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original scientific paper

***Komagataeibacter intermedius* V-05: An Acetic Acid Bacterium Isolated from Vinegar Industry, with High Capacity for Bacterial Cellulose Production in Soybean Molasses-Based Medium**

Running head: Bacterial cellulose production from soybean molasses

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SUMMARY

Research background. Despite the great properties of bacterial cellulose, its manufacture is still limited due to difficulties in production at large-scale. These problems are mainly related to low production yields and high overall costs of the conventional culture media normally used. Reversing these problems makes it necessary to identify new cheap and sustainable carbon sources. Thus, this work aimed to isolate and select a high cellulose-producing *Komagataeibacter* strain from vinegar

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industry, and study their potential for bacterial cellulose synthesis in an industrial soybean co-product, known as soybean molasses, to be used as fermentation medium.

Experimental approach. For one isolated strain that exhibited high level of cellulose production in the standard Hestrin-Schramm medium, the ability of this biopolymer production in a soybean molasses-based medium was determined. The produced membranes were characterized by thermogravimetric analysis, X-ray diffraction, infrared spectroscopy, water holding capacity and rehydration ratio for determination of its characteristics and properties. The selected strain was also characterized by genetic analysis for determination of its genus and specie.

Results and conclusions. An isolated strain was genetically identified as *Komagataeibacter intermedius* V-05 and exhibited the highest cellulose production in Hestrin-Schramm medium (3.7 g/L). In addition, the production by this strain in soybean molasses-based medium was 10.0 g/L. Membranes from both substrates were similar in terms of chemical structure, crystallinity and thermal degradation. Soybean molasses proved to be a suitable alternative medium for biosynthesis of cellulose in comparison with standard medium. In addition to providing higher production yield, the membranes showed great structural characteristics, similar to those obtained from standard medium.

Novelty and scientific contribution. In this research, we have isolated and identified a *Komagataeibacter* strain which exhibits a high capacity for cellulose production in soybean molasses medium. The isolation and selection of strains with high capacity of microbial metabolites production is important for decreasing bioprocess costs. Furthermore, as there is a necessity today to find cheaper carbon sources that provide microbial products at a lower cost, soybean molasses represents an interesting alternative medium to produce bacterial cellulose prior to its industrial application.

Key words: isolation, fermentative process, soybean, co-product, polysaccharide

INTRODUCTION

Acetic acid bacteria (AAB) belong to the family *Acetobacteraceae*, which includes several genera and species. They are strictly aerobic, Gram-negative or Gram-variable, catalase positive, ellipsoidal to rod-shaped cells that can occur as individuals, or in pairs or chains. AAB are involved in the production of several products, such as vinegar, kombucha, gluconic acid, sorbose and bacterial cellulose (BC). This group of bacteria is described as nutritionally-demanding microorganisms, and is difficult to isolate and cultivate in artificial media, particularly for strains isolated from environments with high levels of acetic acid (1).

Bacterial cellulose is an extracellular polysaccharide consisting of a chain of β -(1 \rightarrow 4) linked D-glucose units, secreted from various species of bacteria. The most common and attractive strain is

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Komagataeibacter xylinus, a member of the family *Acetobacteraceae*, due to its ability to produce large amounts of cellulose and to consume a variety of sugars and other compounds as carbon sources. In recent years, the microbiological production of cellulose has attracted interest, due to the unusual properties and characteristics of BC. Unlike cellulose synthesized by plants, which normally contains impurities, BC is free of lignin, hemicellulose, pectin and proteins, is highly organized and crystalline, with a huge water absorbing and holding capacity, and presents interesting physical properties, such as higher tensile strength and elasticity. The excellent biocompatibility and biodegradability of BC also plays an important role in its use as prosthetic tissue (1,2).

High productivity in microbial processes typically depends on the isolation and selection of highly producing microorganisms, and their cultivation in an efficient culture medium. The nutrient source of a fermentation medium influences the growth and metabolism of cells. Therefore, the productivity of a fermentation process is strongly influenced by the culture medium composition, including the carbon and nitrogen sources, growth factors and inorganic salts (3). Carbon and nitrogen sources are the highest-cost components of the medium for any fermentation process. As the problems associated with bioprocesses are typically productivity and production cost, there is a need to develop and use low-cost carbon sources, in order to produce microbial metabolites at a lower cost and higher production yield for industrial applications (4,5). The Hestrin-Schramm (HS) is a common and effective medium for BC production, but is limited in its ability to obtain high productivity in a large-scale production system due to its high cost (6). Thus, many studies have been dedicated to finding alternative substrates with lower cost. The use of agricultural residues such as: fruit and vegetal peels (7,8), milk whey (4,9), and beet molasses (9) have been shown to be promising alternative media.

Soybean molasses is an industrial co-product, generated in the production of soy protein concentrate from defatted soybean meal. It is obtained by precipitating soybean protein using an ethanol extracting solution, resulting in a brown syrup containing a high percentage of carbohydrates (approximately 70 % soluble solids), as well as lipids, proteins, fibers and minerals. A large portion of this by-product is used for animal feed or discarded (10). However, due to its composition, the soybean molasses can also be used as a fermentation medium to obtain microbial metabolites and products, such as bioethanol (11), vinegar (10), and exopolysaccharides (12).

In this work, we aimed to isolate strains of AAB from vinegar industry, and evaluate the ability of BC synthesis by using the soybean molasses as a carbon and nitrogen source, followed by characterization of the BC produced in this substrate.

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MATERIALS AND METHODS

Materials

Soybean molasses were supplied by Selecta soybean industry (Araguari, Brazil), in the concentrated form (approximately 70 °Bx). Strains of AAB were isolated from fruit vinegar broth obtained from the “Tecnologia em Saúde” food industry (Assis, Brazil). Yeast extract, peptone and bacteriological agar were purchased from Himedia (Mumbai, India). Other chemical reagents used for elaboration of culture media were purchased from Synth (Diadema, Brazil).

