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Antimicrobial activity against *Xanthomonas albilineans* and fermentation kinetics of a lactic acid bacterium isolated from the sugar cane crop

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Xanthomonas albilineans is a pathogen that causes leaf scald disease in sugarcane (*Saccharum officinarum* L.) This disease causes the death of seedlings and consequently results in economic losses for sugarcane growers. The objective of this work was to isolate a lactic acid bacterium with antimicrobial activity against *X. albilineans* from sugarcane crops and to evaluate its antimicrobial activity and its lactic acid production kinetics, biomass yield, and substrate consumption in three different fermentation substrates. To isolate the lactic acid bacterium, samples were collected from different parts of infected and non-infected sugarcane plants of var. CC85-92. *Lactococcus lactis* ssp. *lactis* was isolated from the leaves of healthy crops, and showed *in vitro* antimicrobial activity against the pathogen. Batch fermentations of this isolate (at 32 ºC, agitation of 100 rpm, and pH 6) were performed using a commercial substrate (MRS), a commercial substrate supplemented with glucose (MRSG), and a substrate produced from agricultural crop residues (ACR). The highest antimicrobial activity was 5.83 mm in the ACR substrate after 6 h of fermentation. The maximum biomass production of 3.37 g L^{-1} and the maximum lactic acid production of 12.1 g L^{-1} were obtained in the MRSG substrate. The lactic acid production did not show any significant differences between the substrates. This lactic acid bacterium showed antimicrobial activity against *X. albilineans* and is thus a biological alternative for the control of leaf scald disease in sugarcane.

Key words: ACR, *Lactococcus lactis* ssp. *lactis*, leaf scald, *Saccharum officinarum*.

INTRODUCTION

Leaf scald is a disease that affects sugarcane crops (*Saccharum officinarum* L.) The pathology is caused by the Gram-negative bacterium *Xanthomonas albilineans*, which causes the occlusion of xylem vessels and prevents the differentiation of chloroplasts (Huerta Lara et al., 2009). This disease has three states. In the latent phase, no symptoms are evident. The chronic phase appears at 3-mo of age and disappears after approximately 3-mo. The chronic phase is characterized by stem necrosis and the presence of white lines parallel to the leaf midrib. The acute phase is characterized by the immediate death of the seedling (Rott et al., 1995). Leaf scald is one of the most serious and devastating disease affecting sugarcane crops worldwide (Fontaniella et al., 2007). This disease has been detected in 57 countries and, in its acute phase, decreases the juice yield and quality in the sugar industry; in fact, losses exceeding 90% have been reported (Swings and Civerolo, 1993; Destefano et al., 2003). In Colombia, the presence of this disease was detected in the Codazzi Cesar region in 1993 and in the geographic Valley of the Cauca River in 1994; in both locations, this disease affects the plants of the widely used sugarcane var. CC 85-92 (Victoria, 1994).

Xanthomonas albilineans is transmitted through the use of contaminated seeds and the use of cutting material that is contaminated by air and irrigation (Saumtally et al., 1995). Currently, several methods are being used for its control, such as the development of resistant varieties, including 'RB73-9735', 'CP72-2086', 'Q-96', 'CP74-2005', 'RD75-11', and 'MEX79-431' (Huerta Lara et al., 2003); disinfection of seeds through heat treatment and immersion of cutting materials in chemical products (Victoria, 1994); and the use of bio-treatments with *Gluconacetobacter diazotrophicus* (Piñón et al., 2002; Arencibia et al., 2006).

The use of lactic acid bacteria (LAB) may be a biological alternative for the treatment of leaf scald in sugarcane because these bacteria produce antimicrobial peptides called bacteriocins and other substances, such as lactic acid, hydrogen peroxide, and reuterin, that have shown activity against Gram-positive and Gram-negative bacteria. Antimicrobial peptides are under consideration as new substitutes for conventional pesticides and antibiotics (Keymanesh et al., 2009).

