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Dextran synthesis by native sugarcane microorganisms

Síntesis de dextrano por microorganismos nativos de caña de azúcar

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Abstract

The sugarcane agri-food industry boosts the Mexican economy in producing regions. Basing its relevance in sugar production yields. However, by-products are not widely exploited leaving an opportunity for diversification. In this study, three microorganism isolates (A, B, and C) were obtained from sugarcane kefir; the morphology of isolates B and C corresponded to the lactic acid bacterial genus *Leuconostoc*. Thus, we examined the potential for these isolates to produce EPSs, like dextran, a molecule with applications in pharmaceuticals, industrials, and foods. The experiment was performed adjusting the active culture concentration to 1 $\times 10^6$ colony-forming units (CFU)/ml, the culture was maintained at 37°C in agitation at 150 rpm. The obtained EPSs were purified by ethanol and cold acetone precipitation. The results showed that B and C bacterial isolates had the capacity to produce EPSs (14 g/L for isolate B and 32 g/L for isolate C) after 24 h. Fourier-transform infrared spectroscopy (FT-IR) characterization indicated that the EPS was dextran. Further, the produced biopolymer had high solubility in water, avoided freezing at -4°C, and boiled at 85°C.

Keywords: biopolymers, dextransucrase, dextran, extracellular polymeric substances, sucrose.

Resumen

La industria agroalimentaria azucarera impulsa la economía mexicana en las regiones productoras. Basando su relevancia a los rendimientos de producción de azúcar. Sin embargo, los subproductos no se explotan ampliamente, lo que deja una oportunidad para la diversificación. En este trabajo, se aislaron tres microorganismos (A, B y C) a partir de tibicos de caña de azúcar; la morfología de los aislamientos B y C correspondió a la bacteria acido láctica del género *Leuconostoc*. Por lo tanto, examinamos el potencial de estos aislamientos para producir EPS, como el dextrano, una molécula con aplicaciones en productos farmacéuticos, industriales y alimenticios. El experimento se realizó ajustando la concentración del cultivo a 1 $\times 10^6$ unidades formadores de colonias (UFC)/ml, el cultivo se mantuvo a 37 ° C en agitación a 150 rpm. Los EPS obtenidos se purificaron por precipitación con etanol y acetona fría. Los resultados mostraron que los aislamientos B y C tenían la capacidad de producir EPS (14 g / L para el aislamiento B y 32 g / L para el aislamiento C) después de 24 h. La caracterización por espectroscopía infrarroja por transformada de Fourier (FT-IR) indicó que el EPS era dextrano. Además, el biopolímero producido tenía una alta solubilidad en agua, evitaba la congelación a -4 ° C y hervía a 85 °C.

Palabras clave: biopolímeros, dextransacarasa, dextrano, exopolisacáridos, sacarosa .

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1 Introduction

Agricultural industries that generate basic products are particularly important for the economy. However, in recent years international competitiveness and market price diminution has spurred the exploration of value chain opportunities to increase profitability and sustainability. In this scenario, the African sugarcane industry has been exploring biopolymer production as an alternative aiming for product diversification to increase competitivity and profitability (Higgins *et al*., 2007; Thomson *et al*., 2017). The sugarcane agri-industry presents a great opportunity for economic and industrial development in many sugarcane-producing countries. However, this development can only be achieved if there is a multidisciplinary approach, including economic, environmental, agronomical, engineering, financial, and strategical goals that include variables in an integrative, qualitative, and quantitative way (Pippo and Luengo 2013; Aguilar-Rivera 2017). Sugarcane cultivation produced a remarkable amount of product during the last decade. In 2010, more than 1,685,000 tons were processed, which generated 23,600,000 tons of bagasse (dry basis) (Pippo and Luengo 2013). During the 2018/2019 zafra in Mexico, the sugar mills in the country received 56,990,367 tons of sugarcane and produced 6,898,253 tons of sugar; there was a 2,358,513-ton sugar surplus (CONADESUCA 2019). The characteristics of agri-industrial residues and biomass generated during sugar production can be transformed into value-added products by applications of suitable chemical and biotechnological strategies (Solomon 2011; Iqbal *et al*., 2013) and improve production reducing costs being this techniques economically advantageous in relation to plant EPS extraction (de Melo Pereiraa *et al*., 2019).