Isolation of the microorganisms

The culture media used for isolation were MYP (25 g/L mannitol, 5 g/L yeast extract and 3 g/L peptone) and HS (20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L Na₂HPO₄ and 1.15 g/L citric acid). Aliquots of fermenters were transferred to flasks containing the media described above, and incubated at (30±0.5) °C at 120 rpm. After turbidity, 0.1 mL of each diluted medium was transferred to Petri dishes containing a double layer of MYP agar (addition of a 0.5 % agar layer and a 1.0 % agar overlay) (13). Pure colonies with similar morphological characteristics were obtained by using the streak plate method. The selected colonies were re-streaked several successive times until a pure culture was obtained. After purification, the well-isolated colonies were submitted for Gram staining, for selection of Gram-negative and rod-shaped bacteria.

Biochemical characterization

Oxidation of ethanol and acetic acid tests were carried out by colony inoculation in Petri dishes containing Carr medium (30 g/L yeast extract, 20 g/L ethanol, 0.022 g/L bromocresol green and 10 g/L agar). In this medium, the oxidation of ethanol generates acetic acid and changes the colour from green to yellow. Overoxidising capacity was observed by the return of green colour after extended incubation period (1). Oxidation of lactate was observed in DSM agar (1 g/L glucose, 1 g/L sorbitol, 2 g/L mannitol, 3.3 g/L yeast extract, 10 g/L proteose peptone, 15 g/L calcium lactate, 1 g/L KH₂PO₄, 0.02 g/L MnSO₄•H₂O, 0.03 g/L bromocresol purple, 0.29 µg/L brilliant green and 15 g/L agar). This medium changes the colour from yellow to purple, as a result of lactate utilisation, causing a pH increase (1). Production of catalase was observed after addition of 3 % (v/v) hydrogen peroxide (Anidrol, Diadema, Brazil) on bacterial colonies grown in MYP agar. Bubbles formation indicated presence of catalase. Production of indole from tryptophan was observed after growth in tryptone-containing medium (10 g/L tryptone) and addition of 0.1 mL Kovac's reagent (Probac, São Paulo, Brazil). A red colour formed on the surface of the broth indicated a positive result, while a yellow colour indicated a negative result. Ketogenic activity was detected by covering an inoculated agar

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plate (30 g/L yeast extract, 30 mL/L glycerol and 20 g/L agar) with Fehling's solution (Anidrol, Diadema, Brazil). Around positive colonies, an orange-colored halo of CuO was formed. Production of cellulose is described in a separate section below. The strain exhibiting the highest cellulose production in this step was selected for further experiments, including genetic characterization, biosynthesis of BC in soybean molasses-based medium, and characterization of the BC produced.

Extraction and amplification of DNA

For genetic characterization, DNA from an isolated strain was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen Life Technologies, Eugene, USA). Extracted DNA was amplified using the Platinum PCR SuperMix Kit (Invitrogen Life Technologies, Eugene, USA), according to the following conditions: 45 μ L of each reaction containing PCR SuperMix (Thermo Fisher Scientific, Waltham, USA), 1 μ L of each primer (10 nM) and 3 μ L of DNA template (approximately 50 ng). All products were analyzed by electrophoresis in a 2 % agarose gel with Safer Dye (KASVI, São José dos Pinhais, Brazil) in 0.5X TBE buffer (89 mM Tris, 89 mM boric acid; 2 mM EDTA) (Thermo Fisher Scientific, Waltham, USA), pH 8.4, and visualized under ultraviolet light. Molecular size was estimated by comparison with a 100 bp ladder.

Molecular typing of isolated strain

Amplification and sequencing of the 16S rRNA gene were performed for the selected strain in a randomized manner (14). The products of 16S rRNA gene amplification were purified with a Purelink Genomic DNA extraction kit (Invitrogen Life Technologies, Eugene, USA), quantified using a QubitTM Fluorometer (Invitrogen Life Technologies, Eugene, USA), and sequenced on a ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, USA) using primers fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'). The contigs were obtained by CAP3, and sequence quality was analyzed visually in BioEdit software v.7.2.5 (15). The identity was compared with all sequences deposited in the non-redundant database of GenBank using the BLAST program (16). The alignment was created in the BioEdit program using the clustalW package (17). The phylogenetic tree built using the Maximum Likelihood method (18) and the MEGA 7.0.18 program (19).

Characterization of soybean molasses

Moisture content was determined by drying in oven at 105 °C (Odontobrás, Ribeirão Preto, Brazil) until constant dry mass. Crude protein was quantified on the basis of total nitrogen content (conversion factor equal to 6.25) by Kjeldahl method (Kjeltec 8400, Foss, Hilleroed, Denmark). Total

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ash was determined by heating at 550 °C in a muffle furnace (Quimis Q318M24, Diadema, Brazil). Lipid content was determined by the solvent extraction method using chloroform/methanol/water 2:2:1.8 (v/v/v) (Anidrol, Diadema, Brazil) (20). The content and composition of carbohydrates was determined by high performance liquid chromatography (HPLC), using a liquid chromatograph (Shimadzu, Tokyo, Japan) coupled with a refractive index detector (RID-10A, Shimadzu, Tokyo, Japan). The samples were diluted and filtered through a 0.22 µm PVDF membrane (Millipore Millex, Dublin, Ireland). The elution was performed on Aminex HPX-87P analytical column (300 x 7.8 mm, 9.0 µm, Bio-Rad, Hercules, USA), in an isocratic system using ultrapure water as mobile phase, at a flow rate of 1.0 mL/min. The quantification of sugars by HPLC was performed using known concentrations of standards (Sigma-Aldrich, San Luis, USA) of glucose, fructose, galactose, sucrose, raffinose, stachyose and xylose. Fibers were estimated by difference.

