PGTF
THE PEREZ-GUERRERO TRUST FUND FOR ECONOMIC AND TECHNICAL COOPERATION AMONG DEVELOPING COUNTRIES

FINAL REPORT
2008-2010

Code of the project: 00061568

Title of the project: "Increase in sugar production by microbial inhibition of *Leuconostoc* sp. and other bacteria (Phase II)" ISPLI- II

Coordinator: Cuban Research Institute for Sugar Cane By-products (ICIDCA).

Description of the consortium:

ICIDCA	ICIDCA	Cuba
EEAOC	EEAOC	Argentina
	IBT/UNAM	Mexico
UNAERP	UNAERP	Brazil

Head of the Project: Dra. Georgina L. Michelena Alvarez, ICIDCA

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I. Organization of the ISPLI (Phase II) Project

a) Project objectives:

This project is a follow up of a previous one funded by PGTF (cf. project INT/03/K09), which result in the development of a microbial inhibitor (called inhibitor "S") that not only inhibited the microbial activity but also the sucrose losses taken place by the deterioration of the cane juices in the clarifier during the stops of the milling are also decreased.

In this project the following consequences were brought:

1. To develop and validate the product (inhibitor S) in the sugar industry.
2. To have a commercial product that allows reducing the sucrose losses for deterioration of the cane juices.
3. To develop toxicological studies needed for the commercial registration of the product.
4. To register the product and to undertake market studies.
5. To design a technological scheme of application in the sugar mill based on the microbiological interaction between *Leuconostoc* and other bacteria with microbial metabolites, defining dosage, conditions and application points specific for each sugar mill.
6. To generalize toward other sugar mills the use of the product. To extend validation of the product.

The outputs determined by the studies of the project aim at a reasonable balance among economic and environmental considerations. This goal was partially supported by the project funds of national institutions and enterprises related and associated.

This result extends to other sugar mills with the objective of obtaining validation of the most representative use in the sugar industry with a view to achieve the introduction of the product in the usual practice of the disinfection process and during the stops of the sugars mills.

b) Project Outputs.

The outputs of the project were:

1. To generalize toward other sugar mills the use of the product. To extend validation of the product with a technological methodology for the application in sugar mill based on the microbiological interaction of the *Leuconostoc* and other bacteria.

2. Optimization of fermentation conditions for the production of metabolites with inhibitory characteristic, testing different sugar substrates as: cane juice, juice of the filters, intermediate mollasses of the process, mollasses, etc. and other substrates sub-products of the dairy industry and oil.

3. Commercial brand of the products with inhibitory characteristic of the *Leuconostoc* and their properties is being registred. A patent and a publication were gotten.

The integrative nature of the project allowed that these results lead to the achievement of the proposed to medium term objectives that is in definitive, the increase of the production in the sugar mill and the sugar efficiency.

c) Project Activities

The activities of the project were distributed in 7 work packages, one of them being the coordination task.

Work Pack.	Work package title
0	**Project Management**
1	Microbial metabolites production from *Botryodiplodia* and *Actynomicetes* using sugar by-products. Optimization of fermentation.
2	Scale up of the microbial production of inhibitory metabolites
3	Chemical characterization of inhibitory microbial metabolites.
4	Registration and patents of the developed products. Exploratory search of market
5	Enzymatic studies of inhibition of the *Leuconostoc* starting from glucosyltransferases
6	Generalization toward other sugar mills. Extention of the industrial validation of the product.
7	Economical evaluation. Pre-feasibility study.

The partners have been working in the work packages since the First Project Meeting.

II. Evaluation of the objectives of the ISPLI (Phase II) Project

All the objectives conceived have been achieved. The work team climate was very good among all participants. Some dissemination activities have been done.
In the meetings the results achieved were discussed and some proposal for a CYTED, SETCIP and FAPESP Project were prepared looking for other financial contributions.

National projects in each country were also supporting the project tasks. The collaboration of some institutions which are not part of the project consortium is well valued. These institutions are:

- University National of Tucumán, Argentina
- Instituto de Bacterias Lácticas,CERELA, Tucumán, Argentina
- UNESP Araraquara, Brasil
- Centro Nacional de Energías Renovables, CIEMAT, Madrid, España
- Universidade Federal do Amazonas – UFAM, Brazil
- INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France

In the following pages the main technical activities of the Project are described.

In the sugar production stage from the mill station until the clarification of the juice, an important cause for the sucrose destruction is the microorganisms action accompanying sugarcane. The sucrose loss and the dextran formation are associated with the deterioration of the sugarcane. For years, this has been a problem that the industry has faced, becoming a challenge to improve the quality of the sugar. In this work, the effect of EVIPOL on sugar cane disinfection is presented, in the microbial tenor decrease and the indexes of purity, which impacts directly in a bigger yield of sugar.

Universidade Federal do Amazonas - UFAM;
Instituto Cubano de Pesquisa dos Derivados da Cana-de-Áçucar - ICIDCA/HAVANA-CUBA.
Universidade de Ribeirão Preto – UNAERP

Jasmonates are micro molecules that play a role in signaling the biosynthesis of secondary metabolites in plants, with various bioactivities, including the regulation of plant growth. Among these jasmonates, the (-)-jasmonic acid and (+)-7-isojasmonic acid are the most frequently found in plants. Other than plants, microorganisms such as the *Botryosphaeria* fungi are capable to produce jasmonic acid and other jasmonates. Thirty-six strains of *Botryosphaeria* isolated as tropical plant pathogens, such as: citrus, mango (*Mangifera indica*), mogno (*Swietenia macrophylla*), papaya (*Carica papaya*), cupuaçu (*Theobroma grandiflorum*) and timber were analyzed in order to verify their jasmonate production capacity. The derivatives produced after the cultivation of strains in liquid mediums and the selective extraction of filtrates free of mycelial mass were initially evaluated by Comparative Thin-Layer Chromatography (CTLC). Strains with a chemical profile of biotechnological interest were submitted to High Performance Liquid Chromatography (HPLC) for a quantitative evaluation of jasmonate production. Through the analysis of the molecular regions of the rDNA ITS, it was possible to identify all isolates studied as *Botryosphaeria rhodina.* The alignment of the DNA sequences between regions ITS1 and ITS4 showed slight polymorphism among the 33 lineages which were grouped into nine halotypes without any association with geographical origin as well as with jasmonate production.

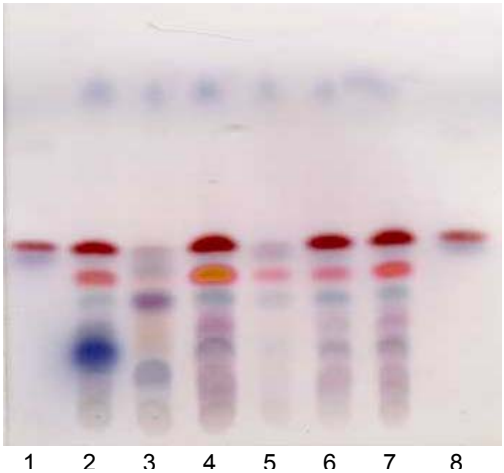
RESULTS AND DISCUSSION

JA production by the analyzed strains, evaluated by means of TLC can be observed in Table 2 and Figure 1.
Among the isolates screened for their potential to produce JA, 26 demonstrated production capacities. The quantitative analysis, by HPLC, showed the majority of lineages produce only traces of JA (Table 1). On the other hand, Mg01, Mg03, Mg12 and Kifn 3.1, revealed higher concentrations and may be considered as promising strains, where Mg03 and Kifn 3.1 were the most productive, with values of 123.6 and 283.2 mg/L respectively.

Table 1 – Jasmonic Acid Production by *Botryosphaeria sp.* strains

Strains	JA concentration (mg/L)	Strains	JA Concentration (mg/L)
Mg 01	99,0	Mg 26	4,4
Mg 03	123,6	Mg 27	4,2
Mg 06	21,8	Mg 28	1,65
Mg 08	4,7	Mg 29	1,2
Mg 09	4,2	IRO 2	Nd
Mg 10	4,4	Kifn 1.2	20
Mg 12	58,15	Kifn 1.3	50

Mg 14	8,3	Kifn 2.2	50
Mg 16	6,20	Kifv 2.2	Nd
Mg 17	1,75	Kifn 3.1	283,2
Mg 18	Nd	C 81	Nd
Mg 20	3,43	C 83	Nd
Mg 21	20,1	C 3.4.7.9	Nd
Mg 22	2,0	C 3.1.8.4	Nd
Mg 23	3,0	C 1517	400
Mg 24	2,43	C 2334	500
Mg 25	1,93		



1	JA standard
2	Mg 03
3	C 83
4	Kifn 3.1
5	Mg 16
6	C1517
7	C 2334
8	JA standard

Figure 1 – TLC of ethyl acetate extracts from fermentation broth of *B. rhodina* strains

In the chromatogram, JA appears with a retention time close to 10 minutes, and close to it, other substances appear, possibly jasmonates, (+)-7-isojasmonic acid ((+)-7-isoAJ) and cucurbitates, substances with biological activity similar to that of JA (11) (Figure 2).