Lactic acid bacteria and their metabolic compounds have been previously used for product innovation and to solve problems in different industries, including the food industry (Cintas et al., 2001; O'Sullivan et al.,

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2002). Some LAB, such as *Lactococcus lactis*, have demonstrated great success in the inhibition of different non-desirable microorganisms such as *Clostridium botulinum*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Listeria monocytogenes* (Yang and Ray, 1994; Vallejo et al., 2009). This success is due to the production of various bacteriocins, including nisin, which exhibits a broad inhibition spectrum and is considered safe by the US Food and Drug Administration (FDA), and lacticins 3147 and 481, which have a more reduced spectrum (López et al., 2008). Other LAB-produced bacteriocins that have been successfully tested in the food industry are pediocin PA-1 and sakacin P, which are responsible for the inhibition of a reduced but important spectrum of pathogenic microorganisms (Dolz, 2008). Furthermore, Kazemipoor et al. (2012) report that the antimicrobial activity of LAB may be due to the action of other compounds such as organic acids and hydrogen peroxide.

In the sugarcane industry, LAB exhibit negative and positive effects. Among the negative effects is the reduction of the percentage of sucrose in the milling process input by *Leuconostoc* sp. and *Lactobacillus* sp., which are exopolysaccharide-producing bacteria (Serrano-Galvis, 2006). Some of the prominent positive effects include an increase in the degradation of organic residues from the harvest and an increase in the population of beneficial microorganisms in the soil (Cleveland et al., 2001). Based on their degradation of organic matter, LAB can be used as bioremediators and antimicrobial agents against various pathogens; thus, LAB can be an alternative to the conventional and developing treatments against *X. albilineans*, which is the causal agent of leaf scald in sugarcane.

The aim of this study was to first isolate a LAB with antimicrobial capacity against *X. albilineans* from sugarcane crops. The bacterium was then evaluated on three different fermentation substrates: a commercial substrate (MRS), a commercial substrate supplemented with glucose (MRSG), and a substrate composed of agricultural crop residues (ACRs). The potential use of this bacterium was assessed according to its antimicrobial activity, biomass formation, lactic acid production, and substrate consumption kinetics in addition to its kinetic parameters, biomass yield, and product yield.

MATERIALS AND METHODS

Isolation and identification of a lactic acid bacterium (LAB) with antimicrobial activity against *Xanthomonas albilineans*

To isolate a LAB with antimicrobial activity against *X. albilineans*, samples were collected from sugarcane crops 'CC 85-92', which is highly susceptible to leaf scald (Victoria et al., 1995). The samples were obtained from 5-mo-old crops, affected and not affected by sugarcane leaf scald. Crops belonged to the Sugarcane Research Center (CENICAÑA). The samples were collected through sterile swab smears on the surface of the leaf and the stem binding site. In addition, smear samples were obtained from the cross-section of stems that were affected and of stems not affected by the disease. The collected samples were placed in tubes with a 0.1% peptone solution and transported in refrigerated containers to the Bioconversions Laboratory of the Campus-Palmira of the National University of Colombia.

Serial dilutions of the collected samples were plated on De-Man, Rogosa and Sharpe agar (MRS; De Man et al., 1960) supplemented with alanine blue (2 mL of a 0.3% alanine blue solution per liter) and incubated at 37 ºC for 72 h. Those colonies suspected to contain lactic acid-producing bacteria (which assimilated the alanine blue) were re-streaked on the same medium until pure cultures were obtained. Subsequently, the antimicrobial activity of these cultures was tested against *X. albilineans*, and the LAB that showed the highest inhibition diameter against the pathogen was selected. The selected bacterium was biochemically identified using the BD BBL Crystal Gram-Positive ID Kit (BD, Franklin Lakes, New Jersey, USA) and preserved in MRS broth and 40% glycerol solution at -20 ºC until further use.

Xanthomonas albilineans isolation and test of pathogenicity

To isolate *X. albilineans*, leaves and stems of 5-mo-old sugarcane 'CC 85-92' plants were collected from the CEÑICAÑA crops. The samples were transported and stored under refrigerated conditions until further use.

The leaf samples were washed with a 2% sodium hypochlorite solution and distilled water. The samples were cut into 1 cm^2 squares and macerated until a leachate was obtained. Serial dilutions from 10^0 to 10^{-6} were obtained, and each dilution was streaked in Nutrient Agar Medium (Oxoid Limited, Hampshire, UK). Plates were incubated at 27 ºC for 72 h. Those colonies that showed the typical morphological characteristics of *X. albilineans* (Table 1) were re-streaked in the same medium until a pure culture was obtained. The presumptive colonies of *X. albilineans* were plated in Wilbrink solid media (5 g Bacto Peptone, 10 g sucrose, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 2.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g Na_2SO_3, and 15 g Bacto agar in 1 L distilled water) (Wilbrink, 1929) and YDC media (20 g calcium carbonate, 10 g yeast extract, 15 g agar-agar, and 20 g dextrose in 1 L distilled water). The colonies were confirmed morphologically according to the characterization protocol described by Jiménez et al. (2004) (Table 1). The strains that exhibited the typical features in the three culture media were cryopreserved in nutritive broth and 40% glycerol solution, at -20 ºC for subsequent pathogenicity and antimicrobial tests. The cryopreserved pure cultures of *X. albilineans* were inoculated on 'CC 85-92' sugarcane cuttings using the

Table 1. Characteristic features of a pure culture of *Xanthomonas albilineans* in different growth media.

