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Sequential production of two biopolymers-polyhydroxyalkanoate and levan by microbial fermentation

Produção sequencial de dois biopolímeros-polihidroxialcanoato e levana por fermentação microbiana

Producción secuencial de dos biopolímeros-polihidroxialcanoato y leván por fermentación microbiana

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Abstract
The sequential production of polyhydroxyalkanoates (PHA) and levan was investigated by microbial fermentation using agro-industrial residues. PHA production was carried out by *Cupriavidus necator* DSMZ 545 in two steps: a) bacterial growth, using pure (MN) and hydrolyzed (MH) molasses and; b) accumulation of PHA, using standard glycerol (GP) and crude glycerol (GB). The fermented remaining from the C. necator growth stage was separated from the PHA-containing biomass and used in the subsequent production of levan by *Bacillus subtilis* (natto) Takahashi. The medium containing MH+GP generated a rate of 15 mg of PHA, while the medium based on MH+GB the rate of PHA was almost twice as high (28.4 mg). FTIR spectroscopic analysis of PHA indicated stretching vibrations characteristic for a PHB-like molecule. In turn, the levan produced in this study was precipitated with different volumes of ethanol, generating levans with different molecular weights. The results of the characterization of this fructan by chromatography showed that it was predominantly constituted by fructose units. In addition, the dynamic and kinematic viscosity values for levan were similar in the analyzed concentrations and, therefore, did not provide any clues about the molecular weight of this biopolymer. Finally, the sequential production process of PHA and levan, as a second bioproduct, represents an elegant alternative to reduce the total costs of PHA production.
Keywords: Polyhydroxyalkanoates; Levana; Fermentation; *Cupriavidus necator*; *Bacillus subtilis* natto.

Resumo
A produção sequencial de polihidroxialcanoatos (PHA) e levana foi investigada por fermentação microbiana usando resíduos agroindustriais. A produção de PHA foi realizada por *Cupriavidus necator* DSMZ 545 em duas etapas: a) crescimento bacteriano, usando melaço puro (MN) e hidrolisado (MH) e; b) acumulação de PHA, usando glicerol padrão (GP) e glicerol bruto (GB). O fermentado remanescente da etapa de crescimento de *C. necator* foi separado da biomassa contendo PHA e usado na produção subsequente de levana por *Bacillus subtilis* (natto) Takahashi. O meio contendo MH+GP gerou uma taxa de 15 mg de PHA, enquanto o meio à base de MH+GB a taxa de PHA foi quase duas vezes maior (28,4 mg). A análise espectroscópica por FTIR de PHA indicou vibrações de estiramentos característicos para uma molécula do tipo PHB. Por sua vez, levana produzida neste estudo foi precipitada com diferentes volumes de etanol, gerando levanas com diferentes pesos moleculares. Os resultados da caracterização desta frutana por cromatografia mostrou que a mesma era constituída predominantemente por unidades de frutose. Além disso, os valores de viscosidade dinâmica e cinemática para a levana foram semelhantes nas concentrações analisadas e, portanto, não forneceram indícios acerca do peso molecular desse biopolímero. Por fim, o processo de produção sequencial de PHA e levana, como um segundo bioproduto, representa uma alternativa elegante para reduzir os custos totais de produção do PHA.
Palavras-chave: Polihidroxialcanoatos; Levana; Fermentação; *Cupriavidus necator*; *Bacillus subtilis* natto.

Resumen
Se investigó la producción secuencial de polihidroxialcanoatos (PHA) y leván mediante fermentación microbiana utilizando residuos agroindustriales. La producción de PHA fue realizada por *Cupriavidus necator* DSMZ 545 en dos pasos: a) crecimiento bacteriano, utilizando melaza pura (MN) e hidrolizada (MH) y; b) acumulación de PHA,

utilizando glicerol estándar (GP) y glicerol crudo (GB). El resto fermentado de la etapa de crecimiento de C. necator se separó de la biomasa que contenía PHA y se usó en la producción posterior de levan por *Bacillus subtilis* (natto) Takahashi. El medio que contenía MH+GP generó una tasa de 15 mg de PHA, mientras que el medio basado en MH+GB la tasa de PHA fue casi el doble (28,4 mg). El análisis espectroscópico FTIR de PHA indicó vibraciones de estiramiento características de una molécula similar a PHB. A su vez, el leván producido en este estudio se precipitó con diferentes volúmenes de etanol, generando leván con diferentes pesos moleculares. Los resultados de la caracterización de este fructano por cromatografía mostraron que estaba predominantemente constituido por unidades de fructosa. Además, los valores de viscosidad dinámica y cinemática del leván fueron similares en las concentraciones analizadas y, por tanto, no aportaron pistas sobre el peso molecular de este biopolímero. Finalmente, el proceso de producción secuencial de PHA y leván, como segundo bioproducto, representa una alternativa elegante para reducir los costos totales de producción de PHA.
Palabras clave: Polihidroxialcanoatos; Levana; Fermentación; *Cupriavidus necator*; *Bacillus subtilis* natto.