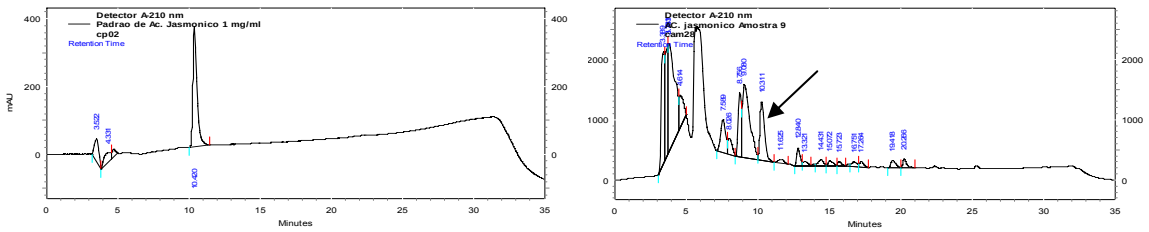


Figure 2 – HPLC profile a) JA standard, b) kifn 3.1 strain.The arrow points to JA peak in the analysed sample.

Influence of by-products Industrial Substrate as Carbon Sources in the Jasmonic Acid Production by *Botryodiplodia*.

Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar
Unidade de Biotecnologia Vegetal, Universidade de Ribeirão Preto - UNAERP

***Jasmonic acid production**:* JA was produced from *Botryodiplodia* sp. 83, Mg3, Kj3.1 and 2334 in 50 mL of medium in a flask of 250 whole volume with the following medium composition: sucrose 50 g/L, yeast extract 1 g/L, KH_2PO_4 2 g/L and traces with pH fixed in 5,5 with NaOH (1M). The use of cane molasses, starch and cellulose was assayed. Cellulose effect on the culture medium was set up according to the following experimental design:

	Low	Medium	High
Sucrose, g/L	0	25	50
Cellulose, g/L	0	25	50

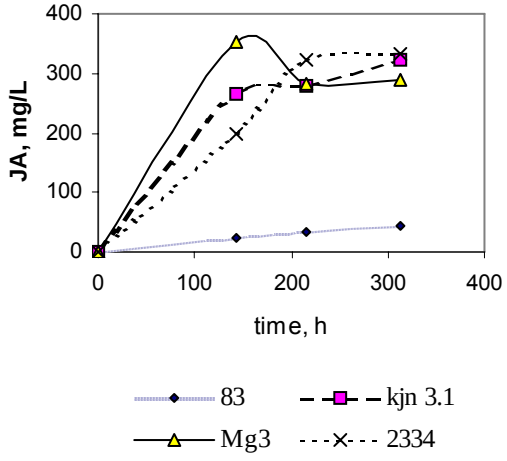
The inoculum was obtained in superficial growth on Petri dishes with 25 mL EMA seed from mycelium of inclined tubes. The Petri dishes were incubated for 5 days at 30°C. 1/8 of mycelial dish was put in each erlenmeyers. Fermentation was carried out at 30 ^{o}C for 13 days.

Jasmonic acid determination:
Mycelium was separated by vacuom filtration with Whatman 4 filter. Aliquotas of 5 mL from filtrated broth was fixed at pH 3.0 with HCl (4M) and were extracted with ethyl acetate (1:1). The fractions with JA were dehydrated with anhydrum sodium sulphate and rotoevaporated at 50^{O}C. For AJ determination an HPLC chromatograph HPLC SHIMADZU (LC-10AD) was used with a reverse phase column C_{18} Supelcosil (25 cm x 4,6 mm id, 5μm) with detector of variable wave lenght. A flux of 0.85 ml/min was used, wave lenght 210 nm and MeOH:acetic acid 0.1 %, 40:60. It was injected 20 uL.Biocrom software for Chromatography version 1.1 was used.

Experimental design and statistical analysis:
The effect of cellulose and sucrose in the culture medium was analysed according to the experimental design and it was processed by a computation statistical program (Statistical 5.0, 1996).

Kinetics of JA production shown in Fig. 3, indicates dependence with the strain during the production time.

Fig. 1 JA production from *Botryodiplodia* in submerged static culture.



It was obsserved at 144 h JA concentrations higher than 200 mg/L. In Table 2 is presented the productivity and yields for all strains assayed. It was observed that the maximun productivity was achieved in 2,21 mg/L-h by Mg3, two-fold higher than others strains. This results is at the same level that reported by Gunther and col. (Gunther et al, 1990).

Table 2. JA production in submerged static fermentation.

Strains	C máx$_{AJ}$ (mg/L)	P_{AJ} (mg/L-h)	$Y_{P/S,}$ (%)
83	45	0,144	0,09
kjn 3.1	322	1,03	0,64
Mg3	354	2,21	0,71
2334	334	1,07	0,66

In Table 3 is shown the effect of the use of sugar industrial substrate on JA production in strain 83 and 2334.

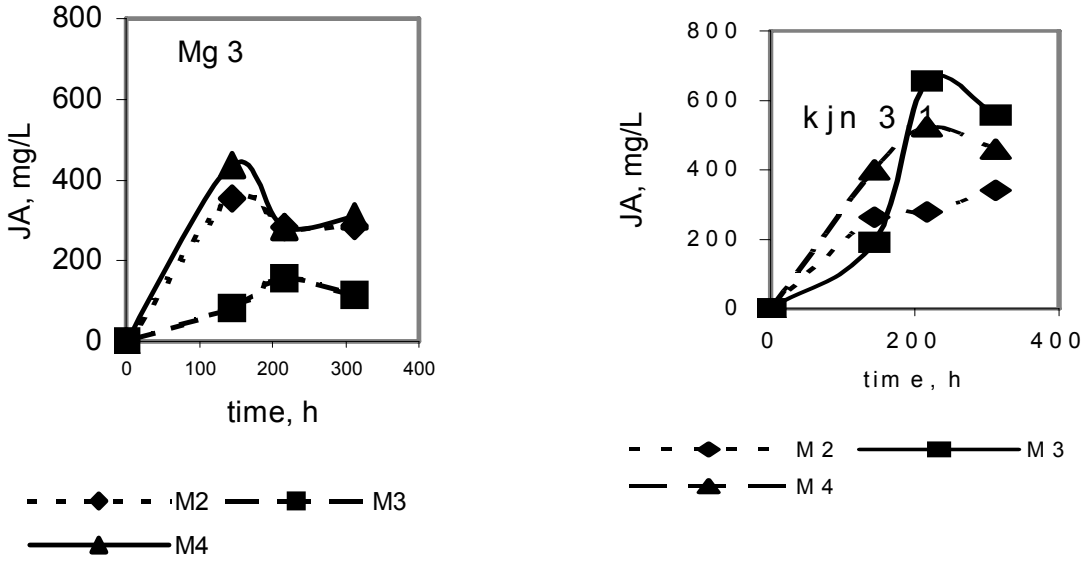
Table 3. JA production using sugar industrial substrate.

Strain	JA, mg/L
2334 sucrose	280
2334 cane juice	184
2334 mollases	45
2334 clarified mollases	103
83 sucrose	45
83 cane juice	102
83 mollases	298
83 clarified mollases	123

In strain 83 the highest JA production in mollases substrate was obtained with a production 6.6 times higher than the sucrose substrate. This fact confirms the difference observed by others autors in the cell metabolism of Botry*odiplodia* according to the substrate. On the other hand, it is demonstrated that mollasses is an excellent industrial substrate, rich in minerals, aminoacids and vitamins. Almazán and col. (Almazán,1982) had reported the factibility of sugar cane mollases as carbon sources in yeast.

Starch as substrate in the secondary metabolites production has been proved by other authors indicating its preference for achieving better productions in sugar limiting conditions. Figure 4 shows an assay with strain 83 and 2334 using diferent mediums.

It was observed higher JA concentrations by kjn 3.1 in 215 h in M3 with mollases. Mg 3 was increase in the productivity in M4.



In table 4 it is indicated the productive parameters for kjn 3.1 and Mg3 on different substrate. It can be seen that in M3 in kjn 3.1 and in M4 in Mg3 the productivity is higher than M2 in the same strain. This increase is 50 % higher to achieve than in M2.

Table 4. Productive parameters in JA production using M2, M3 and M4 in strain KJn3.1 and Mg3.

