contamination reaching 5.00 log CFU/ml after three days of incubation. The filtration step was therefore not fully effective to remove all microorganisms initially found in the sap concentrate. However, this contamination didn't influence pH or viscosity values at 23°C after three days.

Properties of corresponding syrup

Following inoculation and fermentation with isolates A, 2 and N, concentrates were evaporated into syrups at lab-scale. All syrups corresponding to concentrate fermentation at 4°C and controls are similar regarding °Brix, string length and viscosity (Figure 3). Indeed, average viscosity of a syrup without viscosity defect range from 120 to 160 cP. However, in two cases (*Leuconostoc* incubated at 15°C) evaporation was interrupted due to the extreme viscosity of the boiling solution making the evaporation very difficult forcing us to stop the evaporation. Fur-

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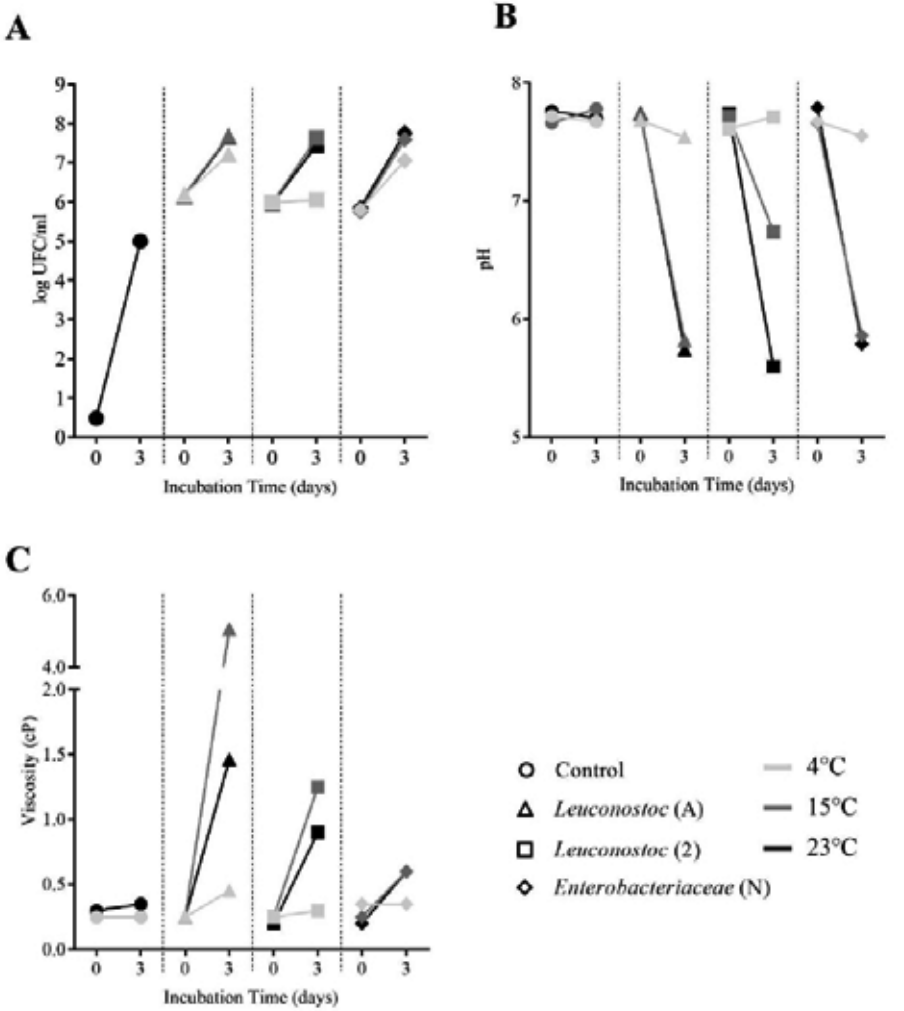

Figure 2: Aerobic mesophilic bacteria count profiles (A), pH (B) and viscosity (C) of sterile concentrate inoculated at 106 UFC/ml with three different bacterial isolates, incubated at three different temperatures over three days.

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thermore, syrup corresponding to fermentation with *Leuconostoc* A at 23°C was too viscous to show a measure by the viscometer in spite of its complete evaporation to 66°B. Fermentation with L. *mesenteroides* isolates produced ropy syrup with strings up to 30 cm long at 15°C and 23°C. Only *Enterobacteriaceae* N gave syrups with shorter strings with 4 and 6 cm long when incubated at 15 and 23°C respectively, making it conform to current regulations and marketable.