1. Introduction

Over the past two decades, the use of petrochemical plastics has increased and become an integral part of capitalist society. The low cost and versatility of these plastics are the main characteristics for their application in various products, with plastic packaging being the main one (Albuquerque & Malafaia, 2018; Alves et al., 2022). Despite its wide application, the increased production and extensive use of these plastics have brought serious problems to today's society. For example, the excess plastic waste generated recently has been discarded and accumulated in landfills, causing irreparable environmental impacts (Geyer et al., 2017; Samui & Kanai, 2019).

In 2020, the total production capacity of petrochemical plastics was estimated at around 367 million tons (Plastics Europe, 2021). Of this total, about 9% by weight was recycled and about 12% was incinerated to generate energy. The remainder was deposited in landfills or released into natural habitats (Geyer, Jambick & Law, 2017). Among natural habitats, the oceans have annually received about 0.48 to 1.27 million tons of plastic waste, and this number could double in 10 years (Samui & Kanai, 2019).

Due to the environmental impacts caused by synthetic plastics, many polymer industries have invested in the production of bioplastics. One of the most promising groups of bioplastics are polyhydroxyalkanoates (PHA). PHA are polyesters synthesized by various bacteria as an intracellular reserve of carbon and energy, with *Cupriavidus necator* DSMZ 545 being the most productive (Albuquerque & Malafaia, 2018). Unlike synthetic plastics, PHAs can be biodegraded by microbial action (Alves et al., 2022). However, bioplastic production is still very low (about 2.42 million tons), and within this group PHA represents about 1.8% of the total production capacity (European Bioplastics, 2021).

On the other hand, levan is an unusual polysaccharide formed by fructose monomers, which are linked by β-(2,6) bonds. It is produced by some species of plants and various microorganisms, with *Bacillus subtilis* (natto) Takahashi being the bacteria that produces the highest yields (Shih et al., 2005; Shih et al., 2010; Shih et al.; 2011). Levan is synthesized from sucrose by the action of the enzyme levansucrase (EC 2.4.1.10), an enzyme that hydrolyses sucrose into glucose and fructose. The glucose generated is used in the normal metabolism of the bacteria and the fructose is used in the polymerization of levan (Öner, Hernández & Combie, 2016; Lima et al., 2020). The diversity of levan applications is illustrated by its potential use in the food, cosmetics, biomedical and pharmaceutical sectors (Öner et al., 2016; Siqueira et al., 2020; Siqueira & Öner, 2023).

In turn, the sequential biosynthesis process by microbial fermentation of one or more bioproducts represents an elegant strategy for reducing the total production costs of these biopolymers. In addition, the microorganisms used are considered a viable resource, since they enable the rapid and ecologically correct production of bioproducts. Added to this, the use of renewable resources in controlled fermentation conditions makes it possible to fully use the substrate without wasting by-products in the process. The sequential fermentation process was used Shih et al. (2010) for the production of levan and

ethanol. Shih et al. (2011) also obtained levan and poly-ε-lysine by the same process. However, to the best of our knowledge, sequential fermentation production of levan and PHA has not yet been reported.

In this sense, the objective of this work was to investigate the viability of PHA and levan production by *C. necator* DSMZ 545 and *B. subtilis* (natto) Takahashi, respectively, from the sequential fermentation process using molasses and glycerol as alternative substrates.

2. Methodology

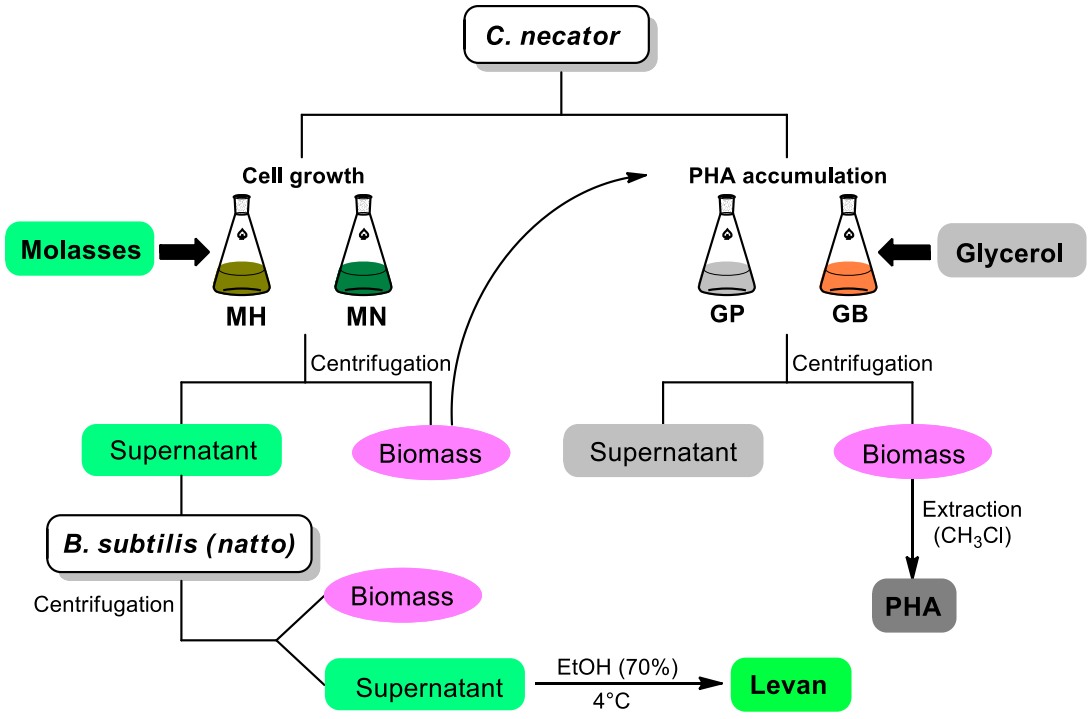
2.1 Microorganisms and culture medium reagents