Growth media	Feature
Nutritive Agar Medium	Bright, round, and yellow colonies. Moderate growth.
YDC Agar (yeast extract, calcium carbonate, and dextrose)	Bright, round, and yellow colonies. Good growth.
Wilbrink Agar	Bright, round, and yellow colonies. Moderate growth.

aluminum capsule methodology proposed by Huerta Lara et al. (2003). The seedlings were subsequently monitored for 20 d, 25 °C of temperature, at 65% relative humidity, until the onset of symptoms. Thereafter, the seedlings inoculated with *X. albilineans* that exhibited disease symptoms were selected. A longitudinal cut was made, and a smear with a sterile swab was obtained. Serial dilutions in 0.1% peptone water to a minimum dilution of 10^{-6} were obtained. Each dilution was plated on XAS medium (Davis et al., 1994). The same culture conditions were maintained, and successive cultures were grown until a pure culture was obtained.

Fermentation kinetics and antimicrobial activity of the selected LAB

Substrates and fermentation conditions. Nine batch fermentations were performed using three fermentation substrates: a commercial substrate (MRS), a commercial substrate supplemented with 40 g L^{-1} glucose (MRSG), and juices from agricultural crop residues (ACRs), which were supplemented with 4 g L^{-1} yeast extract. The ACRs, which were collected from 7-mo-old sugarcane seedbeds, were selected as a substrate because these residues provide good C, N, and vitamin sources that can contribute to the growth of LAB. Furthermore, these residues might prove to be suitable alternative, low-cost substrates for the production and commercialization of the microorganism (Brizuela, 1986; Godon et al., 1993). The ARC substrate was supplemented with yeast extract to provide equivalent levels of N as those obtained in the MRS and MRSG substrates.

Fermentations were performed in 1000 mL Erlenmeyer flasks with a 400 mL workload. The Erlenmeyer flasks were maintained without aeration under 100 rpm ellipsoidal agitation in a shaker (Incubating Orbital Shaker, VWR, Radnor, Pennsylvania, USA) for 48 h at 32 ºC. The strain was adapted to fermentation conditions over three generations using an inoculum corresponding to 10% of the substrate volume. The fermentation was periodically adjusted to pH 6.0 using a 1 M NaOH solution (Mol Labs, Bogotá, Colombia).

Antimicrobial activity kinetics. The antimicrobial activity tests were performed after 2, 4, 6, 12, 24, and 48 h of fermentation in the previously described substrates. The agar diffusion method described by Serna Cock et al. (2010) was used to measure the antimicrobial activity. The antimicrobial activity was measured in two growth media (Nutritive Agar and Wilbrink Agar) with a thickness of 5 mm. Central wells with a 1.7 cm diameter were made in the plates using a sterile borer. The plates were inoculated with 100 μL 1 × 10^8 UFC mL^{-1} *X. albilineans* suspension. Similarly, 5 mm thick 1.7 cm diameter MRS agar circles were aseptically collected and placed in the central wells. These circles were inoculated with 60 μL of the fermented samples, which were collected at each time point and for each fermentation substrate mentioned above. The plates were incubated at 27 ºC for 24 h. Subsequently, the antimicrobial activity was determined by measuring the diameter of the inhibition halo over the pathogen growth. The antimicrobial tests against *X. albilineans* were performed in duplicate for each fermentation time and each fermentation substrate.

Biomass formation kinetics, substrate consumption, lactic acid production, biomass yield, and product yield

To determine the biomass formation, the substrate consumption, and the lactic acid production kinetics, 5-mL samples were collected from the fermentations with each of the substrates after 0, 2, 4, 6, 12, 24, and 48 h of fermentation (time 0 corresponds to the initial substrate conditions). Samples were centrifuged at 5000 rpm and 4 ºC for 10 min (Centrifuge 5804R, Eppendorf, Germany).

