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Use of Leuconostoc Mesenteroides to Produce a Dextran Bioflocculant

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In this study, we aimed to determine the in vitro activity of *Leuconostoc mesenteroides var. mesenteroides* isolated from sugar-industry effluents to produce a dextran bioflocculant from sucrose as a low-cost substrate.

L. mesenteroides strains present in residual cane juice from a sugar factory were isolated and biochemically identified using Mayeux, Sandine, and Elliker agar (MSE) as a selective medium. The strain number 3 (LM03) was biochemically identified as *L. mesenteroides var. mesenteroides,* which was used for this study. The concentration of dextran was quantified by dry weight, the morphology and purity were evaluated using Fourier-transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDS). Flocculation was evaluated via turbidimetric assays in different pH ranges from sugar-industry effluents and doses of dextran.

To evaluate the flocculant activity according to the effect of pH, a jar test kit from Phipps and Bird, USA, was used with the sample recollected from the effluent (sugar industry). The pH of the samples was adjusted to 7, 8, 9, 10 and 11, with a dose of 40 ppm (dextran dose) at a fast and slow speed of 150 and 50 rpm, respectively. To evaluate the influence of the dose of dextran, values of 5, 20 and 40 ppm were used with fast speeds of 180–150 rpm and slow speeds of 30–50 rpm, respectively.

The strain (LM03) was able to produce the highest concentration of dextran (26.87 g/L) in 76 h of incubation. The presence of dextran was identified in the MSE agar after incubation and characterized by FTIR, SEM, and EDS. Besides that, we observed that the best flocculation activity was observed at a pH of 9 and a concentration of 40 ppm of dextran, with a fast agitation speed of 150 rpm for 5 min and a slow agitation speed of 50 rpm for 15 min, achieving 77.7% removal of turbidity from the sugar factory effluent.

L. mesenteroides was responsible for the bioflocculation of dextran in different sugar-industry effluents.

Keywords: residual cane, bioflocculant, *Leuconostoc mesenteroides*, dextran, sucrose, sugar factory.

Introduction

Industrial effluents treatment requires removal of suspended solids for purification and possible reuse. The removal of solids can be achieved by gravitation (a very slow process), coagulation (charge-dependent process) and flocculation (non-charge dependent, but a faster process) (Sashidhar et al., 2015). Wastewater can contain two types of solids, those that settle and those of the colloid type (that do not settle due to gravity). These suspended particles are a major concern worldwide since they affect water quality in terms of its clarity, photosynthesis and an inadequate oxygen environment, making the water unsuitable for wildlife (Wei et al., 2018; Kinyua et al., 2016).

The particles contained in colloidal solids which are stabilized by equal charges on their surface, prevent them from colliding with each other and forming larger masses, called flocs. To colloid destabilization, methodologies such as coagulation and flocculation are necessary, which generally require the application of chemical agents and mixing techniques (Wei et al., 2018). Coagulation and flocculation are processes that normally require the use of inorganic salts and synthetic flocculants. However, the incorporation of these additives has a trade-off of increasing the metal concentration in the effluent and producing chemical decomposition which can potentially produce neurotoxic substances. For instance, flocculants such as aluminium sulphate, ferric chloride and polyacrylamide, are widely used for their effectiveness and low price, but their biodegradation is difficult and even if they degrade they can produce carcinogenic monomers (Vasilieva et al., 2019; Sudha et al., 2017).

Metal-based additives have showed a negative long-term effect on human health; for example, the presence of aluminium in the brain has been associated with senile dementia (Mustapha et al., 2017). In addition, human exposure to aluminium is also linked to the production of encephalopathies, dementia, and neurological disorders (Alexandrov et al., 2018). In recent years, interest has increased in producing new bioflocculants as an alternative to synthetic flocculants, which are harmful to health (Salehizadeh et al., 2018). The main benefits of using bioflocculants lie in produced high molecular weight polymers with stronger flocculant activities, being non-toxic to the environment, and an ecological treatment technology (Zhao et al., 2016; Zhao et al., 2017).