The bacterial strain of *C. necator* (DSMZ 545) was obtained from the culture collection of the Department of Antibiotics at the Federal University of Pernambuco (UFPEDA 0604). *B. subtilis* (natto) Takahashi was purchased from GEM Cultures (Ft Bragg, CA, USA). The molasses used in the production of PHA was supplied by the Pernambuco company Empermel (Siqueira, & Houllou, 2022a). Residual glycerol (GB) was supplied by the Caetés Pilot Experimental Plant of the Northeast Strategic Technologies Center (CETENE), Pernambuco, generated with a yield of 99.5% during biodiesel production. All reagents and inputs used for the culture medium were analytical grade and will be described along with the experimental procedures.

2.2 Cultivation conditions

The cultivation of PHA with *C. necator* DSMZ 545 was carried out in two steps: i) bacterial growth, using pure molasses (MN) and hydrolyzed molasses (MH) (Siqueira, & Houllou, 2022b); and ii) PHA accumulation, using standard (GP) and crude (GB) glycerol, as shown in Figure 1, below:

Figure 1 - Methodological strategy for obtaining PHA and levan.



Source: Authors.

For the growth stage of *C. necator* DSMZ 545, the following medium was used (Albuquerque et al., 2018; Siqueira, & Houllou, 2022b): molasses (2% °Brix), KH_2PO_4 1.5 g/L, NH_4Fe(III) citrate 0.05 g/L; $CaCl_2.2H_2O$ 0.02 g/L; $MgSO_4.7H_2O$ 0.5 g/L; $(NH_4)_2SO_4$ 2 g/L; $Na_2HPO_4.2H_2O$ 4.5 g/L. Trace elements (1 mL/L), at a concentration of g/L: H_3BO_3 0.3; $CoCl_2.6H_2O$ 0.2; $ZnSO_4.7H_2O$ 0.1; $MnCl_2.4H_2O$ 0.03; $Na_2MoO_4.2H_2O$ 0.03; $NiCl_2.6H_2O$ 0.02; $CuSO_4.5H_2O$ 0.01. As shown in Figure 1, after centrifugation, the biomass of *C. necator* DSMZ 545 was subjected to the PHA accumulation step using GP and GB. MN and MH residues from the growth phase of *C. necator* DSMZ 545 were used in the subsequent production of levan. Cultivation was carried out under controlled temperature conditions of 35 °C, pH 7.0 and agitation at 250 rpm.

Levan production was performed by *B. subtilis* (natto) using MN and MH residues (1.2% °Brix) (Dalsasso et al., 2019) in a semichemical medium with the following composition (g/L): urea (2); yeast extract (5); KH_2PO_4 (1); K_2HPO_4 (8); $MgSO_4.7H_2O$ (1); $FeSO_4.7H_2O$ (0.10); $CuSO_4.5H_2O$ (0.0088); $MnSO_4.H_2O$ (0.0076) and $ZnSO_4.7H_2O$ (0.01) (Shih et al., 2005; Siqueira et al., 2017). Cultivation was maintained at a constant temperature of 37 °C, pH 7 and agitation at 250 rpm (Siqueira, 2019). All materials and culture media were sterilized at 121 °C for 20 min.

2.3 PHA and levan extraction

Initially, the biomass was isolated by centrifugation at 10,000 rpm at 4 °C for 20 min. The extraction of PHAs from the interior of *C. necator* DSMZ 545 cells was performed using combined chloroform and sodium hypochlorite (2:1 v/v), followed by evaporation of the solvent in an oven at 40 ºC for 24 h. The productivity of PHAs was calculated by gravimetric method for the biopolymer obtained after extraction. The percentage of accumulation was determined by the ratio between the amount of PHA produced and the amount of biomass (Albuquerque et al., 2018).

For levan isolation, the fermented broth was centrifuged (10,000 rpm, 20 min at 4°C) to remove. Then subjected to heating in a water bath (100 °C, 10 min) – for enzyme inactivation, and cooled down. The polymer was recovered by precipitation in ice-cold ethanol (70%). The generated pellet was resuspended three times in distilled water and converted into powder by lyophilization (Shih et al., 2005; Siqueira et al., 2017; Siqueira, 2019).