The concentrations of elements Mg, Co, Cr, Cu, B, Mn, Fe, Zn and Mo were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS, Mulgrave, Australia). Plasma was generated using Ar. The main flow rate was 17 L/min and the nebulizer flow rate was 0.21 L/min. Concentration of Ca was determined using an atomic absorption spectrophotometer (Varian AA-140, Mulgrave, Australia), equipped with a deuterium and a monoelementary Ca lamp, operating at a wavelength of 422 nm at 4 mA. Concentration of K was determined using a flame photometer (Micronal B462, São Paulo, Brazil). Concentration of P was determined using a visible spectrophotometer (Micronal AJX-1600, São Paulo, Brazil), at a wavelength of 660 nm, via the phosphomolybdate complex formation method.

Production of cellulose in HS and soybean molasses-based media

Using the cellulose-producing isolated and selected strain, colonies were transferred to flasks containing sterile Hestrin-Schramm (HS) medium and incubated in a bacteriological incubator (B.O.D. TE-391, Tecnal, Piracicaba, Brazil) for approximately 10 d at (30±0.5) °C. From this inoculum, 10 mL was transferred into a 500 mL Erlenmeyer flask, containing 90 mL sterile soybean molasses diluted at 20 °Bx (proportion 1:2.5, soybean molasses 70 °Bx:water) and 2 % soybean ethanol. The flasks were incubated in a bacteriological incubator (B.O.D. TE-391, Tecnal, Piracicaba, Brazil) under static conditions for 14 days at (30±0.5) °C. Cellulose production in standard HS medium was performed using a similar method, by transferring 10 % of inoculum into a 500 mL Erlenmeyer flask containing sterile HS broth, and incubating for 14 days at (30±0.5) °C under static conditions in a bacteriological incubator ((B.O.D. TE-391, Tecnal, Piracicaba, Brazil). The cellulose produced on the surface of each medium was collected and heated in 1 M NaOH solution (Synth, Diadema, Brazil) at 80 °C for 30 min in water bath (Quimis Q215M2, Diadema, Brazil), then washed with distilled water until reaching

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neutral pH. The membranes obtained after treatment were dried at 105 °C in a drying oven (Odontobrás, Ribeirão Preto, Brazil) until achieving constant mass, to determine the respective yields expressed in dry mass.

Consumption of sugars

Consumption of sugars was determined by measuring the remaining sugars in the medium using HPLC, under the same conditions as previously described.

Thermogravimetric analysis (TGA)

Analysis by TGA was performed using a thermal analyzer (Shimadzu TGA-50, Tokyo, Japan). The scans were ramped from 0 to 600 °C at a heating rate of 10 °C/min, under nitrogen atmosphere (50 mL/min). Derivative form of TGA (DTG) was obtained using differential of TGA values. The TGA and DTG curves were expressed as the mass variation as a function of temperature.

X-ray diffraction (XRD)

The XRD patterns of the BC membranes were obtained using a X-ray diffractometer (Malvern Panalytical X'Pert PRO MPD, Almelo, Netherlands) and Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$), at 40 kV and 30 mA. All assays were performed with scan speed of 1 °/min, analyzing the range of 5–40° (2θ). The degree of crystallinity was determined for cellulose I as described previously (21).

Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared spectra were recorded using a spectrometer (Bomen MB-100, Quebec, Canada) over the 4000–400 cm^{-1} range. A total of 8 scans were taken for each sample. The spectra were collected in transmission mode, with a resolution of 1 cm^{-1} .

Water holding capacity (WHC) and rehydration ratio (RR)

For determination of WHC, wet samples were shaken quickly and weighed after being removed from the storage recipient (m_{wet}). The samples were then dried at 60 °C for 48 h in order to completely remove water, and weighed again (m_{dry}). WHC was obtained by Eq. 2 (22):

$$\text{WHC} = (m_{\text{wet}} - m_{\text{dry}}) / m_{\text{dry}} \quad /2/$$

The dried membranes (m_{dry}) were immersed in distilled water until the mass of the rehydrated sample (m_{wet}) no longer increased (approximately 24 h). The rehydration ratio (RR) was calculated by Eq. 3 (23):

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$$RR (\%) = ((m_{\text{wet}} - m_{\text{dry}}) / (m_{\text{wet}} - m_{\text{dry}})) \cdot 100 \quad /3/$$

Bacterial cellulose production rate and production yield

The efficiency of BC production was evaluated after 336 h of cultivation. The BC production rate (Eq. 4), BC production yield (Eq. 5) and substrate conversion ratio (Eq. 6) were calculated, respectively, as (24):

$$\text{BC production rate (g/(L·h))} = m_{\text{BC}} / V \cdot t \quad /4/$$

$$\text{BC production yield (\%)} = ((m_{\text{BC}} / V) / (S_i - S_f)) \cdot 100 \quad /5/$$

$$\text{Substrate conversion ratio (\%)} = ((S_i - S_f) / S_i) \cdot 100 \quad /6/$$

Where S_i is the initial substrate concentration (g/L), S_f is the residual substrate concentration (g/L), m_{BC} is the amount of BC produced (g), V is the reaction volume (L) and t is time of fermentation (h).

Statistical analysis

Results of maximum BC production, WHC and RR of the samples were compared between both media used in BC production by using the statistical software R (25).

RESULTS AND DISCUSSION

Biochemical and genetic characterization

Table 1 shows the biochemical characteristics of the isolated microorganisms. After an isolation step and biochemical tests, seven strains belonging to the family *Acetobacteraceae* (1) were obtained from industrial vinegar fermentation broth by using the isolation technique of double-layer agar. The cultivation on this agar plate, by adding 0.5 % agar and coating with a 1 % agar layer, is considered the most efficient isolation technique, as it provides a wet environment for the formation of acidifying bacterial colonies (13). After biochemical tests, the strains were revealed to be Gram-negative bacteria that exhibited oxidation of ethanol to acetic acid and over-oxidation to CO₂ and H₂O, as well as oxidation of lactate and acid production from glucose. All strains were able to produce catalase, while only five and three of them were able to produce cellulose and dihydroxyacetone from glycerol, respectively.