In Mexico, the sugar industry has great production potential due to its economic and nutritional contribution, but in recent years there have been serious operational and profitability problems (Anaya-Reza 2017). Being the main problem that the Mexican sugarcane industry has been focusing his profitability only by measuring production yields (Aguilar-Rivera, 2019). On the other hand, post-harvest sugarcane deterioration causes sucrose loss mainly by sucrose hydrolysis from endogenous invertase as well as dextran synthesis (Cuervo-Mulet *et al*., 2010) from *Streptococcus*, *Lactobacillus*, and *Leuconostoc* species (Bhatia *et al*., 2009; Naessens *et al*., 2005; Santos *et al*., 2020). In the sugar mill process, a bacterial complex that contains yeast, acetic acid bacteria, and lactic acid bacteria (LAB), including *Leuconostoc* spp., is responsible for dextran formation from sucrose spoilage (Davidovic *et al*., 2015; Hemme and Foucaud-Scheunemann 2004). The polymerization process is catalyzed by dextransucrase, which is produced by *Leuconostoc* spp. This enzyme uses sucrose as a substrate and breaks the bond between glucose and fructose to generate dextran and fructose. Dextran is one of the earliest EPS industrially produced (Selvi *et al*., 2019), formed by consecutive glucose long-chained bonded molecules (Heerden *et al*., 2014; Rani *et al*., 2017; Oropeza-De la Rosa *et al*., 2019). However, dextran produced by *Leuconostoc* spp. has received more attention due to his multitude of functions and potential commercial-scale production (Naessens *et al*., 2005; Selvi *et al*., 2019). Dextran obtained from *Leuconostoc* spp. is crucial in the edible film industry; Davidovich *et al* (2018) made an edible film with a mixture of dextran and sorbitol. Normally, dextran produces massive problems in the sugar extraction process (Cuervo-Mulet *et al*., 2010). However, there may be an opportunity to develop a process for dextran production aiming to sugarcane industry diversification (Sentíes-Herrera *et al*., 2017), since it has diverse industrial applications in the pharmaceutical industry used as drug delivery carriers for therapeutic drugs and emerging uses in the food and textile industries (Moosavi-Nasab *et al*., 2010; He *et al*., 2014; Sharmila *et al*., 2020). The aim of the present study was to study dextran production using *Leuconostoc* spp. strains isolated from kefir as an inoculum and sucrose as a substrate.

2 Materials and methods

2.1 Microorganisms

Bacterial strains were isolated from kefir obtained from the sugar extraction process in the San Jose de Abajo sugar mill. The obtained samples were grown using panela as the carbon source; cultures were maintained for 24 h at 25°C without shaking. Ten μL samples were inoculated for 24 h on Mayeux, Sandine & Elliker (MSE) agar plates that contained (in g L^{-1}): tryptone (10; BD BACTO^{TM}), yeast extract (5; BD Bioxon^{TM}), sucrose (100; BD Bioxon^{TM}), glucose (5; MAYER^{TM}), sodium citrate (1; JT Baker^{TM}), sodium

azide (0.075; QUANTYKA^{TM}) (Cuervo-Mulet *et al*., 2010), and agar (15; BD Bioxon^{TM}) (Cuervo-Mulet *et al*., 2010; Nieto-Arribas *et al*., 2010).

2.1.1 Strain purification

Bacterial strains were inoculated on MSE plates using the Drigalski procedure (Hedderich *et al*., 2011) and maintained at 37°C for 24 h to obtain pure cultures. Mucoid colonies that grew on MSE agar (Sarwat *et al*., 2008) and showed similar structural morphology to *Leuconostoc* mesenteroides (Gram-positive staining) were selected for dextran production by submerged culture.

2.1.2 Strain preservation

For bacterial preservation, the isolates were inoculated on De Man, Rogosa & Sharpe (MRS) agar plates and incubated for 24 h at 37°C (Davidovic *et al*., 2015; Paulo *et al*., 2012; Han *et al*., 2014). Biomass produced on MRS agar plates was harvested and inoculated in 250 mL Erlenmeyer flasks that contained MRS liquid medium (without sucrose) for biomass augmentation. The culture was maintained at 37°C for 24 h at 150 rpm. Biomass was recuperated by centrifugation at 15,000 rpm for 15 min. The pellet was suspended in sterile water and washed twice with water. Plastic spheres were immersed in the resuspended biomass-to allow the microorganisms to adhere to the spherical surface- and were placed in cryovial tubes with sterile water and maintained at 4°C (Rojas-Tapia *et al*., 2013; Holzapfel *et al*., 2015). Preserved strains were used for extracellular polymeric substance (EPS) production. After reactivation on MRS agar plates for 24 h at 37°C, activated strains were inoculated in 250 mL Erlenmeyer flasks that contained 50 mL MRS liquid medium and maintained at 37°C for 24 h at 150 rpm. The produced biomass was considered as inoculum (Nieto-Arribas *et al*., 2010).