2.4 Characterization of biopolymers

The PHA was characterized by Fourier transform infrared spectroscopy (FTIR) in a VERTEX 70 spectrometer (Bruker Optics, USA) under dry air and room temperature (25°C). The PHA spectra obtained from pure and residual glycerol were analyzed in the wavelength range of 4000-400 cm^{-1} resolution (Albuquerque et al., 2018; Siqueira, 2019).

Levan was characterized by thin layer chromatography (TLC) and viscosimetric analysis. CCD characterization was performed according to the protocol by Dahech et al. (2013). Briefly, aqueous solutions of levan (1%) were treated with oxalic acid solutions (0.5%, 2 mL) at 100 °C and neutralized. The elution system consisted of a mixture of chloroform/acetic acid/water (6:7:1, v/v/v) and the developing solution consisted of a solution of sulfuric acid (5%, v/v) in methanol.

The levan viscosity was determined using a Stabinger Vicometer SVM 3000/G2 viscometer (Anton Paar, Graz/Austria), duly calibrated. This equipment provides viscosity measurements in a range between 0.2–20000 mPa·s for dynamic viscosity with automatic temperature control (Djuríc et al., 2017).

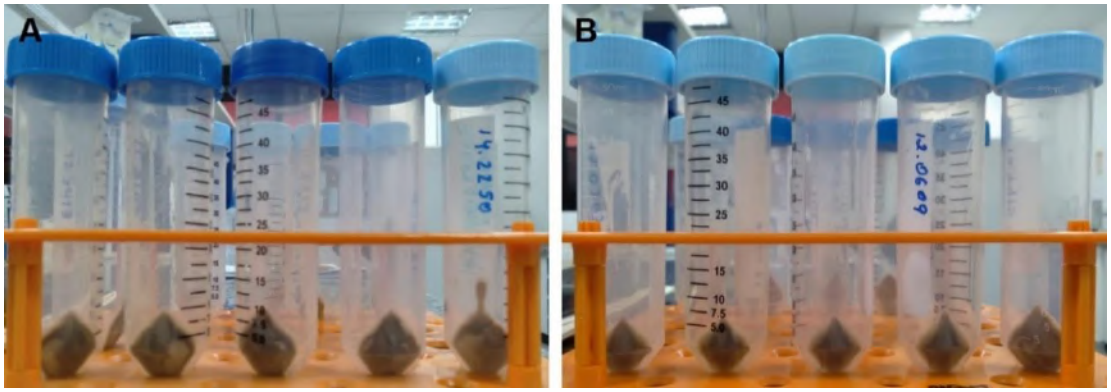
3. Results

3.1 Evaluation of PHA biosynthesis by *C. necator* DSM 545

The PHA production assays by *C. necator* DSM 545 were performed using molasses as a carbon source. Molasses is a by-product of the sugar industry rich in sucrose. In turn, *C. necator* preferentially uses glucose as a carbon source to produce PHA (Alves et al., 2022). In this sense, a comparative assay was performed to observe bacterial growth in MN and MH

(Dalsasso et al., 2019; Siqueira & Houllou, 2022b). The biomass of *C. necator* DSM 545 was higher in the medium containing MH, as shown in Figure 2, below:

Figure 2 - Biomass of C. necator grown on hydrolyzed molasses (A) and non-hydrolyzed molasses (B).



Source: Authors.

As seen in Figure 2, the wet biomass of *C. necator* DSM 545 was higher in medium containing MH (3.88 g of cells), compared to medium with MN (3.41 g of cells). Based on this result, further assays were performed using only MH in the bacterial growth step (Dalsasso et al., 2019; Siqueira, & Houllou, 2022b). The second step of the process, PHA accumulation, was carried out using GP and GB as a carbon source. In this step, phosphorus and nitrogen salts were removed to create a stressful environment for the microorganism, an important factor for PHA accumulation (Albuquerque & Malafaia, 2018).

With these results in hand, new tests were carried out with the combination of MH+GP and MH+GB substrates. The results of these tests are shown in Figure 3.