The biomass was determined using the dry weight (AOAC, 1990). In addition, non-centrifuged samples were used for the plate count according to ICMSF (2002). A linear model was established to correlate the biomass concentration obtained by the plate counting method (CFU) and the concentration obtained by the dry weight (g mL^{-1}).

The supernatant was used to determine the substrate consumption. This measurement was assessed by the concentration of reducing sugars, which was determined through spectrophotometry (Genesys 10 UV, Thermo Scientific, West Palm Beach, Florida, USA) using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The lactic acid production was measured by reflectometry (RQflex Plus 10 Reflectoquant, Merck, Darmstadt, Germany). These measurements were obtained from the supernatant. A sample was collected and filtered through a 0.45 μm filter (Titan, Northbrook, Illinois, USA).

The biomass yield ($Y_{x/s}$), the product yield ($Y_{p/s}$), and the substrate consumption (SC) were calculated using Equations [1], [2], and [3], respectively:

$$Y_{x/s} = \frac{Y_o - X}{S_o - S} \; g \; g^{-1} \qquad [1]$$

$$Y_{p/s} = \frac{P}{S_o - S} \; g \; g^{-1} \qquad [2]$$

$$CS = \frac{(S_o - S) * 100}{S_o} \; \% \qquad [3]$$

where *So* is the initial reducing sugar concentration (g L^{-1}), *S* is the final reducing sugar concentration (g L^{-1}) until the time at which *P* is maximal, *P* is the maximum lactic acid concentration (g L^{-1}), *Xo* is the initial biomass concentration (g L^{-1}), and *X* is the final biomass concentration (g L^{-1}).

Statistical analysis

Completely randomized design with three replicates was used. The effect of substrate on the antimicrobial activity, formation of biomass, lactic acid production, and substrate consumption was measured (response variables). Three modes of substrate, MRS, MRSG, and RAC were used. Measurements were taken at 0, 2, 4, 6, 12, 24 and 48 h of fermentation. The yields of biomass ($Y_{x/s}$) and product ($Y_{p/s}$) were measured at 48 h fermentation. Additionally, differences between antimicrobial activity using nutrient agar and agar Wilbrink of LAB against *X. albilineans* were evaluated.

ANOVA technical for repeated measures over time was used to process the data. Tukey's multiple comparison test was used to compare means between treatments. SAS (System Analysis Software) version 9.3 for data processing was used.

RESULTS AND DISCUSSION

A LAB with antimicrobial activity against *X. albilineans* was isolated from healthy leaves of sugarcane 'CC 85-92'. The bacterium was biochemically identified as *Lactococcus lactis* ssp. *lactis* (Table 2).

Table 2. Biochemical identification of *Lactococcus lactis* ssp. *lactis* using the BBL™ Crystal™ Gram-Positive ID Kit.

Test	Result
4-MU-β-D-Glucoside	Negative
L-Valine-AMC	Negative
L-Phenylalanine-AMC	Positive
4-MU-α-D-Glucoside	Negative
L-Pyroglutamic acid-AMC	Negative
L-Tryptophan-AMC	Positive
L-Arginine-AMC	Positive
4-MU-N-Acetyl-β-D-glucosaminide	Negative
4-MU-Phosphate	Negative
4-MU-β-D-Glucuronide	Negative
L-Isoleucine-AMC	Negative
Trehalose	Positive
Lactose	Negative
Methyl α-D-glucoside and methyl β-D-glucoside	Negative
Sucrose	Positive
Mannitol	Positive
Maltotriose	Positive
Arabinose	Negative
Glycerol	Negative
Fructose	Positive
p-Nitrophenyl-β-D-glucoside	Positive
p-Nitrophenyl-β-D-cellobioside	Negative
Proline and Leucine-p-nitroanilide	Positive
p-Nitrophenyl-phosphate	Positive
p-Nitrophenyl α-D-maltoside	Positive
o-Nitrophenyl-β-D-galactoside and p-Nitrophenyl-α-D-galactoside	Negative
Urea	Negative
Esculin	Negative
Arginine	Positive

Isolation and pathogenicity of *Xanthomonas albilineans*

The bacterium *X. albilineans* was isolated from infected leaves of sugarcane 'CC 85-92' using Nutritive Agar and Wilbrink- and XAS-selective media. The bacterium was then morphologically compared, as described by Jiménez et al. (2004) and Huerta-Lara et al. (2009), they described the growth of *X. albilineans*, with the following morphological characteristics, circular colonies, bright yellow, convex, non-mucosal, and diameters of less than 0.5 mm, these characteristics were consistent with the characteristics of the colonies isolated from leaves of sugarcane 'CC 85-92'. Jiménez and Contreras (2008) found that in the growth medium XAS, the pathogen has increased growth, compared with growth in medium Wilbrink. In the growth medium Wilbrink grows, besides *X. albilineas*, a saprophyte microorganism, which is associated with the cultivation of sugar cane, this makes difficult the isolation of the pathogen.