2.2 Strains characterization

Bacterial strains were characterized according to their morphological, biochemical, and physiological features (Aman *et al*., 2012). Strain characterization was performed with Gram staining and catalase and oxidase tests (Nair *et al*., 2005). For physiological tests, the strains were evaluated for their ability to produce exopolysaccharides in MRS liquid medium Leemhuis *et al*., 2013).

2.3 Dextran production by submerged culture

Dextran production was accomplished by submerged culture using a 250-mL Erlenmeyer flask that contained 200 mL MSE liquid medium. Dextran production was realized by adjusting the inoculum concentration to 1 $\times 10^6$ colony-forming units (CFU) mL^{-1} (Srinivas *et al*., 2014), followed by incubation at 150 rpm on an IKATM KS260 orbital shaker for 24 h at 37°C.

2.3.1 Sugar determination

Total carbohydrate levels were determined by the phenol-sulfuric method (Dubois *et al*., 1956), and reducing sugars were analyzed with the 3,5-dinitrosalicylic acid method (DNS) (Miller *et al*., 1959; glucose (MEYERTM) was used as standard in both analyses. Reducing and total sugars were analyzed at initial and final submerged culture. Additionally, after purification, dextran was quantified using the Dubois method (1956).

2.3.2 Dextran purification and characterization

Dextran was recovered from the culture medium by separating biomass and insoluble matter by centrifugation at 15,000 rpm for 15 min at 4°C (SAVANT^{TM} SC210A) to obtain a clear supernatant. Dextran was precipitated from the supernatant using a 2:1 ratio of chilled ethanol (96%) to culture broth. The mixture was maintained at 4°C, and dextran was recuperated by centrifugation at 15,000 rpm at 4°C for 15 min. Dextran was precipitated by redissolving in sterile water (10 mL) twice to ensure that there was no remaining fructose or sucrose. The dextran was treated three times with 30 mL chilled acetone, precipitated with chilled ethanol, and maintained at 4°C at all steps. The mixture was centrifuged at 15,000 rpm for 15 min at 4°C; finally, it was dried at 40°C for 18 h (Sarwat *et al*., 2008; Vettori *et al*., 2012). Dextran solubilization was analyzed by adding 1 g purified dextran in 100 mL distilled water; mixtures were shaken at 150 rpm in an orbital shaker at 25°C. The dissolution time was measured as the point at which there were no visible solids in suspension (Paulo *et al*., 2012).

2.3.3 Fourier-Transform Infrared spectroscopy analysis

The FTIR spectra for the native dextran from *Leuconostoc* spp. isolated from cane juice were

generated using a Bruker^{TM} Infrared Spectrometer Vertex 70 (with a resolution of 3 cm^{-1}) in the attenuated total reflection (ATR) sampling mode. A diamond crystal with a single reflection was used; the transmission percentage values of this kind of plate are greater than 25. The measurement region was in the middle of the infrared spectrum, from 400 to 4000 cm^{-1}. The sampling mode was equipped with the OPUS^{TM} program for data acquisition. For spectra analysis, Origin^{TM} 6.1 program (OriginLab Corporation, USA) was used (Lenshin *et al*., 2011). Commercial dextran of ~200,000 daltons (Sigma-Aldrich®) produced by *L. mesenteroides* was used as a control.

3 Results and discussion

3.1 Strain isolation and purification

Three bacterial strains were isolated from sugarcane kefir; they were identified as A, B, and C. Gram staining results indicated that there were strains that corresponded to the morphological characteristics of *Leuconostoc* in plates B and C, as described by Hemme and Foucaud-Scheunemann (2004) and Davidovic *et al*. (2015) *Leuconostoc* are Gram-positive bacteria with short coccobacilli morphology grouped in pairs or short chains and are negative for the catalase test. These characteristics were consistent with the results obtained in the analyzed isolates (Table 1). The strains exhibited a high survival capacity in MRS agar. According to previous works, MRS agar was used as the standard for cellular development (Davidovich *et al*., 2015; Paulo *et al*., 2012).