Dextran is a molecule that has gained greater interest within bioflocculants. It is a complex unit of a branched polysaccharide polymer chain of various lengths, from 1000 to 2 000 000 Da (Predescu et al., 2018). Dextran has a rubbery or muciform consistency and is made up of at least 50% of glucose units linked by α-1.6 bonds, with branches linked at α-1.3 although it can present other linked α-1.2 or α-1.4 (Li et al., 2019). In addition, it is naturally formed in the production of cane sugar (*Saccharum officinarum L.*) where a great variety of microorganisms are found, such as bacteria that ferment sucrose and produce lactic acid, called lactic acid bacteria (LAB) (Zafar et al., 2018). LAB such as the genera *Leuconostoc, Lactobacillus, Streptococcus* and *Weissella* species mainly produce dextrans; however, these are different in the type of glycosidic bond, the degree and type of branching, chain size, molecular weight and shape of the polymer chain. L. *mesenteroids* has the gene that translates enzymes such as invertase and dextransucrase, which are responsible for transforming sucrose into glucose and fructose; and consequently, forming dextran (Lim et al., 2018; Rizzello et al., 2019).

Bioflocculants can be obtained from the extracellular components of the microbial metabolism, comprising biopolymers that include proteins, carbohydrates and lipids (Salehizadeh et al., 2018). Its application has considerable potential both for conventional water purification, as well as for biodegraded sludge in agriculture and effluent treatment; furthermore, they can be applied in subsequent processes of the fermentation industries (Xu et al., 2017; Okaiyeto et al., 2016). Villota et al. (2018) showed that 3 species of bacteria (*Pseudomonas luteola, Bacillus coagulans* and *Bacillus amyloliquefaciens*) presented bioflocculating activity with values of 67%, 60.5% and 41%, respectively, to which no adjuvant was applied. For this reason, the objective of this research was to produce a dextran bioflocculant by *Leuconostoc mesenteroides* from sucrose in order to use it as a low-cost substrate and evaluate its in vitro flocculant activity in effluents from the Azucarera Cartavio S.A.A. industry.

Materials and Methods

Samples and isolation of L. mesenteroides var. mesenteroides

Residual cane stalks with the presence of a gummy substances were collected. These samples were processed in Casa Grande Agricultural Company's Raw Material Quality Control Laboratory (Casa Grande, Peru).

An aliquot of 1 mL of residual sugar cane juice was taken and serial dilutions were made until reaching the dilution of 10^5; then, 100 μL of the last dilution was taken and inoculated by duplicate using the surface seeding technique and streak on MSE agar incubated at 30°C for 48 h at pH 6.7 (Zafar et al., 2018). Four bacterial strains were selected according to the highest growth rate, gum production, circular colonies, smooth surface, translucent, cream colour and viscous consistency; seeding each isolated colony in MSE broth at 30°C for 48 h at pH 7.2 and kept under refrigeration (Caro, 2013).

The biochemical identification was performed according to Bergey's Manual of Systematic Bacteriology.

Production of dextran by L. mesenteroides var. mesenteroides

Inoculum and fermentative process

To obtain the inoculum, the Caro's method was used. Mayeux broth (900 mL) with sucrose (100 g/L) and 100 mL (10% v/v) of the bacterial inoculum (1.6×10^8) was added in an aerated stirred bioreactor (Applikon) (Fuentes et al., 2013). This bioreactor was hermetically sealed, followed by the process of fermentation at a pH of 7.0 ± 0.2 at 30°C under the following conditions: 0.5 volume of air/unit of medium per min, and 200 rpm for 76 h under conditions of sterile aeration (Pinchi, 2017).

Quantification of dextran

For the obtaining and determination of dextran, Pinchi's method was used (Pinchi, 2017).

Dextran identification and morphological analysis

The bioflocculant was analyzed using a Fourier-transform infrared spectrophotometer (FTIR; Nicolet iS50 Thermo Scientific, USA) to identify the functional representative groups and determine its polymeric nature. The absorbance spectra were recorded at a speed of 16 scans and a resolution of 1 cm^{-1}. The polymer was dried before use in an oven (Mememrt) at 105°C for 1 h to avoid interference from moisture (Dlangamandla et al., 2016). Then, microscopy and energy dispersive X-ray spectroscopy (EDS) analysis were performed on the bioflocculant produced by *L. mesenteroides var. mesenteroides* using a Tecsan VEGA 3 LM equipped with a gold-coated SPI 11430AB system (TESCAN USA).