In the pathogenicity tests, disease symptoms were observed on the sugarcane cuttings 20 d after the inoculation of the pathogen (Figures 1a and 1b). The symptoms were manifested in accordance with the results reported by Rott et al. (1995). Internal stains bright and dark red at the nodes, internodes, and side shoots were evidenced. Likewise, Huerta Lara et al. (2009) studied the pathogenicity of *X. albilineans* in sugarcane plants (Veracruz, Mexico), and they found that the plants had white lines parallel to the midrib of the leaf of the plant. This feature also identifies the disease.

Fermentation kinetics of *Lactococcus lactis* ssp. *lactis*

The biomass production kinetics of *L. lactis* is shown in Figure 2. The biomass production show significant differences between substrates, fermentation times ($P < 0.0001$), and interactions between fermentation times and substrates. The maximum biomass production was 3.37 g L^{-1} in the MRSG substrate after 48 h fermentation; the second and third highest production levels were in the MRS (2.67 g L^{-1}) and ACR substrates (1.55 g L^{-1}), respectively.

The lactic acid production did not exhibit significant differences between substrates ($P = 0.0667$). However, significant differences were found with respect to fermentation time and interaction between fermentation time and substrate ($P < 0.05$). The maximum lactic acid production was recorded in the MRSG substrate after 48 h (12.1 g L^{-1}); the second and third highest lactic acid production levels were obtained in the ACR (9.52 g L^{-1}) and MRS substrates (8.53 g L^{-1}), respectively.

Figure 3 shows substrate consumption kinetics for *L. lactis* ssp. *lactis*. Significant differences were observed with respect to the evaluated treatments, fermentation times, and interactions between fermentation times and

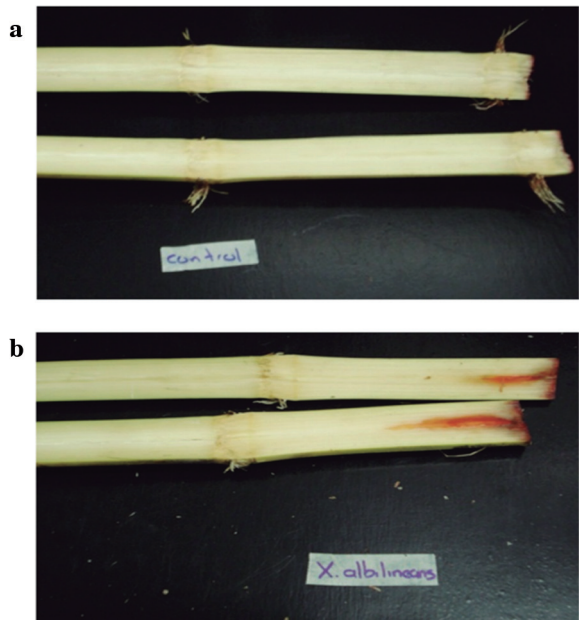


Figure 1. (a) Sugarcane cuttings of 'CC 85-92' uninoculated with *Xanthomonas albilineans*. (b) Sugarcane cuttings of 'CC 85-92' inoculated with *X. albilineans*.

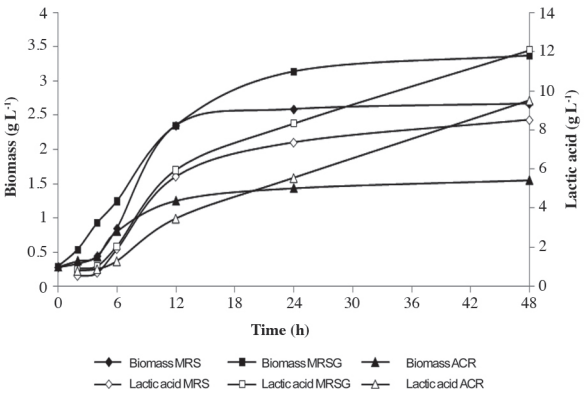


Figure 2. Kinetics of the production of biomass and lactic acid by *Lactococcus lactis* ssp. *lactis* in the MRS (commercial substrate), MRSG (commercial substrate supplemented with 40 g L^{-1} glucose), and ACR (agricultural crop residues) substrates.

treatments. The maximum substrate consumption was observed in the MRS substrate (98.44%); the second and third highest substrate consumption levels were obtained in the ACR (90.68%) and MRSG substrates (72.25%), respectively.