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Table 1

One strain preliminarily identified as “V-05” showed the highest production of cellulose, and was selected for genetic identification, then for testing of BC production in soybean molasses-based medium. For this strain, the evolutionary history in the phylogenetic tree was inferred by using the Maximum Likelihood method, based on the Tamura-Nei model. According to Fig. 1, after genetic analysis of the amplified and sequenced 16S rRNA gene, the phylogenetic position of the selected strain “V-05” was most closely related to the strain *Komagataeibacter intermedius* (GenBank accession no. MN263075). Through genetic and phylogenetic analysis, the strain “V-05” was classified into the genus *Komagataeibacter* belonging to the specie *intermedius*, an AAB first isolated and identified from vinegar samples and Kombucha tea (26).

Figure 1

Physico-chemical characterization of soybean molasses

Table 2 shows the results from soybean molasses physicochemical composition analysis, expressed on basis of dry mass. Carbohydrates are the major components (approximately 70 % m/m) of soybean molasses, mainly sucrose and smaller amounts of oligosaccharides (raffinose and stachyose), as well as monosaccharides (glucose, fructose, xylose and galactose). As in soybeans, K, Ca, P and Mg are the main minerals present in soybean molasses (27). Their high concentration of carbohydrates and other constituents, such as protein, lipids and minerals, makes soybean molasses an attractive substrate for use in fermentative processes. The source of carbon and nitrogen in the cultivation media usually plays an important role in fermentation productivity, as these nutrients are directly linked with the microbial biomass composition and the metabolic products formation (28). In addition, the microorganisms require various metals in ionic form, which are required in varying amounts for their growth. These ions play a biological role – for example, they act as enzyme activators (cofactors), regulate osmotic pressure, and participate in cellular respiration. The significant levels of minerals in soybean molasses also contribute to the development of microorganisms involved in the fermentative process. Mohite *et al.* (29) reported that the concentration of metal ions in the culture medium is an important factor for the production of BC, and also suggested that Mg and Ca have a positive effect on cellulose production.

Table 2

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Production of BC

The strain “V-05” showed high production rate and production yield when soybean molasses was used as fermentation substrate for BC production. The yield obtained in soybean molasses plus ethanol was approximately 3 times higher (10.0 g/L) than in standard HS medium (3.7 g/L), showing that soybean molasses is a potent substrate for BC biosynthesis. The maximum production of BC reached in this study by *K. intermedius* V-05 in soybean molasses-based medium was similar to that obtained by Vazquez *et al.* (30) using glycerol remaining from biodiesel production and corn steep liquor. More results of BC obtained from complex medium and HS can be compared to those from this work in Table 3 (31-40). Mean BC production rate and BC production yield were 0.0298 g/(L·h) and 34.25 %, respectively.

Table 3

The most abundant sugars in soybean molasses, near 90 % (m/m), are sucrose, raffinose and stachyose (Table 1). However, among these sugars, only sucrose is naturally fermentable. Raffinose and stachyose oligosaccharides are not used as a carbon source by AAB, as they do not produce the α -galactosidase enzyme. An increase in fermentable sugars and production yield may be achieved by enzymatic hydrolysis of the α -(1→6)-galactosidic bonds present in soybean molasses oligosaccharides, using α -galactosidase enzyme prior to fermentation (11). Low consumption of xylose is also observed in the fermentative process for BC biosynthesis, attributed to the complexity in metabolizing this carbohydrate (41).

As shown in Fig. 2, the reducing sugars fructose and glucose were the main sugars consumed during fermentation, with 55 % (m/v) and 70 % (m/v) of the initial mass consumed, respectively. In preliminary tests, BC production by *K. intermedius* V-05 in soybean molasses without ethanol addition was much inferior (< 1.0 g/L) to production in the medium containing ethanol. It is known that the addition of ethanol at low concentrations increases the production of cellulose. Li *et al.* (42) found that production increased after the addition of ethanol to the culture medium used. Ethanol functions as an additional energy source for adenosine triphosphate (ATP) generation at the early stage of fermentation by acting as precursor for the growth of bacteria, and by allowing glucose to be used only for BC synthesis. Ethanol also functions as an electron donor (42). In this work, ethanol addition improved the production of BC in soybean molasses-based medium.

Figure 2

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Characterization of BC

Thermal decomposition curves and the respective DTG curves of the membranes are shown in **Fig. 3**. From the obtained curves, showing mass percentage as a function of temperature, the parameters of initial thermal decomposition temperature (T_{onset}), temperature where 10 % of mass was lost ($T_{10\%}$), and mass loss at 600 °C were calculated. The membranes produced in HS medium exhibited higher parameters of T_{onset} , $T_{10\%}$ and mass loss at 600 °C ($T_{\text{onset}} = 307$ °C and $T_{10\%} = 300$ °C) than in soybean molasses-based medium ($T_{\text{onset}} = 299$ °C and $T_{10\%} = 285$ °C). This fact may be correlated with the higher uniplanarity and higher crystallinity index of these membranes, which require higher energy for their degradation. The membranes produced in HS medium also present higher mass loss at 600 °C (81 %) than those from soybean molasses-based medium (79 %).

Figure 3

Degradation curves of the BC membranes produced in soybean molasses medium presented similar and typical profiles, compared to when HS medium was used (**Fig. 3**). The downward peaks in DTG curves were in consistence with the maximum mass loss of TG curves. The maximum rate of mass loss was around 350 °C, similar to the reported in earlier studies (34). The thermal decomposition curves of the membranes indicated three distinct mass-loss steps characteristic of BC process showing BC was stable up to a temperature of 250 °C. The first mass-loss event is observed from room temperature (approximately 30 °C) to approximately 150 °C, and is attributed to mass loss due to evaporation of residual water resulting from the drying process. The second mass-loss event is observed over the temperature range from 250 to 400 °C and is attributed to degradation of cellulose (dehydration and decomposition of the glycosidic units). The third and final event extends up to 500 °C, corresponding to the thermo-oxidative degradation of cellulose (43). Earlier studies have reported a similar thermal stability and degradation temperature of bacterial cellulose as observed in this work (2).

