Antimicrobial activity and fermentation kinetics of *Weissella confusa* against *Xanthomonas albilineans*

Actividad antimicrobiana y cinética de fermentación de *Weissella confusa* contra *Xanthomonas albilineans*

Liliana Serna-Cock, Andrés Felipe Camargo-Guarnizo and Carlos Andrés Rengifo-Guerrero

Departament of Engineering, Faculty of Engineering and Management, Universidad Nacional de Colombia - Palmira. Carrera 32 12 - 00 vía Candelaria. Corresponding author: lserna@unal.edu.co

Rec.: 22.01.13 Acept.: 18.09.13

Abstract

The antimicrobial activity of *Weissella confusa* against *Xanthomonas albilineans,* microorganism that produces leaf scald in sugar cane, was evaluated. The fermentation kinetics of lactic acid bacteria in commercial substrate (MRS) and agricultural waste sugar cane harvest (RAC) was measured. Submerged fermentations were performed at 32 °C for 4 hours, pH 6 and agitation of 100 rpm, and every hour the biomass production, substrate consumption and antimicrobial activity was measured. *Weissella confusa* showed antimicrobial activity against phytopathogenic bacteria *X. albilineans* in MRS and RAC as carbon sources, the inhibition halos diameters were 18.94 and 16.77 mm, respectively. *Weissella confusa* although not being isolated from sugar cane, showed excellent growth on the RAC substrate compared with the commercial substrate MRS. Therefore, it can be suggested *W. confusa* as a potential for biocontrol of leaf scald sugar cane, however, should be evaluated the mechanism of action and their biological and economic impacts. The RAC substrate becomes an economical alternative for the production of lactic acid biomass.

Key words: Acid lactic bacteria, agricultural wastes, sugar cane.

Resumen

Se evaluó la actividad antimicrobiana de *Weissella confusa*, una bacteria ácido láctica, contra *Xanthomonas albilineans,* microrganismo productor de la escaldadura de la hoja en la caña de azúcar. Se midió la cinética de fermentación de la bacteria ácido láctica en sustrato comercial (MRS) y en residuos agrícolas de cosecha de caña de azúcar (RAC) provenientes de semilleros con 7 meses de edad. Se realizaron fermentaciones sumergidas a 32 °C, cada hora hasta 4 horas, a pH 6 y 100 r.p.m. de agitación. Cada hora se midieron la producción de biomasa, el consumo de sustrato y actividad antimicrobiana. *Weissella confusa* presentó actividad antimicrobiana contra la bacteria fitopatógena *X. albilineans* en ambos sustratos, los halos de inhibición presentaron diámetros de 18.94 y 16.77 mm, respectivamente. *Weissella confusa,* aunque no fue aislada de caña de azúcar, mostró excelente crecimiento en el sustrato RAC comparado con el sustrato comercial MRS; por tanto, puede ser un posible biocontrolador para la escaldadura de la hoja de caña de azúcar, no obstante, se deben evaluar sus mecanismos de acción e impactos biológico y económico en la producción de biomasa ácido láctica.

Palabras clave: Bacterias ácido lácticas, caña de azúcar, escaldadura de la hoja, residuos de cosecha.

Introduction

Xanthomonas albilineans is a bacterium that is pathogenic, flagellated, Gram negative, G^-, with the capacity of invading the xylem of plant tissue and producing the phytotoxin albidicin. This bacteria blocks chloroplast differentiation and produces white stripes parallel to the main nerve of the leaves of sugarcane, a characteristic symptom of the leaf scald (Hashimi and Birch, 2010). This disease was first detected in 1911 in Australia and nowadays is found widely in USA, Mexico, Venezuela, Cuba, Honduras and Colombia, among others (Contreras *et al.*, 2004). Transmission of *X. albilineans* happens mainly by the cutting tools and irrigation equipment (Blanco *et al.*, 2010). Control methods are based mainly in thermic treatments and in the development of new sugarcane varieties (Victoria-Kafure and Guzmán, 1998; Huerta-Lara *et al.*, 2003).

Lactic acid bacteria (LAB) are a possible alternative to fight leaf scald of sugarcane and have shown to be antimicrobial agents against Gram positive (G^+) and Gram negative (G^-) bacteria and some fungi. Jalali *et al.* (2012) tested the antagonist effect of *Lactobacillus* species against *X. campestris* PTCC 1473, a pathogen of tomato and soybean crops. Hamed *et al.* (2011) evaluated in vivo the efficiency of *L. plantarum* as a biological control against *Fusarium oxysporum* in tomato crops. The success of the LAB with antimicrobial and antifungal effect is achieved mainly because of the production of ribosomal synthesis peptides known as bacteriocins (González *et al.*, 2003). These bacteriocins are regulated by the quorum sensing mechanisms of cellular fluctuation which has two main components: an auto-inducing peptide, in charge of detecting the cell density through chemical signals and, a response regulator that synthesizes, transport and regulate the bacteriocin formation (Kleerebezem, 2004; Miller and Bassler, 2001).