Table 3 shows kinetic parameters calculated from fermentation kinetics in the three substrates tested. The best biomass yield ($Y_{x/s}$) and substrate consumption (CS) were obtained on the MRS substrate; the second and third best substrates with respect to these measures were the MRSG and ACR substrates, respectively. The product yield was highest in the ACR substrate.

Figure 4 shows the linear correlation between the biomass in UFC mL^{-1} and the biomass in g mL^{-1} for each of the substrates at the different fermentation times. We found correlation coefficients of 0.88, 0.93, and 0.94 for the MRS, MRSG, and ACR substrates, respectively. The corresponding correlation equations are shown in the graph.

Antimicrobial activity kinetics of *L. lactis* ssp. *lactis* against *Xanthomonas albilineans*

Figure 5 shows the antimicrobial activity kinetics of *L. lactis* ssp. *lactis* against *X. albilineans* on Nutritive Agar and Wilbrink medium. No significant differences (P = 0.1136) were found in the antimicrobial activity of *L. lactis* against *X. albilineans* on Nutritive Agar with the three substrates tested. In contrast, significant differences were obtained between the three substrates with respect to the growth inhibition of the pathogen on Wilbrink Agar. The highest inhibition diameter (5.83 cm) was observed with the ACR substrate after 6 h of fermentation when Nutritive Agar was used to grow the pathogen. For the MSRG substrate, an inhibition diameter of 5.33 cm was obtained after 24 h of fermentation when Wilbrink Agar was used to grow the pathogen. The inhibition halos against *X. albilineans* increased proportionally to the amount of biomass; however, the larger halos were influenced by the microbial growth speed.

The biological control of *X. albilineans* has been reported using the bacterium *Gluconacetobacter diazotrophicus* (Blanco et al., 2010). Studies that use mixtures of microorganisms, which are called efficient microorganisms (EMs) and include LAB, have also been performed. Efficient microorganisms have shown positive effects against the pathogen of interest in this study (Castro et al., 1995). However, the use of a pure LAB as the biological controller of phytopathogenic bacteria,

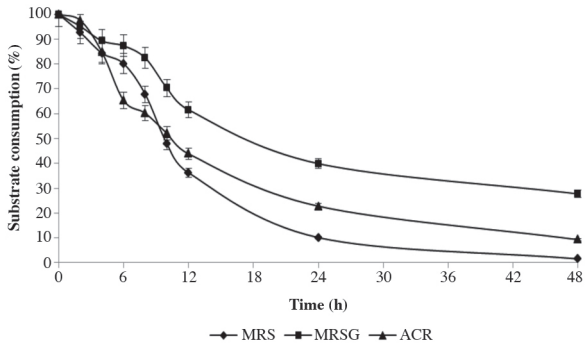


Figure 3. Kinetics of substrate consumption by *Lactococcus lactis* ssp. *lactis* on the MRS (commercial substrate), MRSG (commercial substrate supplemented with 40 g L^{-1} glucose), and ACR (agricultural crop residues) substrates.

Table 3. Kinetic parameters of *Lactococcus lactis* ssp. *lactis* in the MRS (commercial substrate), MRSG (commercial substrate supplemented with 40 g L^{-1} glucose), and ACR (agricultural crop residues) substrates.