Determination of the flocculation activity of dextran at different pH values using physicochemical methods

Beakers were prepared with a consistent volume of the effluent. In each beaker, a dose of 40 ppm of dextran bioflocculant was added, followed by the adjustment of fast and slow homogenizations using a multiple shaker unit, to obtain similar hydraulic conditions for all the samples (Restrepo, 2009). The samples were agitated at a speed of 150 rpm for 5 min to simulate a rapid mixture. Then, the samples were subjected to a slow mix by decreasing the speed to

50 rpm for 15 min. The samples were then allowed to settle for 30 min. This procedure was performed in triplicate and with a target pH ranging from 7 to 11 (Santana et al., 2012; Dominguez 2013).

Determination of the flocculation activity of dextran at different dextran concentrations using physicochemical methods

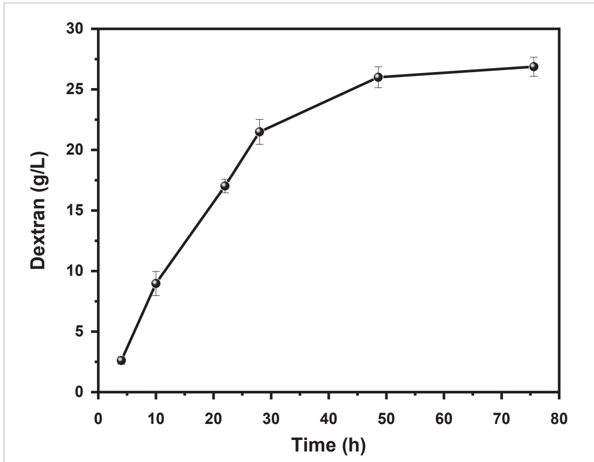
An entirely random experimental design was used with 3 repetitions. The test involved different concentrations (5, 20, and 40 ppm) of dextran as a bioflocculant in effluents from the Cartavio S.A.A. sugar factory in a range of 80–100 ppm, with variable quantities due to the turbidity of the effluent.

The Jarras test method was used (Phipps and Bird, USA) to mix the flocculant with the effluent (Cerna, 2020). Then, respective dextran concentrations were consecutively added to each test at fast agitation (150–180 rpm) for 5 min and at slow agitation (30–50 rpm) for 15 min. After this time had elapsed, agitation was halted, taking care to not break the flocs, and the solution was allowed to rest. Immediately after settling, the turbidity was measured for each test by observing the quality of the flocculant formed during sedimentation and precipitated flocs (consistency and compaction) as well as for the presence of possible flocs in the suspension. Turbidimetric analyses were performed via the nephelometric method using a turbidimeter (Thermo Scientific, Orion AQ 3010 Model) (Cerna, 2020).

Results and Discussion

The obtention of dextran was observed during a period of 76 h and it was observed (*Fig. 1*) that the maximum concentrations of 26.00 g/L and 26.87 g/L were obtained at 46 h and 76 h, respectively, using 10% sucrose in the medium. This suggests that different fermentation times influence dextran production using *L. mesenteroides var. mesenteroides*, as it was similarly reported by Pinchi (24.63 g/L after 50 h , using 10% of sucrose) and Caro (30.73 g/L after 36 h , using 10% of sucrose). The envisioned mechanism of dextran production by *L. mesenteroides* cells obeys the secretion of an inducible enzyme called dextran-amylase, which hydrolyzes sucrose from the medium and results in the release of fructose and glucose, being the glucose the molecule that is oxidized to form dextran. Furthermore, it is noticeable that the behaviour of *L. mesenteroides* was different during the stages of cellular growth because the bacterial cells inoculated in the medium require an adaptation time to the new environment which includes the synthesis of enzymes, ribosomes, and nucleic acids necessary for its growth, and start to generate energy in the form of adenosine triphosphate(ATP) during the fermentation process (Pinchi, 2017).