	Kjn 3.1			Mg3		
	M2	M3	M4	M2	M3	M4
C máx$_{AJ}$ (mg/L)	331	656	525	354	156	438
P$_{AJ}$ (mg/L-h)	1.06	3.03	2.42	2.45	0.72	3.04
Y$_{P/S}$ (%)	0.66	1.31	1.05	0.708	0.31	0.87

It is a normal practical to wait benefits in the metabolites production putting the microorganism in its natural medium for increasing its productive capacity.
On the other hand, the sugar cane bagasse contituiting by cellulose represent a sugar by-product industry of low price and high disponibility. From that, it was verified if strain kjn3.1 native of wood trees increase its JA production in presence of cellulose.
Polinomial models derived from statistical processing of the results obtained in the experimental runs and converted to original variables was the following:

$JA\ (mg/L) = 0{,}1675 + 0{,}1555\ S - 0{,}061\ S^2 + 0{,}074\ S.\ C^2 + 0{,}071 S^2.C$
$r^2 = 0.997$

where S: sucrose concentration, g/L
C: cellulose concentration, g/L

From the model, it can be observed that the increase of both variables has a value that maximizes the JA production. The polinomic model corresponds to the surface shown in Fig. 4. From this, it can be inferred that sucrose and cellulose act in favor to AJ production by strain 3.1. It is demonstrated that this strain is able to metabolize the cellulose and increases the JA production in 15 % comparing when sucrose is using alone. However, the sucrose addition at 50 g/L were decisive for the JA production, not so when cellulose was assayed alone.

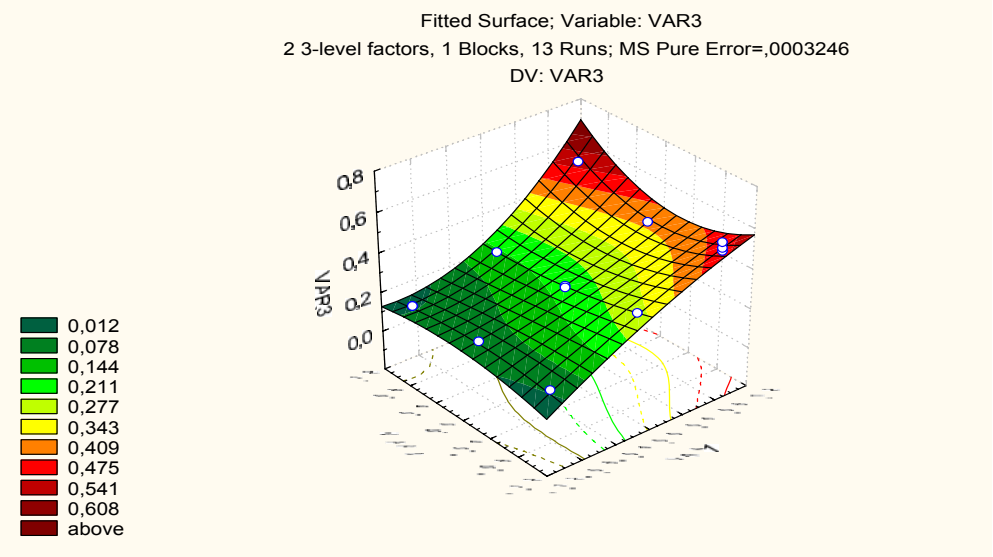


Fig. 4. Surface for polynomic model sucrose-cellulose in the JA production from kjn3.1.

CONCLUSIONS

The advantages of using mollases (M3) and starch (M4) respect to sucrose (M2) in strain kjn3.1 and Mg3 are demonstrated. The maximum increase in the productivity was 50 % higher than the achieved with sucrose (M2).

It was demontrated that kjn3.1 and Mg 3 produce 600 mg /L of jasmonic acid when by-products substrate of industrial are used like: mollases, cellulose and starch as alternative carbon sources. Biotechnology processes with these microorganisms are economically feasible and industrially atractive.

Determination of the bacteriocin effect on L*euconostoc mesenteroides* growth and in the production of dextran

University National of Tucumán, Argentina

Instituto de Bacterias Lácticas,CERELA, Tucumán, Argentina

Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar (ICIDCA)

For this purpose, two strains belonging to the culture collection of ICIDCA, Cuba, were studied, as well as another located in the Reference Center for Lactobacilli (CRL), Argentina. Such microorganisms are detailed in Table 5.

-Microorganisms used

Table 5. Bacteriocine and *Leuconostoc mesenteroides* producing strains that were used in this study

CUBAN STRAINS		
SPECIE	**Strains**	**Origin**
Leuconostoc mesenteroides	B/110-1-2	Dextran Plant
Leuconostoc mesenteroides	B/110-1-3	Dextran Plant
ARGENTINE STRAINS		
Enterococcus mundtii	CRL35	Cheese

-Cultures conditions

The cultures of microorganisms were activated by three successive passages in LAPTg broth media and the one using Leuconostoc without agitation at 30 ° C.

-Culture Media

Media for Leuconostoc **Composition (g/L):** Sucrosse, 20; yeast extract, 5; triptone, 2.5; K_2HPO_4, 2.5; distilled water csp 1L.

Effect of the growth of Leuconostoc mesenteroides in the presence of bacteriocin

The effect of two strains of L. mesenteroides to observe the influence of the presence of the bacteriocin enterocin 35 in the growth of this microorganism, were evaluated. This bacteriocin is produced by Enterococcus mundtii CRL35

To observe this effect, the test was carried out using the spot on Lawn method. Cultures for each microorganism were inoculated at 1% without agitation, 30 º C and the corresponding culture media for 16 hours. For the formation of a suitable lawn of L. mesenteroides strains, the same concentrations used in the previous test bacteriocin activity were kept.

The effect of bacteriocin on the growth of L. mesenteroides strains was seen from a plate containing MRS and L media to a 0.7% agar. Different concentrations were performed (500, 250, 120, 60, 30, 15, 7.5, 3.9, 1.9 and 0.9 mg / mL) of a synthetic bacteriocin enterocin 35 (1 mg / mL) and were deposited on the plates.

Effect of bacteriocin enterocin 35 in the formation of dextrane by Leuconostoc mesenteroides strains

The cultures of the strains of L. mesenteroides were grown by streaking on solid culture media, Medium L-Agar and medium MRS-Agar (replacing glucose by sucrose), with the objective of studying the formation of dextran and to use them as controls.

The 16-hour-cultivation of E. mundtii was centrifuged (Eppendorf centrifuge 5418) to obtain the cell-free supernatant of bacteriocin enterocin 35. Supernatant is used, which is then filtered with 0.22 µ.

We used different dilutions of the bacteriocin 1/5, 1/10 y 1/20 (v/v) which were added to the culture medium. The cultures of L. were striated in order to observe inhibition of the formation of dextran.

RESULTS

Effect of *Leuconostoc mesenteroides* growth in the presence of bacteriocin

The two strains of L. mesenteroides used in this study showed impairment in their growth when the first three dilutions of synthetic bacteriocin enterocin 35 were tested. Despite the observed inhibition halos of these strains in both media, with the exception of strain L. mestenteroides 1-2 in the medium L, the results presented in Table 6 show that they are more resistant to the bacteriocin than the strain of Listeria innocua 7 used as control.

Table 6. Influence of synthetic bacteriocin enterocin 35 in the growth of Leuconostoc mesenteroides strains in L media and MRS

Culture Media	Strains	Inhibition Halo (mm) Concentrations									
		1	2	3	4	5	6	7	8	9	10
L	*Lm* 1-2	N.I	N.I	N.I	N.I	N.I	N.I	N.I	N.I	N.I	N.I
L	*Lm* 1-3	9	7	5	N.I	N.I	N.I	N.I	N.I	N.I	N.I
MRS	*Lm* 1-2	8	5	N.I	N.I	N.I	N.I	N.I	N.I	N.I	N.I
MRS	*Lm* 1-3	10	10	8	N.I	N.I	N.I	N.I	N.I	N.I	
BHI	*Li* 7	21	21	21	21	20	15	10	7	7	7

N.I: No inhibition (number) Lm: *Leuconostoc mesenteroides* Li: *Listeria innocua* 7
Concentrations (µg/mL): **1** (500), **2** (250), **3** (120), **4** (60), **5** (30), **6** (15), **7** (7.5), **8** (3.9), **9** (1.9), **10** (0.9)

On the other hand, it can be seen that in the two methods used, the best results were obtained with the MRS for both strains of Leuconostoc mesenteroides and there is an increased sensitivity of the strain L. mesenteroides B/110-1-3 to the strain B/110-1-2 bacteriocin.

Effect of bacteriocin enterocin 35 in dextran formation

To determine the influence of the bacteriocin on the formation of dextran by Leuconostoc mesenteroides strains, MRS and L media were used, replacing glucose by sucrose to favour the conditions for the formation of dextran in the latter culture medium, which were used as a control for the study with the bacteriocin. The dextran formed in both culture media can be seen in Figure 5.