As shown in **Fig. 4**, the XRD data demonstrated that the culture medium did not influence the crystal organization of the membranes. The membranes produced in both standard HS and soybean molasses and ethanol-based media showed similar crystalline profiles. The samples had two major peaks at 15° and 22.5°. Diffraction peaks at 15 and 22.5° are assigned to the characteristic interplane distances of cellulose I α and I β phases (100I α , 110I β and 010I β planes at 15° and 110I α and 200I β at 22.5°) (44). The observed peaks in this work demonstrate that both BC samples possess typical crystalline forms of cellulose I. The samples produced in soybean molasses-based medium showed crystallinity index slightly higher (71.2 %) than those from HS medium (69.0 %). The values of

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crystallinity index observed in this work for the membranes produced in both media were similar to the reported by other authors in earlier studies (45).

Figure 4

Determination of functional groups of the BC samples was made by analyzing the spectra obtained in wavelength ranging from 400 to 4000 cm^{-1} by FT-IR analysis. As shown in Fig. 5, the functional groups of the samples obtained from both fermentation media were almost the same. The FT-IR spectra of both membranes displayed the main attributes that characterize the cellulose polymer, such as: strong transmission of OH stretching vibrations at 3500 cm^{-1} ; alkane CH stretching and CH_2 asymmetric stretching vibration at 2900 cm^{-1} ; CH_2 symmetric stretching vibration at 2700 cm^{-1} ; OH deformation vibration at 1600 cm^{-1} ; CH_2 deformation vibration at 1400 cm^{-1} ; CH_3 deformation vibration at 1370 cm^{-1} ; OH deformation vibration at 1340 cm^{-1} ; and CO deformation vibration in the range of 1320–1030 cm^{-1} (46). Bands observed at 1640 cm^{-1} (δ s HOH) and 3500 cm^{-1} (ν OH) were attributed to water absorption by the composites, and the band observed at 750 cm^{-1} and 710 cm^{-1} correspond to phases I α and I β , respectively, of the BC samples (47). The results of FTIR analysis are in agreement with studies carried out previously (47) indicating that the substance produced is chemically pure bacterial cellulose.

Figure 5

In relation to hydrophilic properties, BC membranes produced in soybean molasses-based medium exhibited lower WHC (approximately 57 times dry mass), than those produced in HS medium (162 times dry mass). Conversely, the soybean molasses plus ethanol medium provided membranes with higher RR than HS medium (4.4 % and 1.2 %, respectively). The WHC represents the water mass retained per unit of cellulose dry mass. This parameter is directly involved in biomedical applications of cellulose as a dressing material. Appropriate moisture content accelerates the wound healing process and protects against contamination. In addition, it facilitates the penetration of active substances into the wound, allowing more facile regeneration without damaging the newly formed skin (48). Several factors interfere in the hydrophilic properties of the BC membranes such as the drying method used, which can induce structural modifications, including roughness; shrinkage or collapse of some capillary structures (49); the porosity and surface area, as if there are plenty of empty spaces among the BC fibrils, more water may penetrate and adsorb onto the material (48); and the increase of polar groups (50). WHC is a parameter related to never-dried BC while RR represents the degree to which removed water was recovered by the samples. Drying process improves the

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storage and shelf-life of BC, but poor rehydration capacity reduces the utility of dried BC (23). In this work, it was observed that reduction of crystallinity caused an increase in WHC. This fact can be attributed to the larger number of amorphous regions in the structure providing more space for water molecule accommodation, promoting water permeation into the cellulose network (23). Conversely, BC membranes produced in soybean molasses plus ethanol exhibited higher RR, which shows a greater recovery of the water removed during the drying process. Considering that this sample was thicker than those produced in HS medium, the increase of OH hydrophilic groups in the structure may have increased the adsorption capacity of water, both on the surface and inside the cellulose matrix.

CONCLUSIONS

In this work, a new cellulose-producing strain named *Komagataeibacter intermedius* V-05 was isolated from vinegar industry. Besides that, an alternative culture medium composed of soybean molasses supplemented with ethanol showed to be an attractive culture medium for the biosynthesis of BC by this strain. This complex medium provided higher production yield compared to the standard HS medium, the most commonly used medium for BC production. The membranes produced in soybean molasses plus ethanol also presented the same crystallinity usually observed for BC, lower WHC and higher RR than those produced in HS medium, demonstrating that the type of substrate used has an effect on the characteristics of the cellulose obtained.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTION

W.A. Spinosa supervised the work and the experiments. R.J. Gomes, P.C.S. Faria-Tischer, L.V. Constantino and R.T. Chideroli conducted the laboratory experiments and the analysis and interpretation of results. R.J. Gomes wrote the manuscript. C.A. Tischer, M.F. Rosa and U.P. Pereira assisted in conducting the research, providing essential laboratories and equipment and revising the manuscript.

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Table 1. Biochemical characterization of the strains isolated from vinegar samples.

Characteristic	Strains						
	V01	V02	V03	V04	V05	V06	V07
Gram-negative rods	+	+	+	+	+	+	+
Oxidation of ethanol to acetic acid	+	+	+	+	+	+	+
Oxidation of acetic acid to CO ₂ and H ₂ O	+	+	+	+	+	+	+
Oxidation of lactate to CO ₂ and H ₂ O	+	+	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+	+
Acid production from glucose	+	+	+	+	+	+	+
Ketogenesis (dihydroxyacetone) from glycerol	-	-	+	-	-	+	+
Indole production from tryptophan	-	-	-	-	-	-	-
γ (cellulose)/(g/L)	2.2	1.9	2.4	1.9	3.7	0.0	0.0

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Table 2. Physicochemical composition of soybean molasses.