Fig. 1. *Dextran production (g/L) using L. mesenteroides var. mesenteroides isolated from residual cane juice*



The obtained bioflocculant was characterized by FTIR and it evidenced (*Fig. 2*) the functional groups of the biopolymer (dextran). The most intense group can be observed at 3285.13 cm^{-1}, which refers to O–H-axial stretching, while the group in the region of 2923.99 cm^{-1} refers to the C–H-axial stretching. These findings joined to the peak at 1644.25 cm^{-1} which belongs to C=C groups in the carbohydrate structure are the main characteristic peaks of gum produced by microorganisms (Vidal, 2014). In addition, a broad range of signals at 1417.18 and 1339.52 cm^{-1} belong to the C–C stretching, and the C–O angular deformation, respectively.

The polymeric species (dextran) was also confirmed by SEM and EDS, where *Fig. 3a* shows the scanning electron micrographs of the dextran bioflocculant formed by the dextran-amylase enzyme, showing a porous structure similar to that presented by Wang et al. (2014), which is an amorphous bioflocculant structure able to retain water and solid particles. In addition, *Fig. 3b* presents the EDS of the dextran bioflocculant, showing highest abundance of carbon and oxygen which is characteristic of polysaccharides species (Shukla et al., 2011).

Fig. 2. *FTIR spectrum of dextran obtained using L. mesenteroides var. mesenteroides LM03*

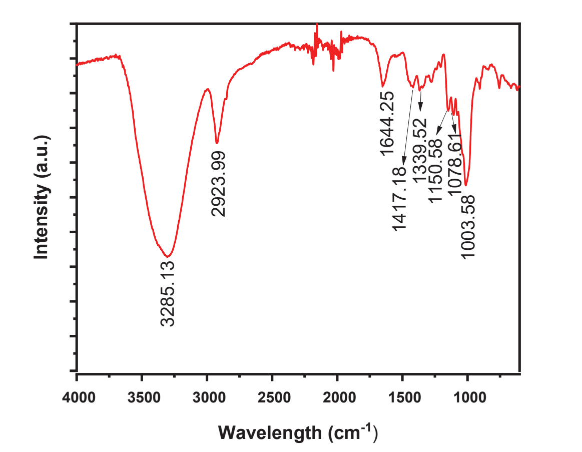
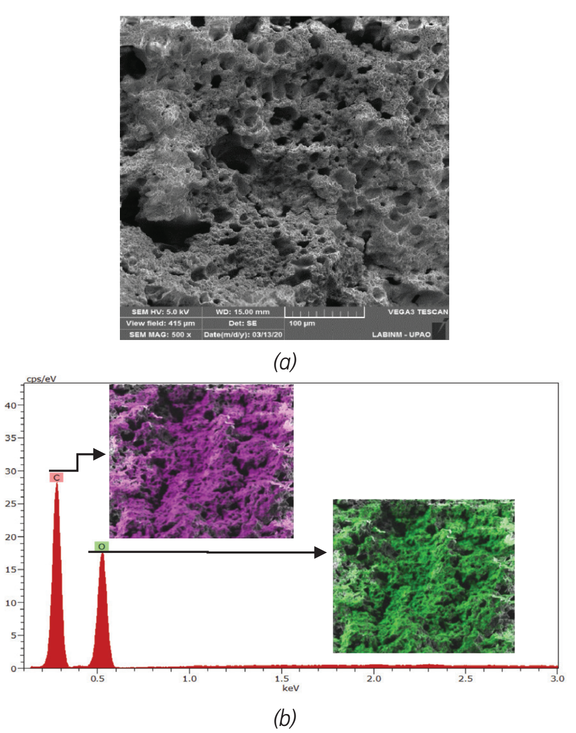
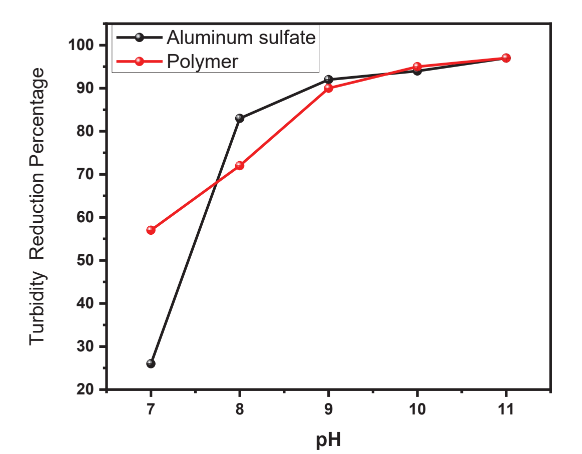