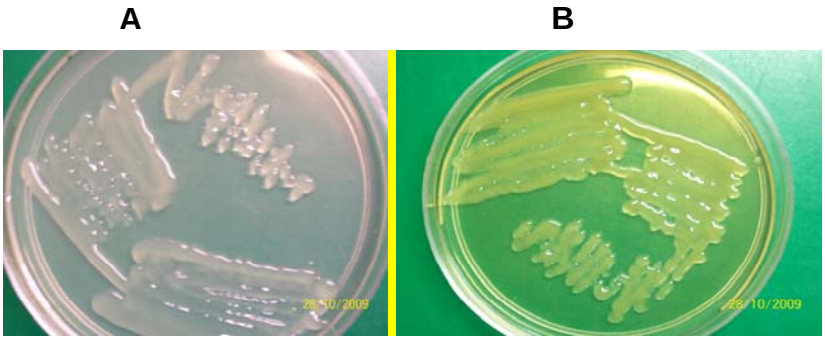


Figure 5: (A) Dextran formation in L media, **(B)** Dextrane formation in MRS with sucrosse media

When assessing the formation of dextran influenced by the bacteriocin, it was shown that none of the strains tested showed inhibition of the formation of dextran in the concentrations of the bacteriocins assessed.

In this sense, it can be concluded that the presence of bacteriocin diminishes the growth of Leuconostoc mesenteroides strains, but not the formation of dextran.

The study was carried out with Botryodiplodia 1517, (highest production strain in the Culture Collection at ICIDCA). Inoculum was prepared in Petri dishs with 25 mL of extract of malt-agar placing grooves of micelium grown inclined tubes for 3 days and incubated by 5 days at 30°C. 10 fragments miceliales of 8 mm were inoculated in erlenmeyers of 500 ml with 100 ml Miersch media used as preinoculum, and incubated at 30°C for 3 days.
The fermentation was in Marubishi bio-reactor of 5 L (MD-3005 L, Marubishi Ltd. Co, Japan, Fig. 6) at 30^{O}C, without agitation (only in the beginning and when the fresh media - 200 mL sucrose solution to 400 g/L) and 0.05 vvm for 15 days. The scale up effect was studied increasing the scale until large bottles of 50 L.

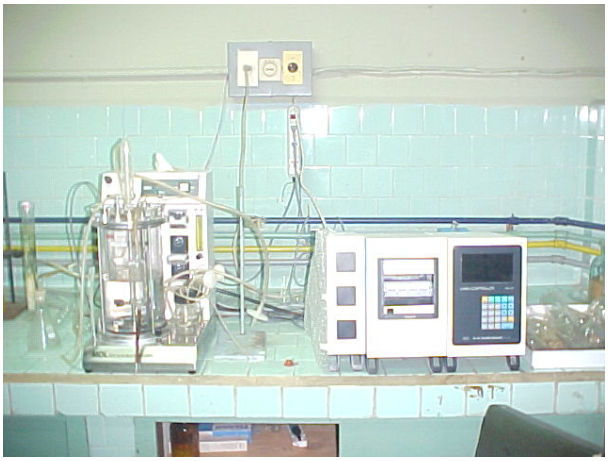


Fig. 6 Marubishi Fermenter (MD-3005 L, Marubishi Ltd. Co, Japón) for bank scale studies.

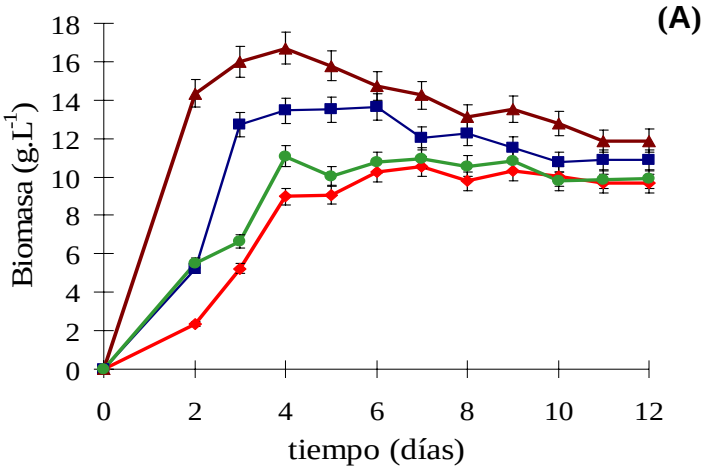
The agitation of the culture grown permits the dispersion of the biomass and provides from dissolved and nutritious oxygen to the cells in growth.
The agitation has been one of the parameters more studied in the jasmonic acid (JA) production with *Botryodiplodia theobromae* because certain speeds of agitation the JA production was affected by the production of other metabolites. Taking out mind these antecedents we proceeded to study the effect of the agitation in the JA production with *B. theobromae* 2334. The growth of the fungus measured by through the dry weight of the biomass, the JA production and the pH variation of the culture at different speeds of agitation (50, 100 and 150 r.min^{-1} with respect to the control without agitation) are shown in Fig.7. Although, the specific speed of growth (μ) was not the same in all the variants (static: μ = 0,670 ± 0,008 d^{-1}; 50 r.min^{-1}: μ = 0,455 ± 0,027 d^{-1}; 100 r.min^{-1}: μ = 0,078 ± 0,016 d^{-1} and 150 r.min^{-1}: μ = 0,339 ± 0,033 d^{-1}), in all the cases the growth reached the stationary phase before or four days of fungus cultivation (Fig.7A). The biggest production of biomass (16,69 ± 0,66 g.L^{-1}) was obtained to a speed of agitation of 100 r.min^{-1}. To 50 r.min^{-1} the concentration of maximum biomass was of 13,65 ± 0,44 g.L^{-1}, value that corresponds to 82% of the biomass taken place in the variant with agitation to 100 r.min^{-1}. To 50 r.min^{-1} and in static the maximum values even were smaller than to 50 r.min^{-1}. The reduction in the biomass concentration after the fourth and sixth day of cultivation were increased in the variants at 100 and 50 r.min^{-1}, respectively.

In all the cases, JA production began when the stationary phase was reached (Fig. 7B), being appreciated two behavior patterns: in the static cultivation, the JA production began to the five days of cultivation reaching a maximum of 867 ± 43 $mg.L^{-1}$ to the ten days of cultivation. In the other variants, JA production reached maximum values of 356 ± 18; 222 ± 11 and 286 ± 14 $mg.L^{-1}$ at 50; 100 and 150 $r.min^{-1}$, respectively; after eight days, and were diminished up to 88 ± 4; 35 ± 2 and 69 ± 3 $mg.L^{-1}$ at 50; 100 and 150 $r.min^{-1}$, respectively, at the end of the experiment.

Regarding the pH of the cultivation media can be appreciated that product of the fungus metabolism (Fig.7C) the pH diminished from 5,5 until values of 3,75 after two days of cultivation, then was increased until values between 6 and 7 for the upset variants and for the variant without agitation reached a value of 8,69 ± 0,43.

The effect of the speed of agitation in the yields of the biomass and JA production (Yx/s), (Yp/s) with regard to the sucrose consumption is shown in Fig.8. The Yx/s reached a maximum value of 0,333 ± 0,003 $gbiomass.g^{-1}$ from sucrose to 100 $r.min^{-1}$, then in descending order the value from Yx/s to 50 $r.min^{-1}$ and lastly the values of 150 and 0 $r.min^{-1}$ without significant differences between the last one. The biggest Yp/s (0,0181 ± 0,0003 g sucrose $AJ.g^{-1}$) was obtained in the static cultivation, while in the other variants the values were inferior with significant differences to the different speeds of agitation.

The agitation of the cultivation of *B. theobromae* 2334 was not favored the JA production (Fig. 8) with regard to the control in static condition. The decrease of the JA production in the upset variants with regard to the control could be related with the production of a polysaccharide extracellular that increased the viscosity of the media cultivation at first sight starting from the day ten and until the end of the experiment. In fact, Günther et al (1989, 1990) reported that the JA production with *B. theobromae* D7/2 in reactor should not surpass the value at 190 $r.min^{-1}$ because the simultaneous production of a polysaccharide extracellular that limits the JA production. In the same way, studies of JA production with this same strain but in static erlenmeyers and with a cultivation media composed by milk serum and oil wastes allowed to reach a JA title of 500 $mg.L^{-1}$ after ten days of fungus cultivation. However, the JA production to a speed of agitation of 250 $r.min^{-1}$ diminished up to 300 $mg.L^{-1}$. Farbood et al (2001) observed in the cultivation of *Diplodia gossypina* ATCC 10936 in erlenmeyer and to speed of agitation of 200 $r.min^{-1}$, the JA production reached a productivity of 170 $mg.L^{-1}.day^{-1}$ but the JA productivity diminished 10 times when the cultivation was carried out in a reactor of 150 L of capacity with constant aeration and periodic agitation to 150 $r.min^{-1}$. These authors also prepared JA to pH 6 in the same reactor with the same strain in controlled conditions of aeration, temperature at 28 °C and speed of agitation of 450 $r.min^{-1}$, reaching a productivity of 90 $mgAJ.L^{-1}.day^{-1}$. This result was not still overcome the productivity of obtained AJ of the cultivations in static erlenmeyers.