	MRS	MRSG	ACR
$Y_{x/s}$, g g^{-1}	0.121	0.078	0.066
$Y_{p/s}$, g g^{-1}	0.434	0.307	0.495

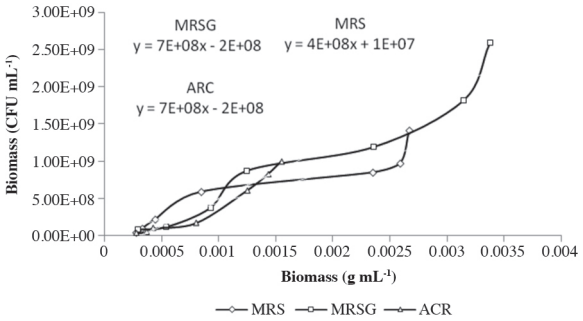
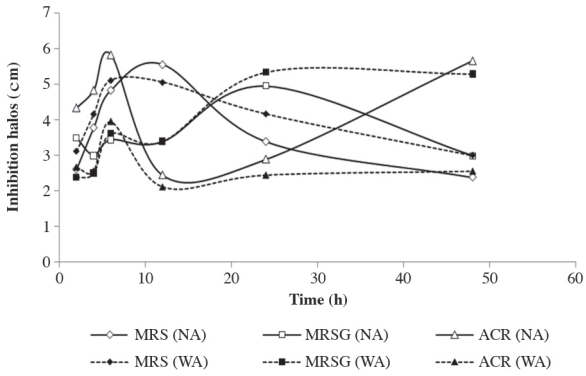


Figure 4. Correlation between the colony forming units (CFU) of *Lactococcus lactis* ssp. *lactis* in the MRS (commercial substrate), MRSG (commercial substrate supplemented with 40 g L^{-1} glucose), and ACR (agricultural crop residues) substrates.



MRS: commercial substrate; MRSG: commercial substrate supplemented with 40 g L^{-1} glucose; and ACR: agricultural crop residues substrates.

Figure 5. Antimicrobial activity kinetics of *Lactococcus lactis* ssp. *lactis* against *Xanthomonas albilineans* grown on nutritive agar (NA) and Wilbrink agar (WA).

such as *X. albilineans*, has not been previously evaluated.

Lactococcus lactis ssp. *lactis* was isolated from sugarcane 'CC 85-92'. This bacterium demonstrated *in vitro* antimicrobial activity against *X. albilineans*. Therefore, it can be considered a potential microorganism for the *in vivo* control of this pathogen. LAB possesses a very broad spectrum of action and has the ability to inhibit both Gram-positive and Gram-negative bacteria (Rattanachaikunsopon and Phumkhachorn, 2010). The action of LAB against Gram-positive bacteria is due to the production of bacteriocins, whereas their action against Gram-negative bacteria is primarily due to the action of organic acids. In fact, Kazemipoor et al. (2012) reported that the antagonistic effect of LAB against Gram-negative bacteria is due to the action of organic acids and other compounds, such as hydrogen peroxide.

Parada et al. (2007) indicate that bacteriocins are not usually effective against Gram-negative bacteria due to the protective effect of the outer membrane of this type of bacteria, which acts as a permeable barrier; however, Todorov and Dicks (2005) found that *Lactobacillus plantarum* strains isolated from molasses, produce bacteriocins which have antimicrobial activity against Gram negative pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*. We observed larger zones of inhibition against *X. albilineans* with the ACR substrate after 6 and 24 h fermentation when Nutritive Agar (NA) was used to grow the pathogen; these inhibition zones had diameters of 5.83 and 53.3 mm, respectively. The largest inhibition diameter obtained using the MRS substrate was observed after 12 h of fermentation (55.5 mm) and after 6 h fermentation (51.1 mm) when Nutritive Agar and Wilbrink Agar (WA), respectively, were used to grow the pathogen. The highest levels of inhibition with the MRSG substrate were observed after 24 h, regardless of whether Nutrient Agar (49.4 mm) or Wilbrink Agar (53.3 mm) was used to grow the pathogen. It is likely that the variations in the inhibition halos between each sampling time obey the particular growth phase of the LAB at that moment and that the inhibitory effect is potentiated in the exponential phase. This hypothesis also applies to the ACR and MRSG substrates, in which high antimicrobial activity was also observed after 48 h fermentation when the pathogen was plated on Nutritive Agar and Wilbrink Agar, respectively.