Those results suggest that factors promoting ropy slime formation in syrup are mainly uncontrolled fermentation of *Leuconostoc* species at higher

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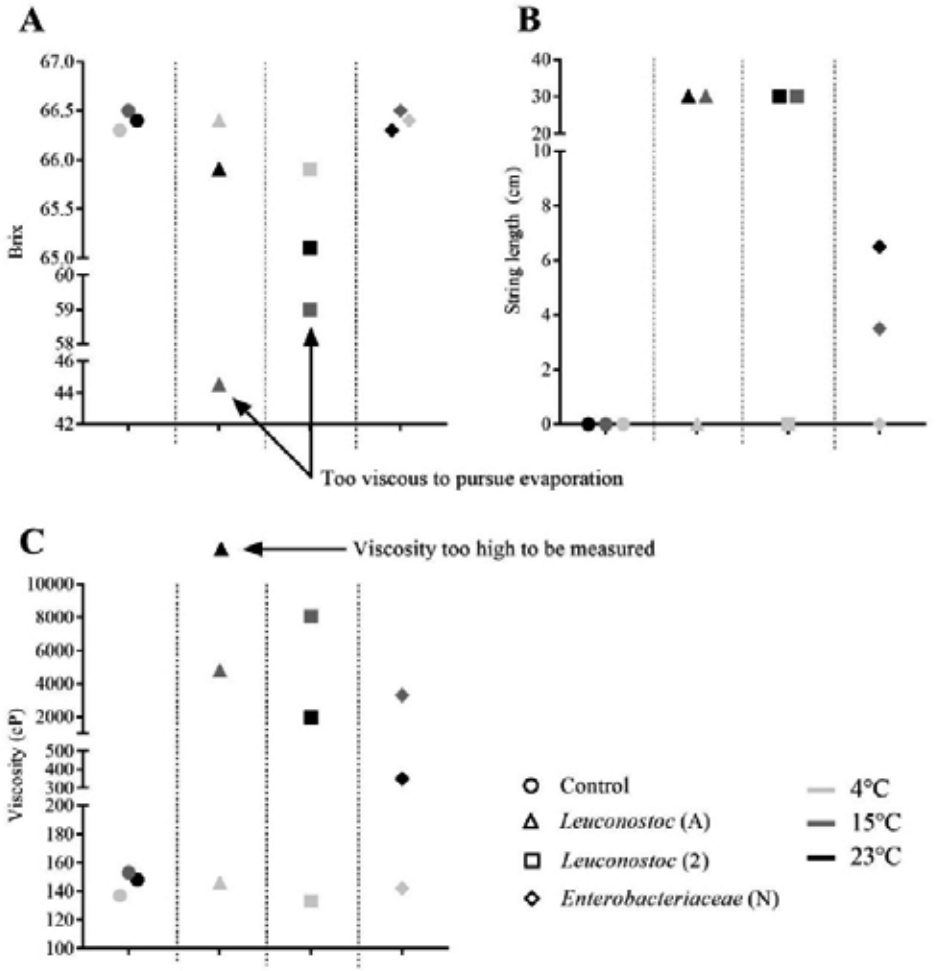

Figure 3: Degree Brix Profiles (A), string length (B) and viscosity (C) of maple syrup produced after inoculating sterile maple sap concentrate at 106 UFC/ml with three different bacterial isolates followed by incubation at three different temperatures over three days.

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temperature (15 to 23°C) in concentrate of 8°B. The slime production of L. *mesenteroides* is well known (Bamforth, 2008; Strausbaugh & Gillen, 2008). They are ubiquitous and can produce ropy slime with or without sucrose and they usually need small concentrations of glucose as carbohydrate source (Korkeala, Suortti and Mäkelä, 1988; Giglio & McCleskey, 1953). Furthermore, a slight increase of sap viscosity can contribute to the production of a highly ropy syrup. Various species in *Enterobacteriaceae* family are also EPS producers such as *Enterobacter agglomerans* which was reported to secrete EPS in concentrated maple sap or *Aerobacter* aerogenes when fermented in diluted maple syrup resulted in a production of highly ropy syrup that could stretch up to 10 feet long (Morin et al., 1993,