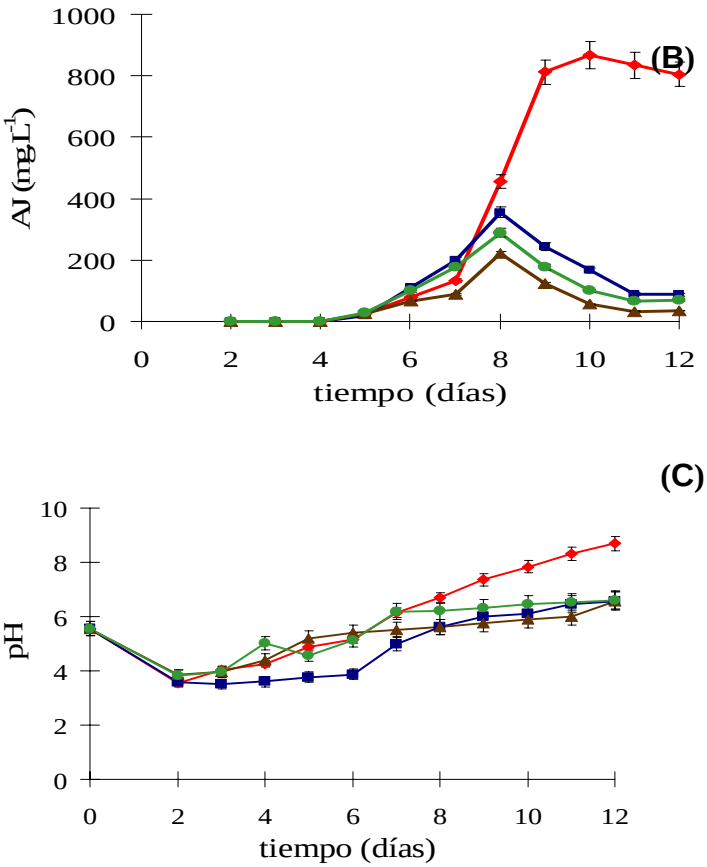


Figure7. Effect of the speed of agitation in the production of biomass (A), jasmonic acid (B) and pH (C) of culture media of *Botrodiplodia theobromae* strain 2334 during twelve days of the fungus cultivation in Miersch modified media in erlenmeyers of 250 mL incubated to 30 ^{o}C and inoculated with 10 mycelium pieces (of 7 mm of diameter), the mycelium inocula was grown in extract of malt agar to 30 ^{o}C, during 3 days of incubation. ♦: 0; ■: 50 r.min^{-1}; ▲: 100 r.min^{-1} y ●: 150 r.min^{-1}. The values average of 3 measures ± the standard desviation are shown.

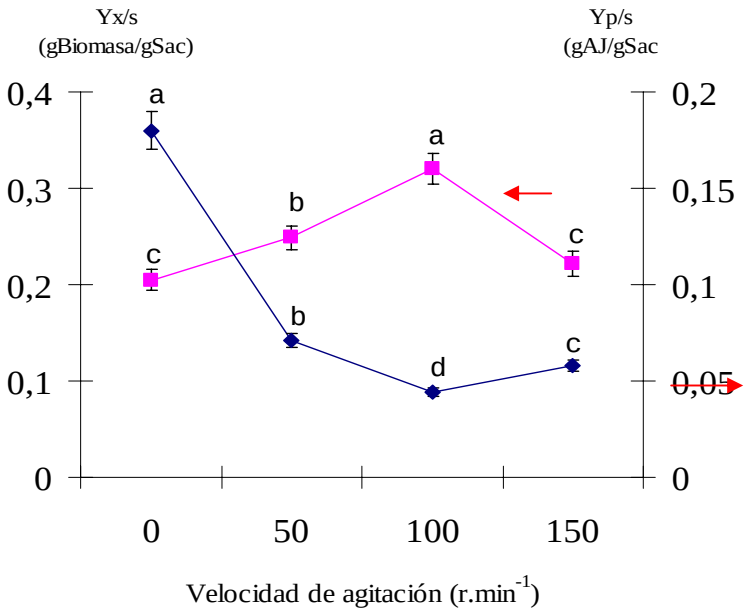


Figure 8. Effect of the speed of agitation in the yields of the biomass production (Yx/s, g of biomass by g of sucrose consumed) and jasmonic acid (Yp/s, g of jasmonic acid by g of

sucrose consumed) of culture media of *Botrodiplodia theobromae* strain 2334 during twelve days of the fungus growth in Miersch modified media in erlenmeyers of 250 mL and incubated to 30 ^{o}C. The inoculation was carried out with 10 mycelium pieces (of 7 mm of diameter) grown in extract of malt agar to 30 ^{o}C, during 3 days of incubation. ♦: Yp/s; ■: Yx/s. Different letters along the curves of the graph denoted significant differences among the medias according to the Test of Tukey ($p < 0,05$). The statistical comparisons were carried out among the yield Yx/s or Yp/s for each speed agitation. The values average of 3 measures ± the standard deviation are shown.

Dhandhukia and Thakkar (2007) reported that the agitation of the media cultivation at 125 r.min^{-1} reduced in approximately three times the JA production with *L. theobromae* MTCC 3068 with regard to the control without agitation. These results suggest that under conditions of agitation of the media culture where there was bigger concentration of oxygen dissolved, the metabolism of *B. theobromae* changed to the production of compound as polysaccharides extracellulars. In that way, Selbmann et al (2003, 2004) in studies of strain selection to the exopolysaccharides production revealed that *B. theobromae* DABAC-P82 showed the best yield. This strain was able to take place up to 17,7 ± 0,8 g.L^{-1} from an exopolysaccharide at the 24 hours of cultivation. The exopolysaccharide production of this strain but in upset reactor showed that the increment of the speed of agitation of 300 to 500 r.min^{-1} increased the yields and exopolysaccharide productivity.
The production of these polysaccharides extracellulars in these strains could be associated to the capsules formation. Diverse functions have been suggested for these capsules in fungi and yeasts. The most acceptable suggestion is the substances protect to the organism of the drying, thanks to the capacity to retain considerable quantities of water. In our case, is probable that the formation of these capsules is a way of the fungus to counteract the stress that causes the agitation of the culture media in the upset variants because the fungus cultivation in liquid and static is the superficial type form.
The reduction in the JA concentrations in the variants with agitation starting from the day eight of fungus growth (Fig. 7B) could be related with the consumption of this compound as source of carbon, because the fungus was in stationary phase. However, in the static cultivation this effect is less accented because the strain was under conditions of smaller stress. Abdala et al (1999) reported in their studies with bacteria were able to synthesize JA that was consumed or metabolized by the cells at the final stage of cultivation.
In the Fig. 8 can be appreciated the effect of the agitation more clearly in the JA production. It was evident that the increment of the speed of agitation up to 100 r.min^{-1} favored the biomass production. However, the yield Yp/s showed a contrary behavior, because the JA production was favored in static and to the speed of agitation of 100 r.min^{-1} where the smallest Yp/s was obtained.
The pH decrease after having lapsed two days of growth of the fungus (Fig. 7C) could be due to the metabolites excretion by the consumption of glucose and fructose of the media and previous enzymatic hydrolysis of the sucrose. With the course of the fermentation of the fungus and the consumption of the ions nitrates, sulfates and phosphates, the pH was increased by the excretion of OH^- ions to the media culture. In the variants with agitation the pH reached values around 6, while in static surpassed the value of 8 (Fig. 7C). This behavior agreed with that reported by Selbmann et al (2003) in their studies of exopolysaccharide production with *B. theobromae* strain DABAC-P82, because the pH was increased in the course of the fermentation until values average of 6,45 ± 0,11. Therefore, the results obtained in our case could also evidence that happened a metabolism change in the variants with agitation with regard to the control without agitation, in which the production of the polysaccharides competes with the JA production.

The agitation study demonstrated the sensibility of this production to the oxygen concentration, being more advantageous the static fermentation system.

This system without turbulence minimizes the convective mass transport, maximizing the effect of the difusional phenomenon. For this reason, it is decisive to consider as approach to keep similar durin scaleup the relationship superficial area/volumen on the production of JA.