The supplementation of the commercial substrate with 40 g L^{-1} glucose visibly increased the production of lactic acid. The lactic acid production in the ACR substrate was slower than that obtained in the MRSG substrate, although good acid concentrations were obtained at the end of the process and likely exhibited an inhibitory effect toward the end of the fermentation. This can be explained from investigations Alakomi et al. (2000) who concluded that the lactic acid can permeate the cell membrane of Gram negative bacteria, and this allows entry of antimicrobial compounds as antibiotics, detergents and bacteriocins. According to Wee et al. (2006), many factors, such as ammonium salts and P and K ions, have a stimulating effect on the growth of the microorganism and on the production of lactic acid. These elements are likely present in the ACR substrate because it is derived from an organic source that is rich in nutrients and supplemented with glucose and yeast extract. According to Cocaign-Bousquet et al. (1995), because these substrates are obtained from plant material, the nucleic acid content provides cytosine, adenine, guanine, and uracil bases that have a growth-promoting effect on *L. lactis*. Effat et al. (2001) indicated that the supplementation of fermentation substrates with other elements, such as glucose and yeast extract, benefits the production of the antimicrobial compound. Nevertheless, some authors report that the sugar concentration considerably affects the lactic acid production of strains such as *L. lactis* ssp. *lactis*. Akerberg et al. (1998) obtained 86 g L^{-1} lactic acid with an initial sugar concentration of 180 g L^{-1}. Serna and Rodríguez (2007) obtained 40.5 and 81 g L^{-1} lactic acid with initial concentrations of 50 and 100 g L^{-1}, respectively.

The biomass production was affected by the type of substrate and the fermentation time. In the MRS and MRSG substrates, higher biomass production levels in CFU mL^{-1} were obtained compared with that in the ACR substrate. This result demonstrates that minor components, such as vitamins, minerals, amino acids, and C sources other than glucose, have an important influence in cell replication (van Niel and Hand-Hägerdal, 1999; Panesar et al., 2007). In addition, the sugar concentration also affected the formation of biomass: the values obtained with the MRS and MRSG substrates were 2.67 and 3.37 g L^{-1}, respectively. This result is explained by the immediate availability of the minor components that are necessary for the cell division of the LAB in the MRS and MRSG substrates. The comparison of the MRS and MRSG substrates indicates that *L. lactis* responds to the increment in C source by increasing its biomass concentration. The lower biomass levels obtained with the ACR substrate are primarily due to the presence of elements in the substrate that can inhibit the microorganism. Several authors, such as Canli and Kurbanoglu (2002), have reported that elements, such as Fe, present in the fermentation substrate can have an adverse effect on the microorganism and inhibit its growth. The presence of a high concentration of salts increases the osmotic pressure in the cells and causes their disruption (Divya et al., 2011). However, studies, such as those conducted by Taskin et al. (2011), show that elements, such as S and P, that are present in the fermentation substrate exhibit a protective effect toward the cell because these favor the formation of the phospholipid membrane and regulate the exchange of substances into the cell. Therefore, although we did not obtain the highest biomass levels with the ACR substrate, a protective effect in the cells was observed due to the consumption of certain elements that are present in this substrate. This protective effect increased the viability and the lactic acid production of the LAB toward the end of the fermentation process.

Several authors, such as Serna and Rodríguez (2005), isolated a strain of *Lactococcus lactis* ssp. *lactis* from the same sugarcane variety that was used in this study and under the same process conditions, with the exception of the agitation speed (120 rpm). These researchers reported a substrate consumption of 90.26% and a lactic acid production of 32.6 g L^{-1} after 48 h fermentation. This result suggests that the effect of agitation on the substrate consumption, lactic acid generation, production of bacteriocins, biomass increase, and antimicrobial effect must be evaluated. Tanyildizi et al. (2012) found that glucose concentration and agitation speed are very important variables in lactic acid production. The agitation of the culture ensures the correct distribution of the nutrients in the medium but can also cause the mechanical disruption of cells.

The ACR substrate induced levels of lactic acid production and antimicrobial activity that were comparable to those obtained with the MRS and MRSG substrates. It can thus be deduced that the ACR substrate, which costs 4.5-fold less than the commercial substrates, can potentially be used for the growth of *L. lactis* ssp. *lactis*.

CONCLUSIONS

Lactococcus lactis ssp. *lactis* could become a biological controller of *Xanthomonas albilineans*, which is the pathogen that causes leaf scald in sugarcane, because the bacterium demonstrated *in vitro* antimicrobial activity against this pathogen. This lactic acid bacterium could be produced in a low-cost substrate that is formulated from sugarcane crop waste because these residues are a good C source for the lactic acid bacterium and do not affect the production of the antimicrobial compounds against *X. albilineans*. In addition, it was demonstrated that the antimicrobial activity of *L. lactis* ssp. *lactis* does not depend on the growth substrate used in the fermentation. In conclusion, the results obtained with *L. lactis* ssp. *lactis* significantly increase the spectrum of applications of lactic acid bacteria in the phytopathology field.

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