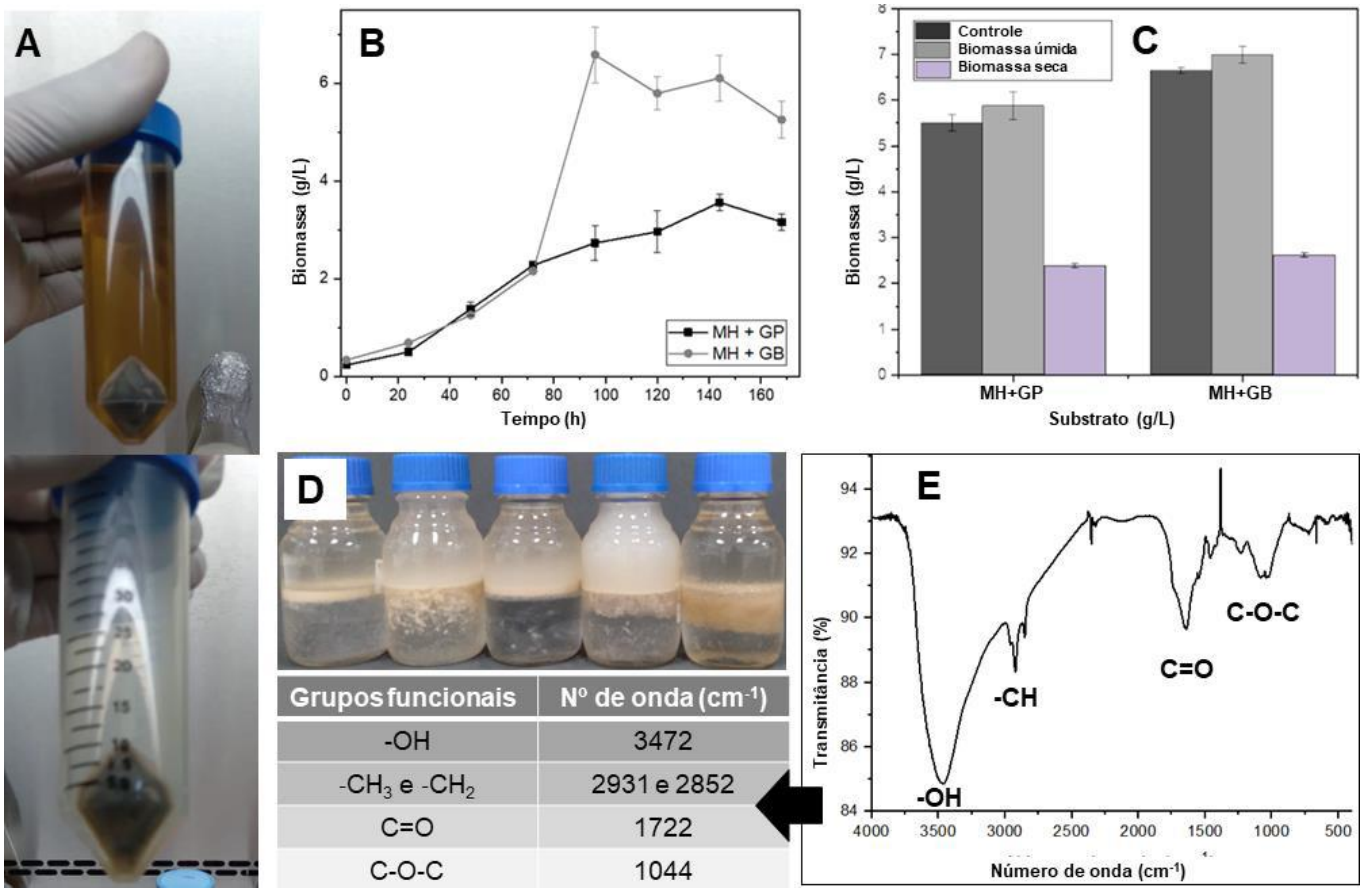
The results presented in the previous figure show that, after the centrifugation process (Figure 3.A) and the return of the biomass to the culture medium containing GP and GB, the growth rate was higher in the GB-based medium (Figure 3. B), as noted earlier (Albuquerque et al., 2018). The final results show that the wet and dry biomass generated by the combination of MH+GP were 5.87 and 2.39 g, respectively. In this condition, the PHA rate was 15 mg. In the medium containing MH+GB, the wet and dry biomass rates were 6.93 and 2.53 g, respectively; while the PHA content was 28.4 mg of PHA. These yields were obtained in the accumulation phase when the salt concentration was reduced to 10% of the total used in the growth phase. In parallel to this experiment, a medium containing MN+MN was used as a control (Dalsasso et al., 2019) (Figure 3.C).

3.2 PHA characterization

Generally, PHA extraction involves the formation of three distinct phases (Figure 3.D). A lower phase formed by PHA soluble in chloroform; an intermediate phase, formed by cell residues and; an upper phase, formed by an aqueous solution of by-products and soluble salts in it (Albuquerque et al., 2018). By decanting the chloroform, it is possible to detect the presence of PHA in this solvent (not shown here).

The PHA was characterized by FTIR (Figure 3.E) and presented a spectrum with typical bands and signals. A broad band between 3100 and 3500 cm^{-1} was observed, attributed to the -OH stretching vibration. Two peaks in the region between 2931 and 2852 cm^{-1}, attributed to the stretching vibration of $-CH_3$ and $-CH_2$, respectively. A peak at 1722 cm^{-1}, corresponding to the C=O stretching group, peak denoting the presence of the identifying carbonyl ester of polyhydroxybutyrate (PHB). Another important peak was verified at 1044 cm^{-1}, attributed to C-O-C stretching vibrations, which indicates the presence of an ester bond found in the PHB molecule (Albuquerque et al., 2018; Vega-Vidaurri et al., 2022).