The figure 9 shows the JA concentrations in static superficial cultivation of Botryodiplodia, until levels of 50L. As it can be observed the biggest productive relationships were in large bottles of 5 L, working to 1 L. Considering the high price of this product (180 USD/250 mg, SIGMA http://sigmaaldrich.com/catalog/search/A/J2500) in the market it is justified the production for this system.

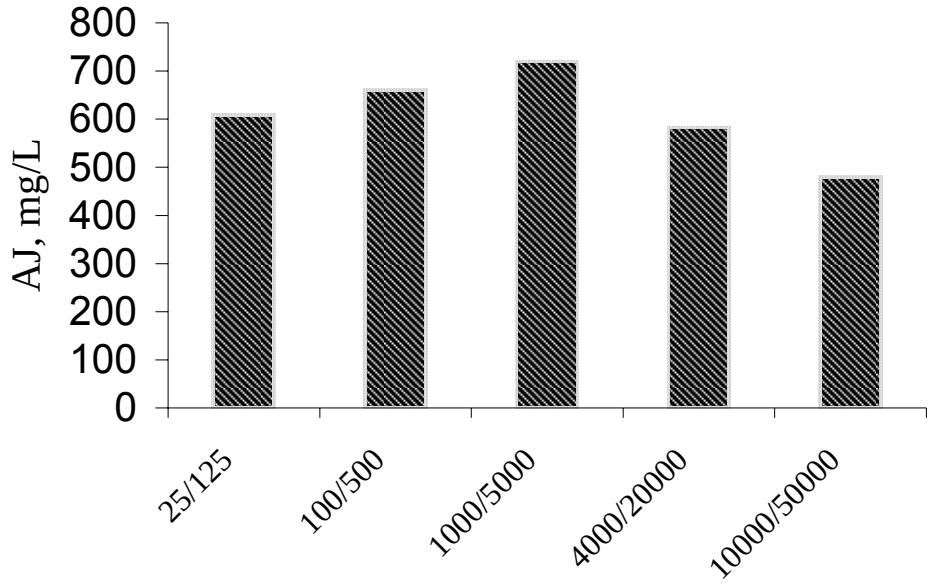


Fig. 9 Scale up Jasmonic acid production in statical culture.

Jasmonic acid (JA) is a regulator of the endogenous vegetable growth, synthesised in a natural way for a great variety of plants. It was isolated in 1971 from *Botryodiplodia* and identified as potent inhibitor of the growth in plants.
JA [3-oxo-2-(2´cis-pentenyl)-ciclopentane – 1- acetate] and their derivatives are distributed in higher plants and microorganism. JA has two enantiomers with two quiral centers in the cyclopentanone ring. It is known natural transformation of cis JA to trans during the isolation and other procedures result a molar equilibrium between trans and cis forms of 9:1.
Phytochemistry studies for the separation, purification, identification and quantification of fermentation broths from Botryodiplodia indicate a diversity of jasmonates and derived that act in the plant growth mechanism.
JA and its related compounds are been separated with relatively difficulty and identificated by chromatography techniques.
Bothryodiplodia theobromae and mutants of *Gibberella fujikuroi* have been reported as JA producers. Nevertheless, little information concerning to the effect of the culture conditions and strain type on the metabolites produced is available.
The aim of this study was to separate and quantify the JA and to characterize the chemistry perfil from *Botryodiplodia* broths.

Experimental

Jasmonic acid production*:* JA was produced from *Botryodiplodia* sp. 83, Mg3, Kj3.1 and 2334 in 50 mL of medium in a flask of 250 whole volume with the following medium composition: sucrose 50 g/L, yeast extract 1 g/L, KH_2PO_4 2 g/L and traces with pH fixed in 5,5 with NaOH (1M). The inoculum was obtained in superficial growth on Petri dishes with 25 mL EMA seed from mycelium of inclined tubes. The Petri dishes were incubated by 5 days at 30°C. 1/8 of mycelial dish was put in each erlenmeyers. Fermentation was carried out at 30 ^{o}C for 13 days.

Sample preparation: Mycelium was separated by vacuom filtration with Whatman 4 filter. Alíquotas of 5 mL from filtrated broth was fixed at pH 3.0 with HCl (4M) and were extraited with ethyl acetate (1:1). The fractions with JA were dehydrated with anhydrum sodium sulphate and rotoevaporated at 50^{O}C.
Thin layer chromatography: Sílica gel 60 G was used support. The solvent system was chlorophorm: ethyl acetate: aceton: acetic acid (40:10:5:1). Jasmonates were detected on thin-layer plates by spraying with acetic acid: sulfuric acid: anysaldehide (100:2:1).
High-performance liquid chromatography: For AJ determination was used na HPLC chromatograph HPLC SHIMADZU (LC-10AD) with a reverse phase column C_{18} Supelcosil (25 cm x 4,6 mm id, 5μm) with detector of variable wave lenght. A flux of 0.85 ml/min was used, wave lenght 210 nm and MeOH:acetic acid 0.1 %, 40:60. It was injected 20 uL. Biocrom software for Chromatography version 1.1 was used.

RESULTS AND DISCUSSION

A TLC procedure was developed for the separation of the metabolites from *Botryodiplodia* broth. Fig 10 shows the separation of the stands and the JA standard. A resolution higher than 1, was obtained when the quaternary solvents system were applied. Rf of 0.56 and 0.7 were reported by JA in the described conditions. In this case, aethyl acetate fraction was applied after developing the separation procedure from *Botryodiplodia* broth. JA had Rf = 0.5. In the strains 2334, 1517, 81, 83, 3184, 3479 other stan appear in Rf = 0.42 and 0.37 and in *Botryodiplodia* 1517 a stand near to JA (in Rf = 0.48) appeared with the anysaldehide revelater was used.

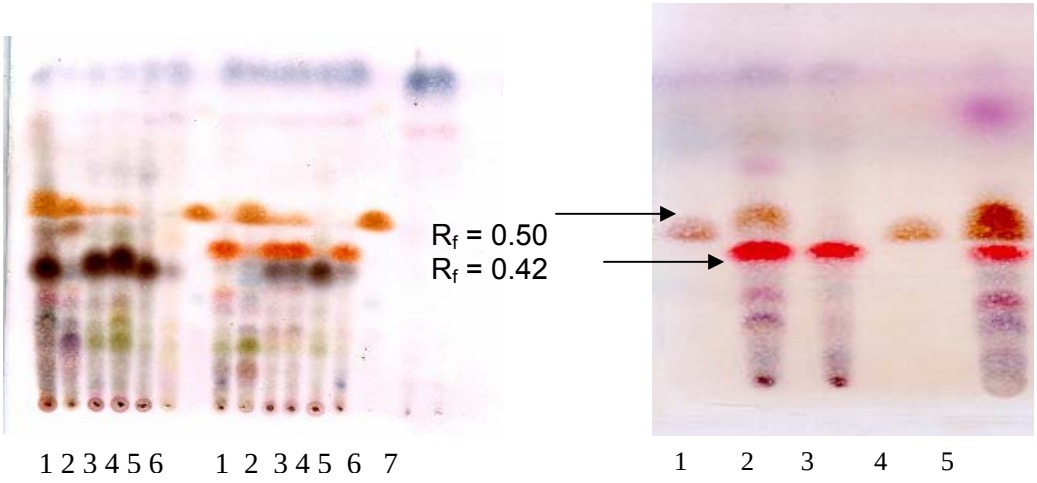
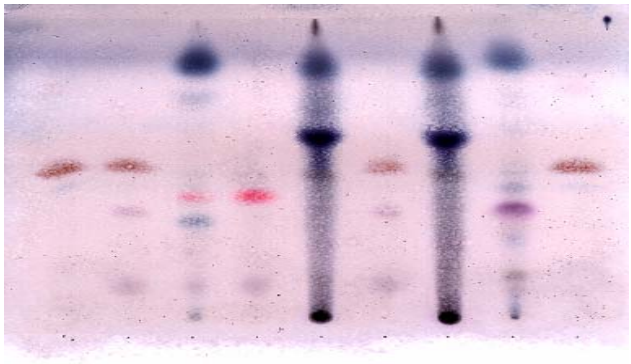


Fig. 10 Chromatogram in TLC with anisaldehyde revelator. a) 1- 2334, 2- 1517, 3- 81, 4- 83, 5- 3184, 6- 3479 (M1) and in the same order (M2), 7- JA. b) 1- JA, 2- 83, 3- 3479, 4- JA, 5- Kijn 3.1.

The profiles indicate the JA production in all of the strains analyzed and at least two products more: a in Rf = 0.42 and other in 0.37 in M1. The strain 3479 does not produce JA, maybe a JA derivative. There are some reports about this system. For example: JA was reported with Rf =0.56 and 0.7 while cucurbic acid was reported in Rf = 0.36 and 0.45.