Component	Content on a dry basis
w(total carbohydrates)/(g/100 g)	70.4±4.4
w(sucrose)/(g/100 g)	30.5±1.7
w(raffinose)/(g/100 g)	14.2±1.3
w(stachyose)/(g/100 g)	19.0±1.1
w(glucose)/(g/100 g)	1.5±0.0
w(fructose)/(g/100 g)	1.8±0.1
w(galactose)/(g/100 g)	0.5±0.0
w(xylose)/(g/100 g)	2.9±0.1
w(protein)/(g/100 g)	10.7±0.1
w(lipids)/(g/100 g)	7.7±0.1
w(ash)/(g/100 g)	6.8±0.1
w(fibers)/(g/100 g)	4.4±0.0
Minerals	
w(potassium)/(g/kg)	36.50±4.20
w(calcium)/(g/kg)	0.70±0.04
w(phosphorus)/(g/kg)	0.30±0.02
w(magnesium)/(g/kg)	0.27±0.01
w(iron)/(mg/kg)	0.31±0.06
w(boron)/(mg/kg)	0.03±0.00
w(manganese)/(mg/kg)	0.02±0.00
w(chromium)/(mg/kg)	0.01±0.00
Cobalt	n.d.
Copper	n.d.
Zinc	n.d.
Molybdenum	n.d.

n.d.: not detected.

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Table 3. Comparison of BC production from diverse complex medium, by-products and agroindustrial wastes reported in literature.

Complex medium	Strain	t/day	γ (BC)/(g/L)	γ (BC in HS)/(g/L)	Source
Carob and haricot bean	<i>Gluconacetobacter xylinus</i> ATCC 700178	10	3.2	-	(31)
Cashew apple juice and soybean molasses	<i>Gluconacetobacter xylinus</i> ATCC 53582	7	4.5	4.0	(2)
Cheese whey	<i>Gluconacetobacter xylinus</i> PTCC 1734	14	3.5	3.3	(9)
Citrus peel and pomace	<i>Komagataeibacter xylinus</i> CICC No. 10529	8	5.7	3.9	(7)
Cotton cloth hydrolysate	<i>Gluconacetobacter xylinus</i> ATCC 23770	14	10.8	5.9	(32)
Date syrup	<i>Acetobacter xylinum</i> 0416	10	5.8	~3.0	(22)
Grape bagasse and corn steep liquor	<i>Gluconacetobacter xylinus</i> NRRL B-42	14	8.0	~2.0	(30)
Orange juice	<i>Acetobacter xylinum</i> NBRC 13693	14	5.9	-	(33)
Overripe Banana	<i>Komagataeibacter medellinensis</i> NBRC 3288	12	4.0	-	(34)
Pecan nutshell	<i>Gluconacetobacter entanii</i>	28	2.8	-	(35)
Pineapple peel juice	<i>Gluconacetobacter swingsii</i> sp.	13	2.8	2.1	(36)
Potato peel wastes	<i>Gluconacetobacter xylinum</i> ATCC 10245	6	4.7	1.2	(8)
Soybean molasses and ethanol	<i>Komagataeibacter intermedius</i> V-05	14	10.0	3.7	This work
Sugar beet molasses	<i>Gluconacetobacter xylinus</i> PTCC 1734	14	4.6	3.3	(9)
Waste beer yeast	<i>Gluconacetobacter hansenii</i> CGMCC 3917	14	7.0	-	(37)
Waste durian shell hydrolysate	<i>Gluconacetobacter xylinus</i>	10	2.7	2.5	(38)
Wastewater from rice wine distillery	<i>Gluconacetobacter xylinus</i> BCRC 12334	7	6.3	~3.0	(39)
Waste glycerol and corn steep liquor	<i>Gluconacetobacter xylinus</i> NRRL B-42	14	10.0	~2.0	(30)
Waste water of candied jujube	<i>Gluconacetobacter xylinum</i> CGMCC No.2955	6	2.2	-	(40)

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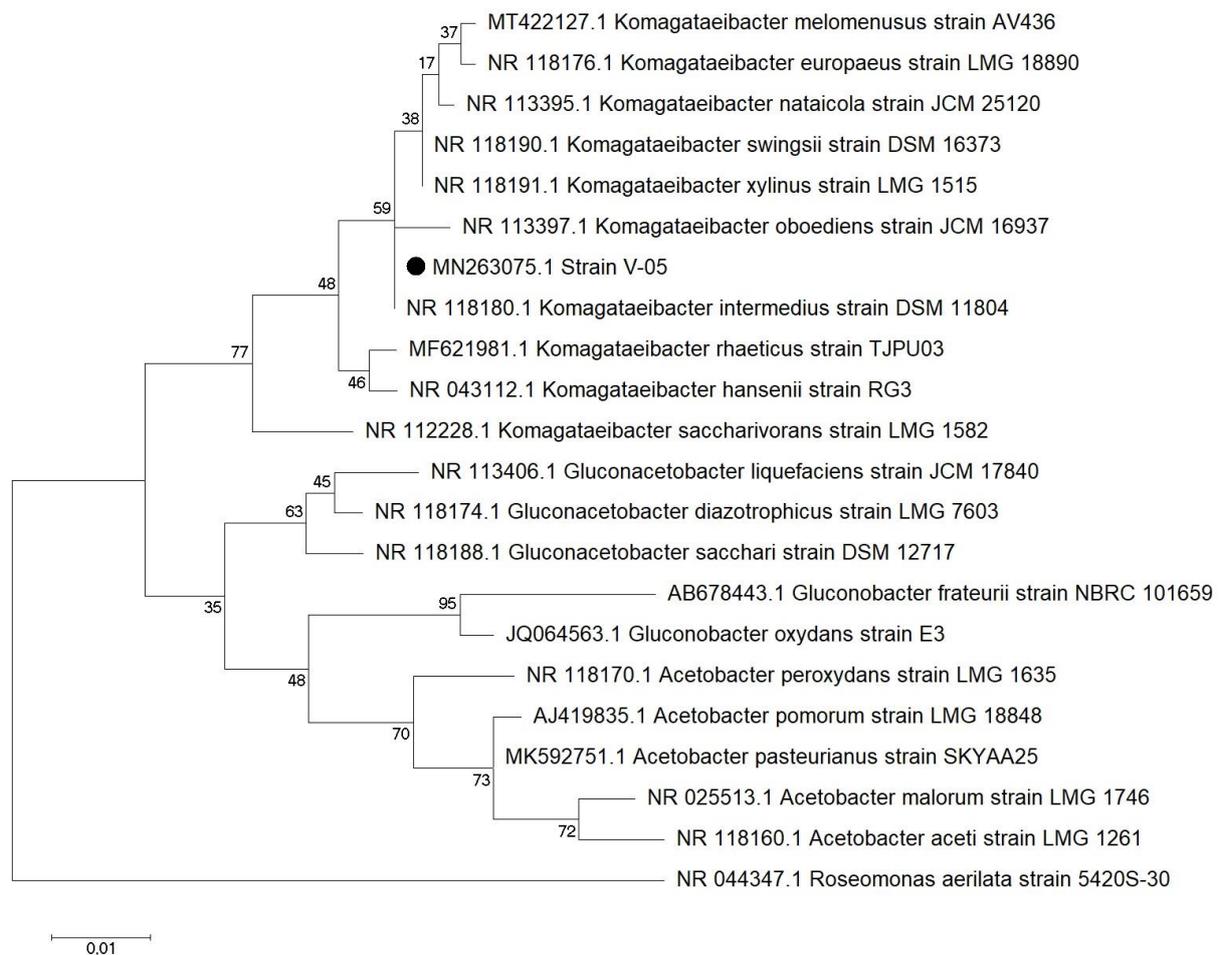