Among the LAB group, the *Weisella* genus has been identified by its potential as antimicrobial agent. This genus takes advantage on the production of Bacterion Like Inhibitory Substances (BLIS) that has been proved against different microorganisms, both Gram- and Gram+, pathogens of plants, animals and humans, among them: *Staphylococcus aureus*, *Streptococus agalactiae*, *Candida albicans*, and *Escherichia coli* (Espeche *et al.*, 2009; Serna *et al.*, 2010). LAB are nutrient demanding microorganisms that need complex nutrient sources for their optimal growth (Savijoki *et al.*, 2006). In the biotechnology industry the substrates for microbial growth often represent the highest cost on biomass and its bioproducts production (Kurbanoglu and Algur, 2002); among them are the peptones and other nitrogen sources like yeast extract (García *et al.*, 2010).

Sugarcane crop produces agricultural residues (SARs) with high potential to be used as substrate source. According to Serna and Rodriguez (2007), these residues have water content close to 75% and nutrients like total sugars, nitrogen, phosphorus, calcium and magnesium, which are essential elements for fermentation substrates for microbial growth.

The objective of this study was to evaluate the kinetics of the antimicrobial activity of *W. confuse* against the plant pathogenic bacteria *X. albilineans* and, to measure the kinetics of substrate consumption and biomass production when the commercial compound (MRS) and the sugarcane agricultural residues (SARs) are used.

Materials and methods

Xanthomonas albilineans isolation

Isolation of *X. albilineans* was done from leaves and stems of 5 months old sugarcane CC 85-92 variety, collected in crops localized in the Colombian Sugarcane Research Center (Cenicaña). These samples were moved to the laboratory and stored under cooling control conditions.

Leaf samples were washed with 2% sodium hypochlorite solution and distilled water; next, they were cut in 1 cm^2 pieces and macerated until a lixiviate was obtained in order to get serial dilutions from 10^0 to 10^{-6}. Each dilution was plated by streaking method in nutrient agar plates (Oxoid, England) which were incubated at 27 °C for 72h. The colonies that presented the typical morphological characteristics of *X. albilineans* (Table 1) were replicated in the same media till getting a pure culture. The promising *X. albilineans* colonies were plated on solid media using Wilbrink agar (5 g bactopeptone, 10 g sucrose, 0.5 g $K_2HPO_4.3H_2O$, 0.5 g $MgSO_4$. $7H_2O$, 0.25 g Na_2SO_3, 15 g Bactoagar, 1 l distilled water) (Wilbrink, 1929), and YDC agar (20 g calcium carbonate, 10 g yeast extract, 15 g agar-agar, 20 g dextrose and, 1 l distilled water). Morphological confirmation of the bacteria was performed according to the characterization described by Contreras *et al.* (2004) (Table 1) and the strains that presented the typical characteristics in both culture media were cryopreserved to perform, later, pathogenicity and antimicrobial activity tests.

Fermentation substrates and conditions

The cryopreserved *W. confusa* strain used was obtained in research performed by Serna *et al.* (2010). This strain was adapted to each one of the substrates (MRS and SARs) during three generations using 10% of the inoculum in respect to the working volume. To produce *W. confuse* and to measure its fermentation kinetics, six batch fermentations were performed using the commercial substrate MRS (DeMan *et al.,* 1960) and other substrate formulated with juices of sugarcane agricultural residues (SARs). SARs came from 7 month old nurseries selected as substrates because they are good sources of carbon, nitrogen and vitamins that contribute to the growth of lactic acid bacteria; additionally they can be alternative substrates of low cost for production and marketing of the microorganism (Godon *et al.,* 1993; Brizuela, 1986). In order to get the equivalent values of nitrogen presented in the commercial substrate MRS, SARs were supplemented with yeast extract. Juices coming from the SARs were characterized for its nutrient value by determining elements such as total nitrogen (Kjeldahl, 1883) and potassium by atomic absorption and, reducing sugar content by the DNS method (3,5-dinitrosalicilic acid) (Miller, 1959). Fermentation was periodically adjusted to pH 6.0 using a NaOH 1M solution (Mol Labs® - Bogotá, Colombia).