In order to determine and quantify the cucurbic acid and its derivate, a preparative TLC of the acetate phase in *Botryodiplodia* kjn 3.1 was developed and the stand that corresponds to Rf for cucurbic acid was separated and readed its optical density to 210 nm. Fig 11 illustrates the results of the preparative TLC of the acetate fraction from *Botryodiplodia* broth and Fig. 12 the espectre obtained by densitometry, in gradient decrease of the polarity.



1 2 3 4 5 6 7 8 9

1. JA
2. Fr_6 in kjn3.1
3. Fr_4 in kjn3.1
4. Fr_5 in kjn3.1
5. Fr_3 in 83
6. Fr_6 in 83
7. Fr_3
8. 83
9. JA

Fig. 11 Preparative TLC of the ethyl acetate fraction from Botryodiplodia kijn 3.1broth. Solvent system: Chlorophorm: ethyl acetate: aceton:acetic acid (40:10:5:1).

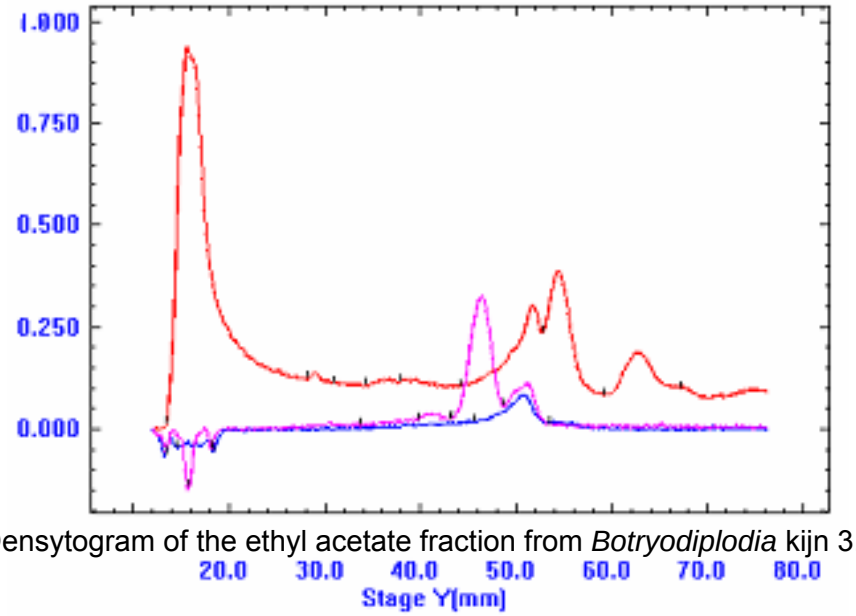


Fig. 12 Densytogram of the ethyl acetate fraction from *Botryodiplodia* kijn 3.1broth

A coincidence in the peaks near to 50 mm was observed. This fact allows suggesting the cucurbic acid presence due to polarity (less than JA). Compounds with polarity lower than JA were observed in strain 3966.

The fractions Fr3 in 83, Fr3 in kjn3.1, Fr6 in kjn3.1 from preparative TLC were characterized by HPLC and uv spectrometry. From these results, some compounds were observed whose characteristics are shown in table 7.

Table 7. Characteristics chromatographics and spectrometrics of the collected fractions in preparative TLC from kjn3.1.

Fraction	Rt in HPLC, min	λ max adsortion in UV, nm
Fr3	15,43	204, 290
Fr4	10.88	210, 300
Fr5	11,08	210, 250, 300
Fr6	12,23	210

It was demonstrated that kjn3.1 produces jasmonic acid, its derivate and cucurbic acid or its derivate.

In order to identify these products, IR spectre was made according show Fig. 13. The difference between carbonil group and hidroxil group in JA and cucurbic acid can be evidenced.

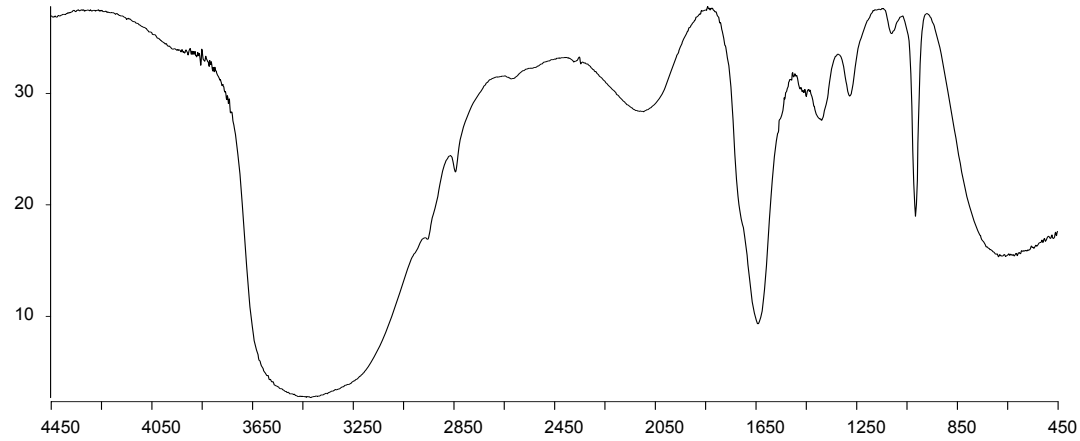


Fig. 13 Infrared spectre from ethyl acetate separation from Botryodiplodia 83 broth.

IR absortions show to be strong in 3400 mm and 1650 mm. These absortions are characteristic of the group –OH intramolecular and –C=O of cetons. There is absorption ~950 mm, characteristic of vinilic group. These results allow suggesting a structure as a cyclopentanone (shown in Fig. 14). These results affirm the Ueda (1994) and Miersh (1987) reports.

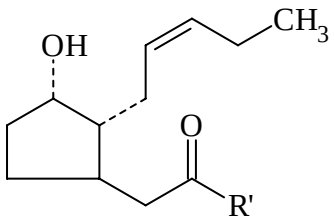


Fig. 14. Cyclopentanone structure.

CONCLUSIONS

The production of JA and its derivatives by TLC and HPLC chromatography in Botryodiplodia strain was evidenced. Other products as cucurbic acid or a product with chemical structure same as ciclopentanone were identified.

Centro Nacional de Toxicologia, CENATOX, Cuba
Oficina Cubana de la Propiedad Intelectual (OCPI)
Cuban Institute of Research on Sugar cane By- products (ICIDCA)

Ecotoxicological evaluation of jasmonates from *Botryodiplodia* for the completion of registration formalities.

a) Acute toxicity of the product in Guppy fish (*Poecilia reticulata)*.

As part of the toxicity studies of this product, the testing on fish was proposed due to their particular susceptibility to environmental pollution. The Guppy fish is a species for conducting standardized Acute Toxicity Studies of 96 hours, as it is regulated by the Office of Pesticides and Toxic Substances of the Environmental Protection Agency (EPA) of the United States, in the guidelines for the Pesticide Toxicity Assessment (EPA, 1996) (1) in the ISO 7346-1 (1998) Standards, according to Directives 91/414/EEC and 2001/36/EC.

Conclusions
According to the results obtained and the conditions under which the test took place, we can conclude that the final product did not cause signs of toxicity or mortality in Guppy fish to the concentration of 100 mg / l (rate of application in agriculture).

b) Acute toxicity by contact in the earthworm *Eisenia andrei.*

Objectives

- To conduct a study of acute toxicity on filter paper contact of the product, using the earthworm *Eisenia andrei* as test organism, as non-target organism, according to the regulations by the Directives of the European Community and the OECD guidelines for toxicity tests.

Conclusions.

- According to the results of the test conditions, it can be stated that the product showed no mortality in any of the test concentrations on the earthworm *Eisenia andrei* in the contact toxicity study.
- The concentrations tested did not cause any behavioral or physiological changes in the test organisms during the study period.

c) Acute toxicity in amphibians: Anuran species *Osteopilus septentrionalis*.

Objective
Conducting Acute Toxicity Studies of 96 h in Amphibians: Anurans *Osteopilus septentrionalis* species, as non-target organisms, according to regulations by the Office of Prevention, Pesticides and Toxic Substances of the Environmental Protection Agency (EPA) United States, in the Guidelines for the Evaluation of Toxicity of Pesticides (EPA, 1996) (EPA, 1975) and 2001/36/EC.