Fig. 3. *(a) SEM micrograph and (b) EDS of the dextran bioflocculant produced using L. mesenteroides var. mesenteroides*



The flocculation of the dextran was evaluated at different pH ranges, and it was found that (*Fig. 4*) as the pH increases, the percentage (%) of reduced turbidity also increases. Using a conventional oxidant salt such as aluminium sulphate at pH of 7, 8, 9, 10, and 11, the percentage of turbidity reduction was 26%, 83%, 92%, 94%, and 97%, respectively. Meanwhile, when dextran was used as a polymer at pH of 7, 8, 9, 10, and 11, the percentages were 55%, 73%, 90%, 95%, and 97%, respectively, suggesting that the values obtained for pH 7 and 8 with dextran have a great potential for the use of dextran as a biological flocculant; being able to potentially replace aluminium sulphate at a low cost and as a green resource. Besides that, a 100% turbidity reduction was achieved at an alkaline pH. The influence of pH in different bacteria was also studied by other authors such as Zhao et al. (2013) where a high flocculation activity of MBF-5 by *Klebsiella pneumoniae* in acidic pH (less than 5) was reported. In contrast, Wang et al. (2015) evaluated the flocculation activity and flocculation mechanism of bioflocculant (XMMBF) produced by *Bacillus licheniformis*, obtaining more than 92% of activity at a pH range of 5–12, with a maximum activity of 97% at a pH of 8, demonstrating that pH is a key factor influencing flocculation activity in different reaction systems (Dlangamandla et al., 2016). Because there was no significant difference between the percentage values of reduced turbidity for pH 9, 10 and 11, pH 9 was used. If the pH is increased, metallic substances may precipitate in the form of hydroxides.

Fig. 4. *Effect of pH on the reduced turbidity percentage in effluents from the Cartavio S.A.A. sugar factory using the Jarras test method*



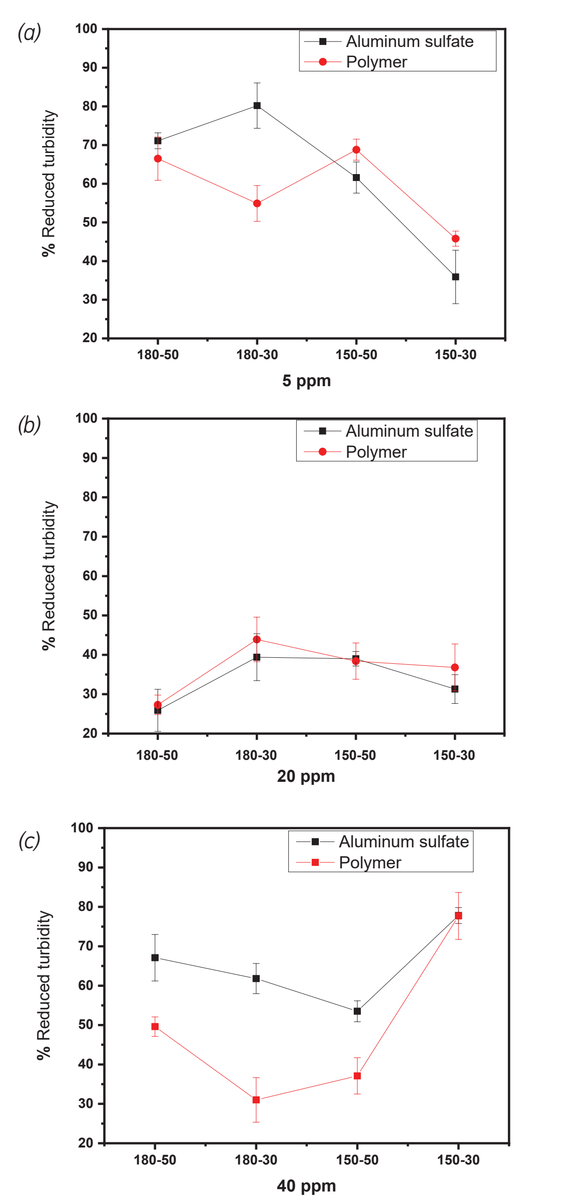
The reduction of turbidity (*Fig. 5*) was evaluated for aluminium sulphate and dextran, finding that 5 and 40 ppm of aluminium sulphate obtained a high percentage (%) of reduced turbidity of 80.2 and 77.8% at agitation speeds of 180–30 and 150–30 rpm, respectively; meanwhile, 5 and 40 ppm of the dextran flocculant obtained reduced turbidity of 68.8 and 77.7% at agitation speeds of 150–50 rpm and 150–30 rpm, respectively. These results are in agreement with those of Villota (2018), who found an increase in the flocculation activity when different bacterial species, such as *Bacillus velescensis, P. luteola* and *B. amloliquefaciens, B. coagulans, Psuedomonas sp.* and *P. aeruginosa*, were used, with activities of 98%, 96%, 92%, 90%, and 81%, respectively. In addition, our results suggest an increase of the flocculant activity at high concentrations (40 ppm), which was similarly observed by Wang et al. (2015) who evaluated the effect of bioflocculant concentration (CBF-F26 in a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6) in a range of 0–40 mg/L and found that the flocculation activity was higher than 90% in the 8–24 mg/L range, with maximum activity at 12 mg/L (96.21% ± 1.19%).