3.2 Strains preservation and viability

The isolated strains were preserved in sterile water using plastic spheres. Strain viability was examined by adding a plastic sphere of each preserved strain in MRS agar. Strains remained active for 9 months, like the findings from Holzapfel *et al*. (2015), they remained active after the experimental stage of this research. This preservation technique was ideal to maintain these microorganisms in stock for long time periods while preserving high cellular activity and the biochemistry and metabolic behavior of the isolated strains. This finding is like that reported by Juven (1979) where a pumice stone was used as the inert preservative substance.

3.3 Submerged culture for EPS production

For isolates B and C, sucrose content in submerged culture decreased 55% (w/v) after 24-h culture. Notably, sucrose consumption by *Leuconostoc* spp. may be used for different purposes because all species within this genus are heterofermentative (Bailey and Oxford 1958). Figure 1 shows the possibilities of different carbon source use under different conditions. Using sucrose as a unique carbon source, *Leuconostoc* spp. may produce dextran as a principal product from its metabolism, and fructose would be the second possible compound generated. In this study, strains B and C produced 14 and 32 g/L dextran, respectively, when using sucrose in submerged culture. This result is superior to the maximum dextran concentration (18.46 g/L) reported by Davidovic *et al*. (2015) and by Long *et al*., (2019) (18.29 g/L), similar to the maximum dextran reported (32.15 g/L) by Han *et al*. (2014) and lower than the dextran produced (35.8 g/L) by Ye *et al*., (2019). Notably, the obtained dextran concentration in submerged culture by the *Leuconostoc* strains B and C were obtained using culture medium without optimization. In other words, the results reflect a high capacity of dextran synthesis even under non-ideal conditions. On the other hand, reducing the sugar concentration in *Leuconostoc* isolates B and C submerged cultures decreased the dextran content from 33.5 to 18% (w/v). This phenomenon is possibly mediated by the glucose produced by sucrose hydrolysis or from the activity of dextransucrase (Han *et al*., 2014).

Table 1.- Bacterial isolates characterization; +: positive reaction, -: negative reaction.

Characteristics	Isolate		
	A	2	D2
Gram stain	+	+	+
Cellular shape	Coccobacilli	Coccobacilli	Coccobacilli
Catalase	-	-	-

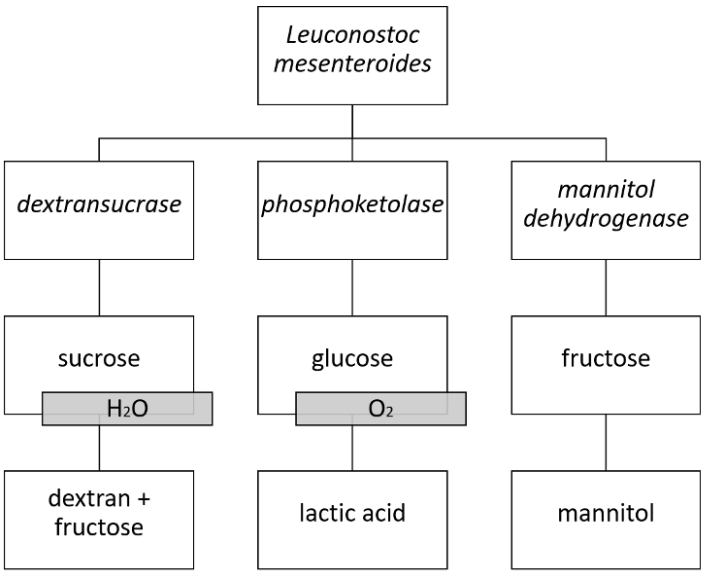

Figure 1. Carbon possible pathways of *Leuconostoc mesenteroides*.