Kinetics for biomass formation, substrate consumption, lactic acid production and biomass yield

To measure the kinetics for biomass formation, substrate consumption and lactic

Table 1. Characteristics of *Xanthomonas albilineans* pure growth in different growth media

Growth media	Characteristics
Nutritive agar	Bright, rounded and yellow colonies. Moderate growth.
YDC agar (yeast extract, carbon, calcium, dextrose)	Bright, rounded and yellow colonies. Good growth
Wilbrink agar	Bright, rounded and yellow colonies. Moderate growth.

Source: Contreras *et al.*, 2004.

acid production 5 ml fermented samples were taken aseptically from each of the substrates at the beginning (hour 0) and at 1, 2, 3 and 4 h of fermentation. The samples were centrifuged at 5000 rpm for 10 min at 4 °C (Eppendorf Centrifuge – 5804R, Germany) to separate the biomass from the supernatant.

Biomass concentration was established by dry weight (A.O.A.C, 1990). In the supernatant the reducing sugar concentration was determined by spectrophotometry with 3,5-dinitrosalicilic acid (Miller, 1959) (ThermoScientific – Genesys 10UV, USA). Lactic acid production was measured by reflectometry *(Reflectoquant* Merck - RQflex Plus 10, Germany*).* Supernatant was filtered (0.45µ) before the measurement (Titan, USA).

The kinetic parameters calculated were biomass yield ($Y_{x/s}$) and substrate consumption (*CS*) using the equations 1 and 2, respectively.

$$Y_{x/s} = \frac{X_o - X}{S_o - S}\ g/g^{-1} \quad (1)$$

$$CS = \frac{(S_o - S) * 100}{S_o}\ \% \quad (2)$$

where, *S0* is the initial concentration of reducing sugars (g/l), *S* is the final concentration of reducing sugars (g/l), *X0* is the initial biomass concentration (g/l) and, *X* is the final biomass concentration (g/l). Additionally, the specific growth velocity µ was calculated using the slope of the exponential phase in the LAB growth curve.

Antimicrobial activity kinetics

The antimicrobial activity tests were performed at the beginning and at 1, 2, 3 and 4 h of fermentation in the previously described substrates.

For these measurements the diffusion in agar methodology described by Serna *et al.* (2010) was used. Plates with 5 mm thick nutritive agar were used with central wells of 1.7 cm of diameter using a sterile cork borer. Plates were massively sown using 10 µl of *X. albilineans* suspension with 1 x 10^8 UFC m/l concentration. In the same way, sterile disks of MRS agar of 5 mm thickness and 1.7 cm diameter were placed in the central wells. Disks were inoculated with 60 µl of the fermented sample taken from each substrate at the different fermentation times. Dishes were incubated at 27 °C for 24 h and later, the antimicrobial activity was determined by measuring the diameter of the inhibition halos on the pathogen growth. The antimicrobial activity tests against *X. albilineans* were done in duplicate for each fermentation time and for each substrate.

Statistical analysis

A unifactorial design with three replicates was used, using as factor the substrate type with two levels, commercial substrate MRS and SARs. Response variables were the kinetics of biomass production, substrate consumption an antimicrobial activity. Response variables were measured at the beginning (time 0) and at 1, 2, 3, and 4 h of fermentation. Additionally, it was calculated the biomass yield ($Y_{x/s}$) and the specific velocity of growth µ from the growth curve slope at the exponential stage. For data analysis the SAS (System Analysis Software) and the Tukey´s ($P < 0.05$) statistical test to compare means were used.

Results and discussion

In Figure 1 are shown the biomass production and consumption of *W. confusa* in the commercial substrates MRS and SARs. Biomass production did not show differences ($P = 0.8882$) between the evaluated substrates (Table 2). In the SARs substrate the highest biomass concentration was produced at the 3 and 4 hours of fermentation, with concentrations of 1.36 y 1.496 g/l, respectively, whereas in the commercial MRS substrate were produced 1.17 y 1.38 g/l of biomass in