There are bacteria that can produce different types of biopolymers by consuming simple or complex substrates to produce dextran; once formed, they locate themselves intra- or extracellularly. The C, N, and P sources are of vital importance for the production of bioflocculants as they interfere in the composition of the floc matrix as well as its structure (Zhao, 2013). Therefore, different dextran concentrations show turbidity; however, the highest dose in this study was 40 ppm, which could be due to factors such as nutrients and the bacterial growth phase and could be the most adequate condition for dextran production.

In recent years, many studies have reported that chemical as well as natural polymeric flocculants demonstrate excellent flocculation yield; however, chemical polymers are non-biodegradable and toxic, whereas natural flocculants, such as dextran, have active groups that biodegrade easily over time (Lee et al., 2014; Li et al., 2018). The wide variety of molecular weights and low probability of degradation in slightly acidic and alkaline conditions suggest that dextran is the ideal candidate for the treatment of wastewater (Li et al., 2016), although large doses of polymers are needed due to its moderate flocculation effectiveness and short lifespan (Kyzas et al., 2014).

Various investigations show that biopolymers perform remediation via chelation, reduction, precipitation, or ion exchange of metals. A practical case is the extraction of heavy metals in the treatment of effluents by the exopolysaccharide of the bacterium *Zooglea ramigera* (Bramucci and Nagarajan, 2000).

Fig. 5. *Reduced turbidity percentage at (a) 5, (b) 20, and (c) 40 ppm using aluminium sulphate and dextran evaluated in vitro in sugar factory industry effluents*



Conclusions

We were able to successfully demonstrate that the polymer produced by *L. mesenteroides var. mesenteroides* was dextran via FTIR and the nature of the porous nature of the polymer by SEM. Furthermore, we were able to prove that time influences the dextran generation by *L. mesenteroides var. mesenteroides*, attaining a maximum value of 26.87 g/L at 76 h. Additionally, pH affects dextran activity, being able to produce a highest flocculant effect at pH of 9.The maximum dextran dose as a bioflocculant was reached at 40 ppm at a fast agitation speed of 150 rpm for 5 min and a slow agitation speed of 50 rpm for 15 min, reducing turbidity by 77.7% from a volume of 600 mL of effluent from the Cartavio S.A.A. sugar factory.

This polymer provides a solution to the environmental problems caused by the treatment of effluents from the sugar industry. Furthermore, it is a low-cost, biodegradable product that will result in the discontinuation of chemical flocculants, which are harmful to human health.

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