Figure 3 - Results of PHA production and characterization by *C. necator*. In A, biomass resulting from the growth step using only hydrolyzed molasses (MH); in B, two-step PHA production curves, highlighting the accumulation step using pure glycerol (GP) and crude glycerol (GB); in C, final biomass (wet and dry) after fermentation; in D, extraction and isolation of PHA using chloroform and NaOH and; in D, infrared spectrum for the PHA obtained.



Grupos funcionais	Nº de onda (cm^{-1})
-OH	3472
$-CH_3$ e $-CH_2$	2931 e 2852
C=O	1722
C-O-C	1044

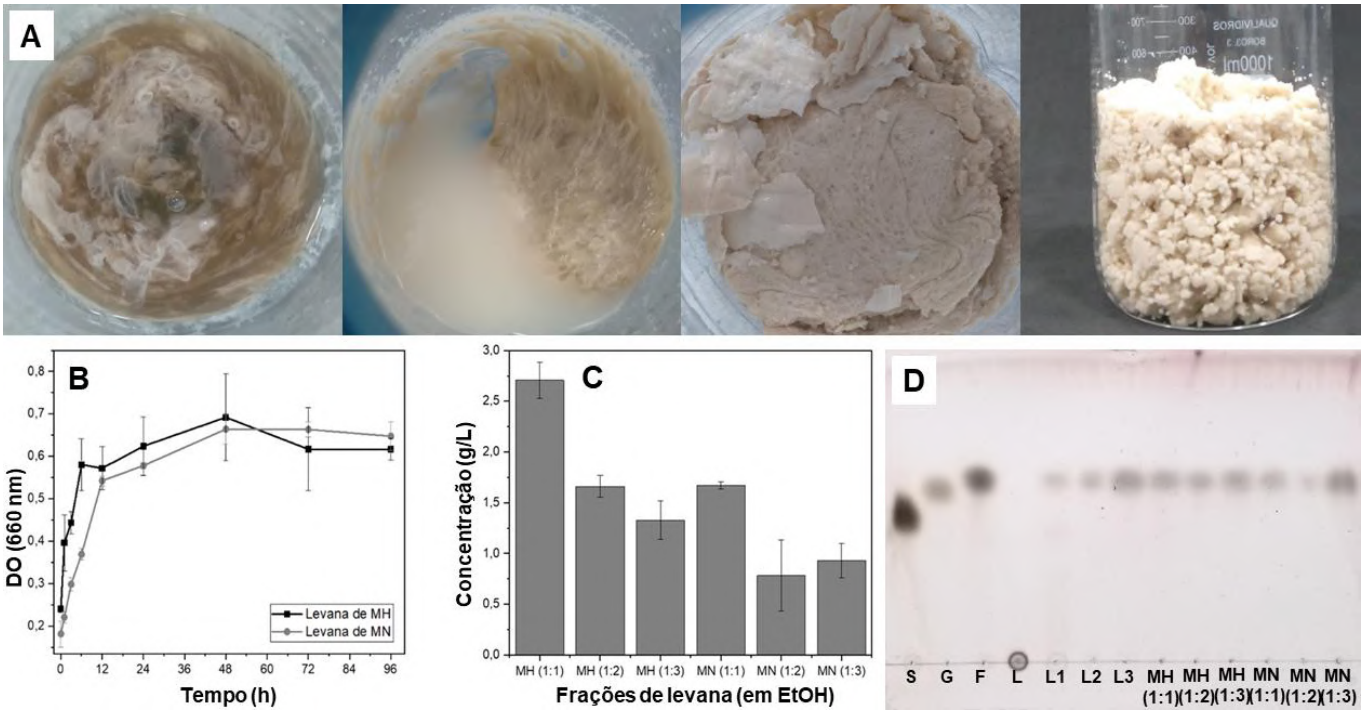
Source: Authors.

3.4 Evaluation of levan biosynthesis by *B. subtilis* (natto) Takahashi

Levan was produced by *B. subitilis* (natto) in a shaker under constant agitation at 250 rpm, temperature of 37 °C and pH below 7.0 (Siqueira, 2019). The presence of levan was confirmed by visual observation from the gradual appearance of an opalescence and an increase in the viscosity of the culture medium (Shih et al., 2005; Shih et al., 2010). Furthermore, when subjected to precipitation with 70% ethanol (4 °C), the formation of a fibrous and compact mesh was observed, which is a typical characteristic of this fructan (Siqueira & Houllou, 2022a) (Figure 4.A).

Figure 4.B shows the growth curves for *B. subtilis* (natto) Takahashi in culture media based on hydrolyzed molasses and non-hydrolyzed molasses (MH and MN). As can be seen, both the bacterial growth curve for the MH-containing medium and the MN-based medium were similar.

Figure 4 - Stages of the processes of production, purification and characterization of levan produced by *B. subtilis* (natto) Takahashi. In A, precipitation of the polymer with cold ethanol and obtaining the pellet; in B, microbial growth curves in medium based on hydrolyzed molasses (MH) and non-hydrolyzed molasses (MN); in C, levans with different molecular weights obtained by varying the ratio of the volume of the fermented broth and the precipitating solvent; in D, the TLC analysis showing the predominance of fructose monomers in the composition of the different levans obtained (Read: S, sucrose; G, glucose; F, fructose; L, standard levan; L1, L2 and L3, hydrolyzed levan in 5, 10 and 30 min, respectively MH (1:1) and MN (1:2), levan obtained from fermented broth containing hydrolyzed and non-hydrolyzed molasses, precipitated with different volumes of ethanol).