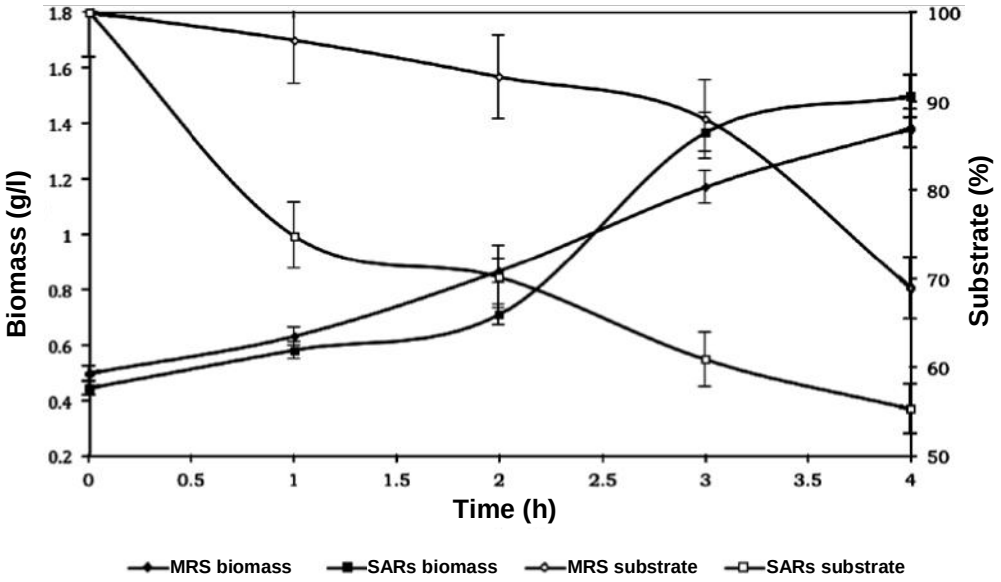

Figure 1. Kinetics of growth and substrate consumption of *Weissella confusa* in commercial substrates MRS and SARs.
Bars in each value indicate the standard deviation of the mean.

the same fermentation times.

Substrate consumption showed differences (P < 0.05) between substrates and the interaction time x substrate. In the MRS broth the substrate consumption rate was slow during the first 3 h of the experiment and it increased in the fourth hour, with a consumption of sugar content close to 30%. On the other hand, in the SARs accelerated sugar consumption was evident at the beginning of the process, with consumption close to 45% by the fourth hour of fermentation. In the Table 2 are included the biomass yields in relation to the substrate ($Y_{x/s}$) being higher with the MRS substrate (0.15 g/g) when compared to the ones obtained by SARs (0.07 g/g). The specific velocity of growth μ was higher with the SARs substrate (0.142/h) compared to the MRS substrate (0.115/h) (Table 3). These results indicate that the biomass concentration was equal in both substrates; however due to the higher sugar content and accelerated consumption in SARs, the specific velocity of growth was higher, reaching equal biomass concentrations to the one obtained with the MRS substrate at the end of the fermentation. The latest indicates that the SARs substrate could be a competitive carbon source for lactic acid biomass production, with a potential to be used as substitute of the conventional media for bacterial growth.

Ossa *et al.* (2010) found similar results to

Table 2. Substrate consumption, biomass production and antimicrobial activity of *Weissella confusa* against *Xanthomonas albilineans*.

Substrate	Substrate consumption	Biomass	Antimicrobial activity
MRS	16.15 a*	0.9146	1.79
SAR	41.04 b	0.9193	1.61

MRS = commercial substrate. SAR = substrate based on agricultural residues of sugarcane harvest.
* Means on the same column followed by the same letter are not different (P > 0.05), according to the Tukey´s test.

Table 3. Kinetic parameters of *Weissella confusa* for the commercial substrates MRS and SARs.

Parameter[a]	Medium	
	MRS	**SAR**
Yx/s (g/g)	0.150	0.070
μ (/h)	0.115	0.142
CS (%)	31.08	44.73

[a]Biomass yield *Yx/s*, specific velocity of growth μ and percentage of substrate consumption.

the ones of this study when they obtained an increase in *L. plantarum* biomass production from 10% to 30% w/v of sugar concentration. Serna and Rodriguez (2007) determined the biomass production for *Lactococcus lactis* spp *lactis*, using agricultural residues of sugarcane with sugar concentrations around 90 and 60 g/l, and the biomass production obtained was 2 and 1 g/l, respectively (Serna and Naranjo, 2005).