Fig. 1. Phylogenetic tree of the strain V-05, representing its relative position in the genus *Komagataeibacter* based on 16S sequences. The Maximum Likelihood method was used for phylogenetic inference, and reliability of the nodes was estimated using 1000 bootstrap replicates. Evolutionary distances were computed using the Tamura-Nei method. The sequence obtained through sequencing was deposited in GenBank under the accession number: **MN263075**

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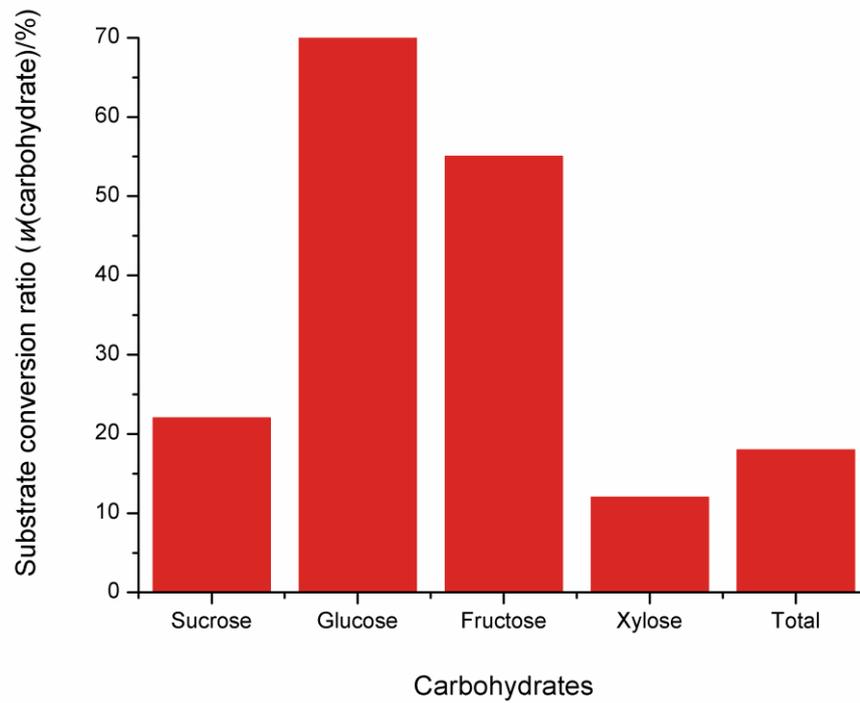


Fig. 2. Consumption of sugars in soybean molasses after a 14 day-fermentation period

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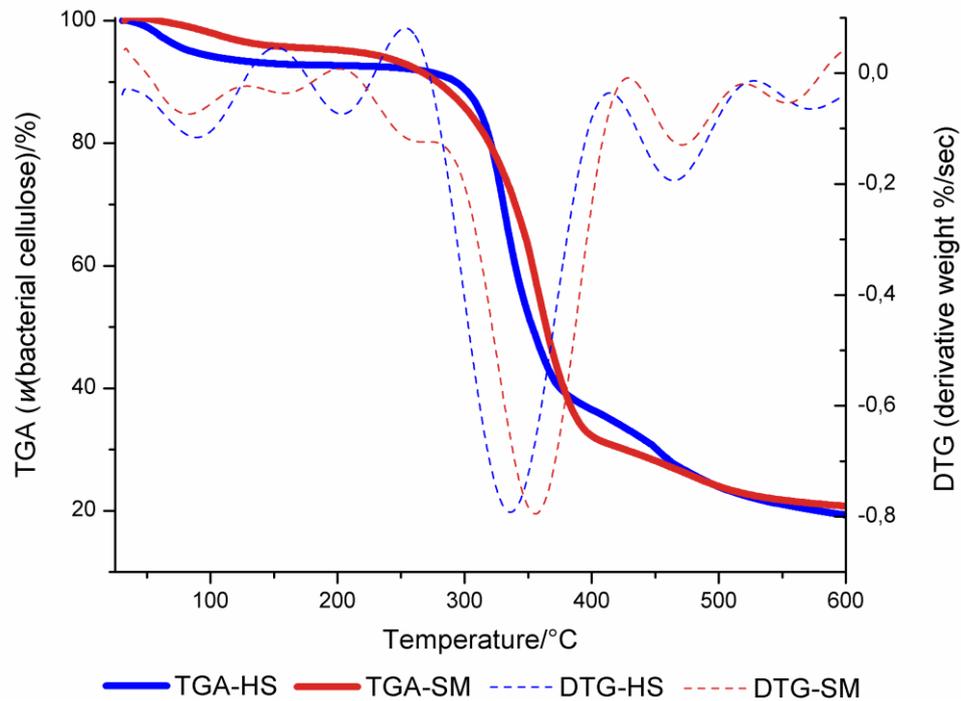