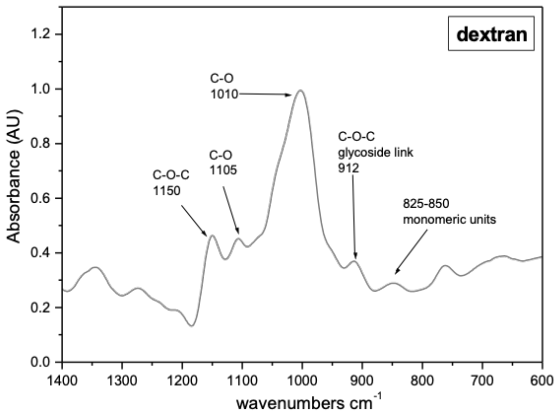

Figure 2. Infrared spectrum of dextran.

Even when other sub-products were not quantified, it is reported that fructose might be converted into other metabolites like mannitol by mannitol dehydrogenase. Non-polymerized glucose fractions can be used as a substrate for lactic acid production by phosphoketolase; mannitol and lactic acid are the main sub-products obtained (González-Leos *et al*., 2020), but CO_2 and acetic acid can also be produced (Heerden *et al*., 2014).

3.4 Fourier-Transform Infrared spectroscopy analysis

Purified EPS samples were used for FT-IR analysis to corroborate that the EPS product was dextran. This method identified the main characteristic dextran structure. Figure 2 shows the dextran standard spectrum in the 1400-600 cm^{-1} region. The spectrum bands represent all the functional groups that constitute this polysaccharide, all of which contribute their functional groups (-C-O-C, -C-O). The major dextran structural groups were detected. The main absorption bands that characterize dextran α (1 $\rightarrow$ 6) exopolysaccharide were found in the region of 1150 cm^{-1}; there are related to the vibrations of the link glycoside C-O-C. The broad peak at 1105 cm^{-1} is relevant to the vibration of the C-O bond at the C-4 position of a glucose residue. The presence of an absorption peak at 1010 cm^{-1} reflects the great flexibility of the dextran chain described at 912 cm^{-1} and indicates the existence of this glycoside link in the alpha (α) conformation (Shingel 2002).

Figure 3 show that the isolate B infrared spectrum was like the C spectrum. Specifically, the line shape was remarkably similar around 600-1400 cm^{-1}, and only slight band shifts were detected. FTIR analysis confirmed the dextran functional groups, and thus the EPS synthesized by strains B and C matched the dextran spectrum characteristic of a polysaccharide. The FTIR spectra of the dextran synthesized by strains B and C was similar to the one obtained by Davidovic *et al*., (2015), Han *et al*. (2014), and Paulo *et al*. (2012), data that indicate a similar structure. Taken together, FTIR analysis confirms that the polysaccharide synthesized in sucrose medium was dextran.

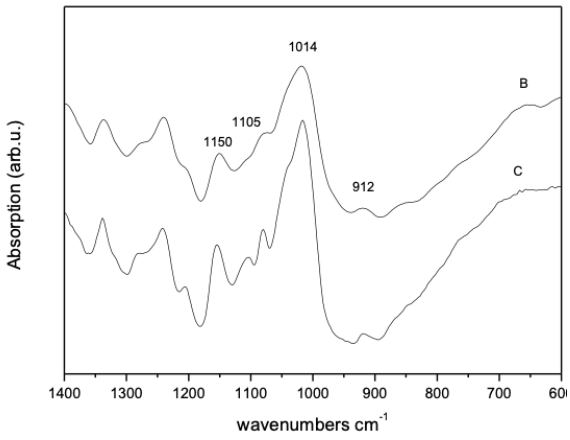

Figure 3. Infrared spectrum of the exopolysaccharides produced by strains B and C.

3.5 Dextran characterization

After purification, dextran produced by *Leuconostoc* isolates B and C showed the capability to avoid freezing. Previous data reported that dextran can prevent crystallization (Yildiz and Karatas, 2018). Heated pure dextran from both isolates boiled at 85°C; this boiling point is reported in the technical specifications for commercial dextran (Guide Chem 2019). Dextran solubilization from isolates B and C indicated total dissolution in distilled water at 25°C after 1 h 17 min; dextran solubility is related to branching degree (Paulo *et al*., 2012). Overall, the characteristics of the dextran produced by *Leuconostoc* isolates B and C indicate potential interest and use for industrial applications.

Conclusions

The isolated strains were biochemically and morphologically related to the *Leuconostoc* genus. Strain preservation in sterilized distilled water allowed good cellular stability and viability; the strains could be reactivated from preservation to produce EPS. Strains B and C exhibited the capacity to produce dextran. This finding opens an opportunity for sugarcane agri-industry diversification.

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