In Figure 2 is observed the antimicrobial activity of *W. confusa* against *X. albilineans* in the substrates used. The statistical analysis (Table 2) indicates that there are no differences between substrates (P = 0.1115). The largest inhibition halo was presented in the commercial substrate MRS with a diameter of 18.94 mm at 4 h of fermentation. In the SARs substrate the highest antimicrobial activity was presented also at 4 h of fermentation with a diameter of 16.77 mm. Moreover, in the MRS substrate the antimicrobial activity was proportional to the time of fermentation when *W. confusa* reaches its exponential activity. These results showed that *W. confusa* in MRS media has an antimicrobial activity against *X. albilineans* and that the antagonist effect was affected by the time of fermentation in the commercial substrate MRS but, this is not true for the SARs substrate in which there were no differences (P > 0.05) in fermentation between the first and forth hour. Afdora *et al.* (2010) found that during the logarithmic phase antibacterial substances

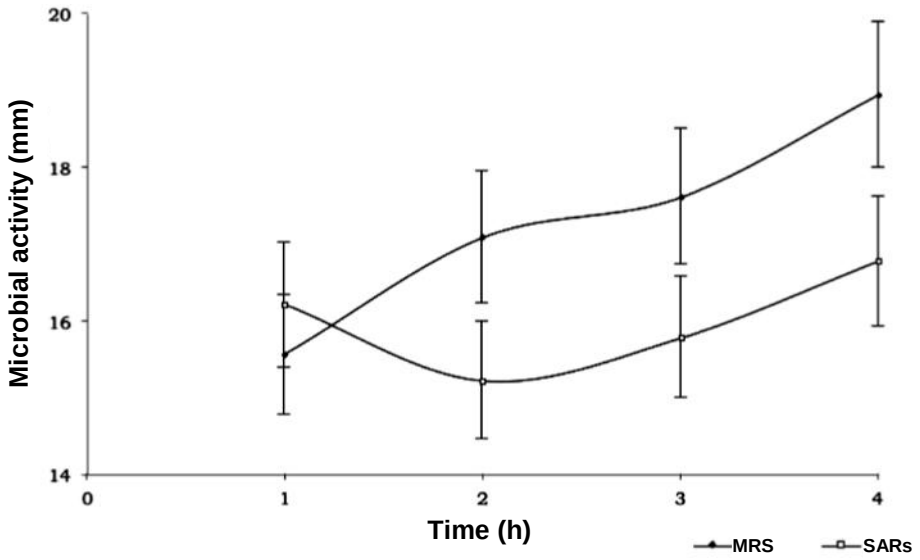

Figure 2. Microbial activity of *Weissella confusa* against *Xanthomonas albilineans* on commercial substrates MRS and RAC.
Bars in each value indicate the standard deviation of the mean.

like lactic acid and bacteriocins are produced, therefore, during that phase is reached the maximum concentration of these metabolites.

In several studies it has been observed that there is an antagonism of the *Weisella* genera against several types of pathogenic microorganism by production of bacteriocins; Pal and Ramana (2010) found antimicrobial activity of a purified bacteriocin from *W. paramesenteroides* against several G^+ and G^- bacteria like *E. coli, P. aureginosa, Salmonella typhimurium* and *Vibrio parahaemolyticus*. Chavasirikunton *et al.* (2006) revealed an antagonisms of the *W. confusa* CP3-1 bacteriocin against *B. cereus*. Srionnual *et al.* (2007) found an inhibitory effect of a bacteriocin produced by *W. cibaria* against pathogen G^+ bacteria and, LeBlanc and Todorov (2011) found antimicrobial activity in bacteriocins from *Weissella* sp. against several pathogenic microorganisms.

Some biological treatments based on bacteriocins derived from lactic acid bacteria have been proved for the treatments of pathogenic microbes for humans (Roldán *et al.,* 2011; Estrada *et al.*, 2005); however, there is no information of lactic acid bacteria with antimicrobial activity against plant pathogenic microorganisms. To date, the biological control of these is done by G^- bacteria, for example: Reinoso *et al.* (2006) and Reyes *et al.* (2011) found that *Basillus subtilis* controls *P. carotovorum* and *Macrophomina phaseolina* (Tassi) Goid which are the causal agents of the soft rot of potato and the rot of corn, respectively. *Gluconacetobacter diazotrophicus* has antimicrobial activity against *X. albilineans* (Piñon *et al.,* 2002; Arencibia *et al.*, 2006), therefore, this work opens a new research and application field for lactic acid bacteria.

Conclusions

- It was found that *W. confusa* has antimicrobial activity against *X. albilineans*, independently of the substrate type used, MRS or SARs.
- The substrate of sugarcane agricultural residues (SARs) demonstrated that it is a good source to grow *W. confusa* compared to the commercial substrate MRS. The inhibitory effect of *X. albilineans* was affected by the time of incubation. In both substrates types the higher biomass concentration was found at the third and fourth hours of fermentation.
- In the future, the studies should be focused on the evaluation of new lactic acid bacteria against plant pathogens, on the inhibition mechanisms, methodology development for biological control of plant diseases and formulation of substrates for growth at low cost.

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