Fig. 3. TGA and DTG curves of bacterial cellulose membranes produced by *K. intermedius* V-05 in soybean molasses (SM) and standard (HS) media

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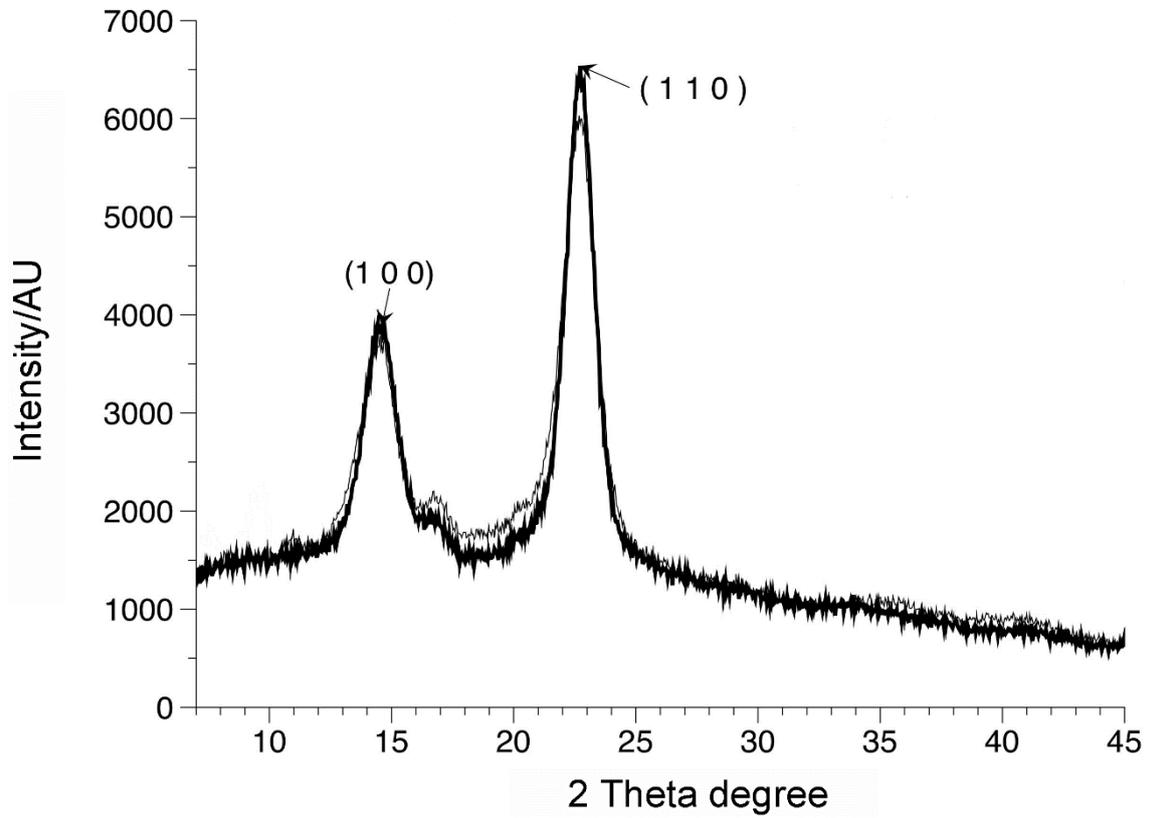


Fig. 4. XRD pattern of the cellulose membranes produced by *K. intermedius* V-05

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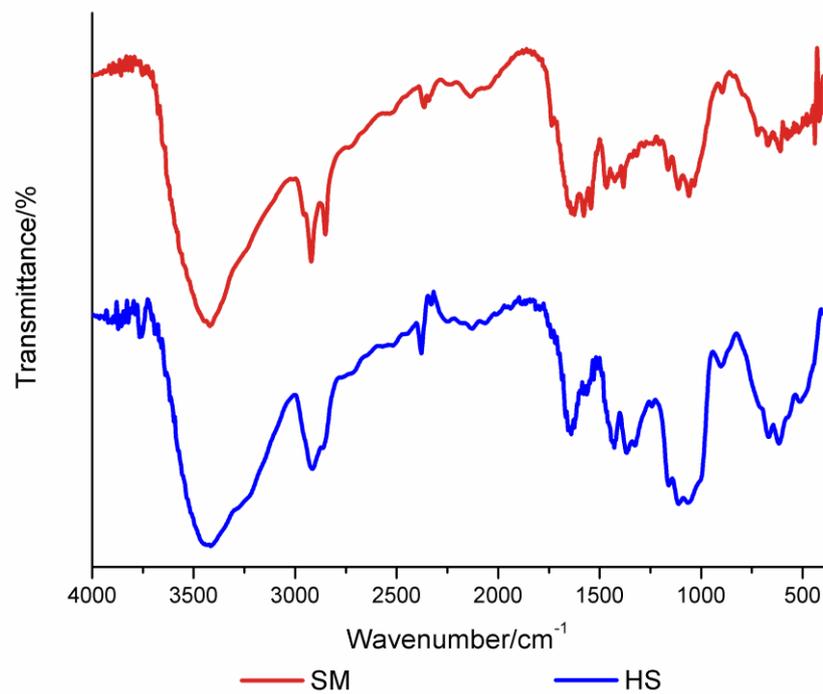


Fig. 5. FT-IR spectra of the cellulose membranes produced by *K. intermedius* V-05 in soybean molasses (SM) and standard (HS) media