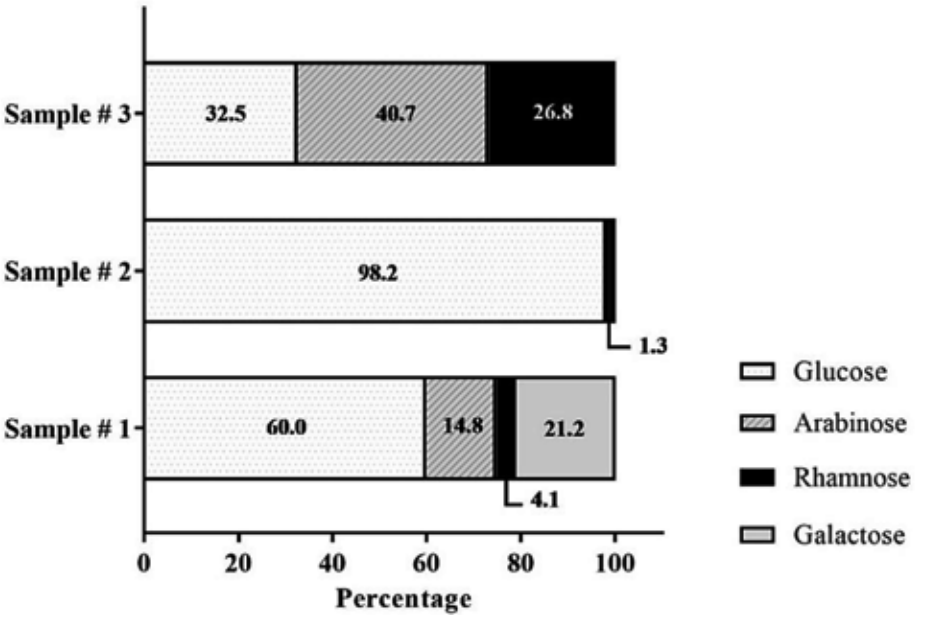

Figure 4: Monosaccharide composition of purified polysaccharides from 3 ropy maple syrup samples.

Fabian & Buskirk, 1935). Storage of sap or concentrate at a lower temperature such as 4°C can be a good way to prevent the growth of slimy bacteria and the production of ropy syrup.

Monosaccharide composition of ropy maple syrups

The polysaccharide (PS) composition of three ropy maple syrups samples retrieved from producers was carried out and each sample contained several PS of different molecular weights (results not shown). Sample #1 showed seven chromatographic peaks with molecular weights ranging from <1000 Da up to more than 800000 Da, suggesting that seven different PS were present in this syrup sample. Sample #2 and #3 showed four and eight chromatographic peaks respectively. This analysis showed that a large variety of PS were present in ropy syrup and each of them could be produced by different microorganisms.

The analysis of monomeric sugars after hydrolysis of PS mix purified from each ropy maple syrup sample showed that glucose was present in each sample, with the highest percentage observed in sample #2 with 98.2%, while it represented 60.0% and 32.5% in sample #1 and #3 respectively (Figure 4). This suggests that polysaccharides of glucose such as dextrans are largely present in ropy syrup. The latter had been reported to be synthetized by lactic acid bacteria (LAB) such as *L. mesenteroides* in sucrose-based medium and in maple syrup (Han et al., 2014; Roberts, 1982; Storz, Darvill and Albersheim, 1986). Dextran was possibly the main PS present in sample #2. Other potential PS present in sample #1 could potentially be galactans and arabinogalactans due to the presence of galactose and arabi-

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nose. Arabinogalactan was previously reported in maple syrup (Adams & Bishop, 1960; Lamport, 1977) and Darvill et al. (1980) explained that PS isolated from primary cell walls of plants are similar to the composition of arabinogalactan present in maple syrup. This leads us to suspect that some (presumably a low portion) PS slimes isolated from ropy syrup can be originated from the maple tree. Sample #3 could include arabinoglucans, rhamnoglucans, and dextrans due to the presence of glucose, rhamnose and arabinose.

CONCLUSION

Slimy PS isolated from maple syrup may be composed of various chemical structures. Some are EPS produced by various bacteria naturally found in the vicinity of the sugarbush such as *L. mesenteroides* or *Enterobacteriaceae*, others could possibly derive from the tree cells. Further chemical analysis on each PS purified would permit to confirm their identification and characterization of slime produced by *L. mesenteroides* and *Enterobacteriaceae* isolates and could provide information on the specific production of EPS by each isolate. Otherwise, the probability to produce a ropy maple syrup increases with the increase of temperature and storage time. A proper handling of sap and concentrate is essential, especially when temperature rises as the sugar season progresses. Controlled storage temperature and cleanliness of sap collection and storage equipment will help prevent ropy syrup production.

Our research hypothesis was that the production of EPS by bacteria in the sap was responsible for the highly viscous texture of the syrup. Some EPS are known and used in other applications in the food industry as texture modifiers or thickeners such as dextran. Such an approach could possibly allow the valorization of this type of non-compliant maple syrup.

ACKNOWLEDGEMENTS

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