Source: Authors.

3.5 Characterization of levan

Levan fractions with different molecular weights were obtained by precipitation in ethanol, varying the relationship between the volumes of broth recovered and the solvent used in the precipitation (Shih et al., 2005; Siqueira, 2019; Lima et al., 2020; Siqueira & Houllou, 2022a). In addition to the variation in molecular weight, a variation in the concentrations of levan can be observed, mainly in the fractions in which the culture medium was MN, as in the samples MN (1:1), MN (1:2) and MN (1:3) (Siqueira & Houllou, 2022a) (Figure 4.C).

TLC analysis showed that the different fractions of levan obtained were completely hydrolyzed with 0.5% oxalic acid in 5 min (100 °C). Total hydrolysis released only fructose as monosaccharide units, which was confirmed by the same retention (Rf) of fructose used as standard (Siqueira, 2019; Siqueira & Houllou, 2022). A sample of levan was used as a control and was initially hydrolyzed at different time intervals (L1, 5 min, L2, 10 min, and L3, 30 min). This assay aimed to find an ideal interval for the partial hydrolysis of levan and, consequently, to observe the formation of fructooligosaccharides (FOS) (Dahech et al., 2013; Djuríc et al., 2017; Siqueira, 2019; Siqueira, & Houllou, 2022).

The viscosimetric analysis of levan (dynamic viscosity, kinematic viscosity and specific mass) are detailed in Table 1, below:

Table 1 - Viscometric analysis of 0.5% and 1.0% (w/v) levan solutions.

Sample	Dynamic viscosity (mPa.s)	Kinematic viscosity (mm^2/s)	Specific mass (g/cm^3)
MH (1:1) 0,5%	1.3281 ± 0.0121	1.3243 ± 0.0121	1.0028 ± 0.0000
MH (1:1) 1,0%	1.6690 ± 0.0049	1.6599 ± 0.0049	1.0059 ± 0.0004
MH (1:2) 0,5%	1.2258 ± 0.0045	1.2219 ± 0.0045	1.0032 ± 0.0000
MH (1:2) 1,0%	1.2826 ± 0.0085	1.2758 ± 0.0008	1.0053 ± 0.0001
MH (1:3) 0,5%	1.0812 ± 0.0032	1.0768 ± 0.0033	1.0040 ± 0.0001
MH (1:3) 1,0%	1.1144 ± 0.0008	1.1055 ± 0.0009	1.0081 ± 0.0001
MN (1:1) 0,5%	1.1010 ± 0.0126	1.0993 ± 0.0126	1.0016 ± 0.0000
MN (1:1) 1,0%	1.1199 ± 0.0018	1.1149 ± 0.0158	1.0045 ± 0.0002
MN (1:2) 0,5%	1.0750 ± 0.0045	1.0743 ± 0.0035	1.0089 ± 0.0001
MN (1:2) 1,0%	1.1018 ± 0.0031	1.0921 ± 0.0032	1.0089 ± 0.0001
MN (1:3) 0,5%	1,0672 ± 0.0013	1.0624 ± 0.0013	1.0045 ± 0.0000
MN (1:3) 1,0%	1.1030 ± 0.0054	1.0938 ± 0.0053	1.0084 ± 0.0001

Source: Authors.

As can be seen in Table 1, there was no significant increase in the viscosity values of levan in both analyzed concentrations (0.5% and 1.0%). Only the 1.0% MH (1:1) samples showed a dynamic viscosity of 1.67 mPa.s and a kinematic viscosity of 1.66 mm2/s; even so, the specific mass of this sample remained similar to the others (Siqueira, & Houllou, 2022a). Other samples, including MH (1:1) 0.5%, MH (1:2) 0.5% and MH (1:2) 1.0% showed both dynamic and kinematic viscosity in the range of 1.20–1.35, but maintained their specific masses around 1.0 without significant variations (Table 1).

4. Discussion

PHA and levan microbial polymers have numerous biotechnological applications because they are biocompatible and biodegradable (Siqueira, 2019; Siqueira et al., 2020; Lima et al., 2020; Alves et al., 2022; Siqueira & Öner, 2023). The production of these polymers can be carried out by many microorganisms, with bacterial production being the most economically advantageous, mainly due to its greater accumulation capacity (Öner et al., 2016). However, the high production cost limits the applications of these biopolymers on a commercial scale (Siqueira & Houllou, 2022a). In this sense, the synthesis of PHA and levan by a sequential process (Figure 1) represents an alternative to reduce the total costs of its production (Alves et al., 2022).

The *C. necator* is by far the most studied bacteria to synthesize PHA (Albuquerque & Malafaia, 2018; Alves et al., 2022). However, its preference for glucose limits the use of substrates rich in other sugars, such as sugarcane molasses, in which sucrose is the predominant sugar. In addition to sucrose, molasses has significant amounts of minerals, such as calcium, magnesium, potassium and iron, essential for the growth of *C. necator* (Siqueira & Houllou, 2022a). In this sense, hydrolysis tests of molasses were carried out to enrich its composition in glucose and favor bacterial growth and PHA accumulation (Akaraonye et al., 2010; Siqueira & Houllou, 2022b). The best PHA rates were obtained by combining MH+GB substrates. The MH rich in glucose must have favored the growth of *C. necator*, while the GB used in the PHA accumulation step was more significant than the GP. This can be explained by the fact that GB from the biodiesel industry presents, in addition to glycerol, secondary compounds that can function as trace elements for bacterial metabolism (Albuquerque et al., 2018).

PHA characterization was performed by FTIR spectroscopy. According to the principle of FTIR spectroscopy, specific chemical bonds absorb specific energies or wavelengths. In this work, the characterization of PHA showed mainly -OH bands, vibrational elongations and $-CH_3$ and $-CH_2$ stretches, glycosidic bond (C-O-C) and carbonyl group (C=O) stretches

typical of PHB-type PHA (Figure 3. AND). All absorption bands were identical to the PHA bands described in the literature (Albuquerque et al., 2018; Vega-Vidaurri et al., 2022).

The production of levan by *B. subtilis* (natto) Takahashi using the supernatant of the growth step of *C. necator* was motivated by the robustness of that bacterium in relation to this one. *B. subtilis* (natto) has a faster growth rate than *C. necator* and this is an important factor because it does not require ultrafiltration steps using a cutting membrane, which are quite expensive (Shih et al., 2010; Siqueira et al., 2017; Siqueira, 2019). The enzyme levansucrase from *B. subtilis* (natto) hydrolyzes sucrose, generating glucose and fructose monomers. The glucose moieties generated in the hydrolysis are used as an energy source for the microbial growth phase, and the fructose units are used in the polymerization of levan (Öner, Hernández & Combie, 2016). Unlike the production of PHA, which is limited inside bacterial cells, levansucrase acts in the extracellular environment, promoting a higher concentration of levan in the culture medium. In this work, *B. subtilis* (natto) was used in its lyophilized form, that is, there was no subculture and adaptability with the culture medium, therefore, in both curves (MH and MN) the presence of the lag phase is not observed (Ozcan, & Öner, 2015) (Figure 4.B).

The characterization of levan was performed by acid hydrolysis accompanied by TLC and viscosimetric analysis. In this work, levan was hydrolyzed with oxalic acid (0.5%) and the TLC result revealed that its composition was predominantly formed by fructose units (Siqueira, 2019; Siqueira & Houllou, 2022a). Differences in hydrolysis patterns can also occur as a result of differences in molecular weight or the degree of branching (Figure 4.C), in the case of branched levans (Djuríc et al., 2017; Siqueira, 2019). The hydrolysis of levan with organic acids (oxalic, lactic, acetic and gluconic) ensures greater formation of fructose monomers; while the use of inorganic acids for this process produces high amounts of fructooligosaccharides and low molar mass levan (Shih et al., 2005; Djuríc et al., 2017; Siqueira, 2019).

One of the common features of polysaccharide polymers is their ability to form highly viscous aqueous solutions even at low concentrations (Stojkoviç et al., 2015). In this study, levan solutions at concentrations of 0.5 and 1.0% (w/v) were analyzed for dynamic and kinematic viscosities (Table 1). However, the viscosity values were not significantly modified as a function of the analyzed concentrations. Benigar et al. (2015) found that levan produced by *B. subtilis* has very similar viscosity at low concentrations (up to 1.0% w/v), and may vary significantly at higher concentrations. The authors explain that at concentrations < 1.0%, the structural chains of levan produced by *B. subtilis* are more flexible than the levan segments of *Zymomonas mobilis* and *Erwinia herbicola*, for example (Benigar et al., 2015). In the case of the levan of this study, the viscosity in the studied concentrations seems not to have been affected by the attractive and repulsive interactions of the different segments of the polymeric chains.

5. Conclusion

A process for sequential production of polyhydroxyalkanoates and levan by microbial fermentation using molasses and crude glycerol as substrates has been demonstrated. The highest rate of PHA was obtained when using the combination of hydrolyzed molasses and crude glycerol (MH+GB) for the bacterial growth and PHA accumulation steps, respectively. FTIR spectroscopy analysis of PHA indicated characteristic stretching vibrations typical for a PHB-like molecule. Levans with different molecular weights were obtained by precipitation with different volumes of ethanol. The total hydrolysis of levan released only fructose monomers as forming units. Finally, the levan viscosity values were similar in the analyzed concentrations and, therefore, were not conclusive about the molecular weight of this fructan. The sequential process described in this study has a greener and more ecologically correct character. Furthermore, obtaining levan as a second product is an alternative to reduce PHA production costs.

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