Conclusion
According to the results of the test conditions performed, the product did not produce signs of toxicity or death at a concentration of 100 mg / l in frog larvae of species *Osteopilus septentrionalis.*

d) Acute toxicity in bees: species *Apis mellifera*
This work had the objective to evaluate toxicity on bees (as part of the ecosystem) that may have topical application of the product only at doses of 0.005 and 3 µg/bee, corresponding to concentrations of 19 and 600 mg / L respectively. We used a total of 225 bees of the species *Apis mellifera*, divided into three experimental groups: untreated control and two groups with the product (minimum and maximum doses).

During the study, the variables analyzed were bee mortality at 4, 24 and 48 hours of the test as well as the signs of toxicity in the same periods of time.

The product produced minimum and maximum doses in the groups, mortality of 36 and 27 percent respectively, biological and statistically unsignificant values. In regard to clinical observations, the animals showed no toxic signs or alterations in their behavior that can be attributed to the administration of the test products.

PATENT STUDY about the topic.
This report is the result of a patent investigation conducted from the art review state to determine the technical solutions that state the biological microbial inhibition of *Leuconostoc mesenteroides.*

To carry out the investigation Patent Databases having a high degree of updating and coverage, were reviewed. Patent Scope (which includes information on patent applications handled by the PCT Treaty), esp@ cenet (which groups international information) and other national offices websites of Industrial Property. Several strategies in the search were combined in which keywords and codes of the International Patent Classification (IPC) were used.

As a result, six patent families (3, 33%) were found, stating technical solutions to achieve this inhibition, most of which involve other isolated bacteriocins from lactic acid bacteria, as it was previously reported in scientific literature, where bactericidal properties against different species regardless of their phylogenetic proximity are attributed. Also, isolating compositions were reported from plant material and mixed with chemical biocides compounds, sometimes including very different culture media of bacteria such as Salmonella, which are involved in the production of sugar.

The overall trend in the protection of technological innovation performance in this field through the first patent shows that the first records on Leuconostoc mesenteroides date from 1937, but is only in the decades between 1950 to 1970 when it started to have some activity, showing moderate peaks of ups and downs. From 1980 on, applications increased, extending maximum peaks at marked times. Specifically, the patents related to the inhibition of these bacteria start to register from 1990 up to now.

Concerning the origin of the technology, the United States stands as leading nation where these inventions have been requested for the first time. Referring to the fate of technology, the United States and Australia have been the highest receptors, coinciding with the facts that they are sugar-producing nations and that they develop research in this field.

Most owners (83.3%) have an invention on this topic and only QUEST INTERNATIONAL FLAVORS Company & FOOD INGREDIENTS COMPANY (U.S.) has two registers. Only a patent belongs to independent inventors, which shows that this research has been conducted primarily in the institutional and business sector.

Search strategies

The databases were consulted taking into account different search strategies based on the use of key words such as:

→*Leuconostoc mesenteroides*
→Microbial or microbian and inhibit or restrain,
→Actinomyces, Streptomyces, Pseudomonas
→Lasiodiplodia or *Botryodiplodia theobromae*, botryodiplodin
→Natural and antimicrobial
→Nisin, bacteriocins,

The following codes of the International Classification Patent (IPC) were also taken into account:
→ A01N63/00- Biocides, products that attract or repel harmful animals, or plant growth regulators containing microorganisms, virus, fungi, enzymes, fermentation products, or substances obtained or extracted from microorganisms or animal substances.
→ A0163/02- Fermentation products or substances extracted from microorganisms or animal substances.
→ A01N63/04- Microscopic fungi or their extracts.
→ A23B5/16- Preservation of food by microorganisms or enzymes
→ C12S3/10- Treatment of materials of animal or plant origin, or microorganisms. Treatment of sugar or molasses.
→ C12R 1:01 - Bacteria
→ C12R1: 04 - Actinomyces
→ C12R1: 38 - Pseudomonas

Results. Patents on microbial inhibition of *Leuconostoc mesenteroides.*

As a result of the study, 180 families of patents on "Leuconostoc mesenteroides", were recovered, of which only six families claimed technical solutions involving metabolites from microorganisms or microorganisms themselves with this inhibitory effect on this lactic acid bacteria.

KR20050023188
This document presents a bacteriocin production by Leuconostoc ciitreum G7, which has antimicrobial activity against several genera of bacteria and an improved stability in heat and under pH changes.

US5817362
This invention claims for a new bacteriocin produced by Lactococcus lactis NRRL18535, which is used and is very useful for food, and presents a broad spectrum of activity against Gram positive bacteria in an optimal range of pH between 2 and 8.
The bacteriocin is a protein of 6000 daltons, which is inactivated by proteases, which inhibits the growth of bacteria selected from: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus carnosus*, *Pediococcus pentosaseus*, *Pediococcus acidilactici*, *Lactococcus cremoris*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus bifermen.*

KR20050023188
This application dealt under the PCT Treaty seeks protection for a product used in the stabilization of the sucrose contained in sugar cane and to preserve the purity of the juice. It can be applied at any stage of sugar production process, although it is preferably to be done in the first extraction. It also reduces the fouling of the material in the flowpipes and sugar mill equipment and avoids the use of antibacterial agents. The reivindicated product consists of a solution of an active agent, a carboxylic acid or its salt, a short chain alcohol, a terpene, and a buffer solution. The active agent can be formol or acetalaldehyde.

US6379720
The invention relates a composition with biocidal properties which contains a mixture of hop extract from a plant Humulus lupulus and certain chemical compounds that show some synergistic action, which is used as a biological control of some types of algae in water systems as streams and municipal and industrial water. It also includes the processing of foodstuffs such as fruit juices, meats, and diffuser systems for the processing of sugar beet.

CA2546301
This document is registered with the Canadian Office and it refers to an antimicrobial composition comprising *nisin* and *poly Lysine*, which act synergistically in order to prevent microbial colonies increase in food without affecting its taste and physical properties.

R E S U L T S O F T H E P A T E N T S T U D Y :
The main inhibition presented in this species is due to the action of bacteriocins produced by lactic acid bacteria species and even by its metabolites.

With regard to the age of technology, the first Leuconostoc mesenteroides related records dating from 1937, where the state of research is emerging in the period between the '50s and 1970s, showing greater activity in registering, with moderate peaks that show moments of ups and downs. But since 1980 the fluctuations increased concerning the number of records, showing maximum peaks in 1990, 1994 and 2004 (10) and 2001 (11) respectively, although from a cumulative point of view, there is a trend to increase the records. Patents specifically related to inhibition of these bacteria begin to register from 1990 up to now.

Concerning the ownership of inventions, although there is no institution that widely stands according to the number of records, we can conclude that the English enterprise QUEST INTERNATIONAL FLAVORS & FOOD INGREDIENTS COMPANY (U.S.), holder of two patent families, is the most prominent, to which the company MICROLIFE TECHNICS INC, holder of a record, has been attached. Other institutions maintain a uniform title of a single registration each. The most important countries in relation to the origin of the technology are the U.S. (78%) followed by Mexico and Korea, with 11%. The destination of the technology is more diverse and includes countries in Europe, Latin America and Asia but the United States and Australia stand out as recipients of technology as they are sugar-producing countries who have conducted research in the sugar industry.

CONCLUSIONS
As a result of the study, 180 families of patents on "Leuconostoc mesenteroides", were recovered, of which only six families claimed technical solutions involving metabolites from microorganisms or microorganisms themselves with this inhibitory effect on this lactic acid bacteria, therefore, a patent about of a biocide from jasmonate salt for inhibition microbial could be obtained.

INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France
Institute of Biotechnology of Mexico, Morelos, Mexico
Cuban Institute for Research on Sugar cane by- products, ICIDCA, Cuba.

The industrial *Leuconostoc* strain B/110-1-2 producing dextran and dextran-derivatives was taxonomically identified by 16S rRNA as *L. citreum*. Its dextransucrase enzymes were characterized according to their cellular location and reaction specificity. In the presence of sucrose, the strain B/110-1-2 produced two cell-associated dextransucrases (31.54 % of the total glucosyltransferase activity) with molecular weights of 160 and 240 kDa and a soluble dextransucrase (68.46 %) at 160-180 kDa. Two open reading frames (ORF) coding for *L. citreum* strain B/110-1-2 dextransucrases were identified. One of them shared a 56 % identity with the alternansucrase ASR of *L. citreum* NRRL B-1355 and with a putative annotated alternansucrase sequence found in the genome of *L. citreum* KM20. The structural analysis (HPAEC-PAD, HPSEC and ^{13}C-NMR) of the polymer and oligodextrans produced by the B/110-1-2 dextransucrases suggest this novel glucansucrase has specificity similar to a dextransucrase but not to an alternansucrase, producing a soluble linear dextran with glucose molecules linked mainly in α 1,6 and α 1,3 with α -1,4 branches. These results enhance the understanding of this industrially-significant strain and will aid in distinguishing between physiologically-similar *Leuconostoc* spp. strains.

Dextransucrases (EC 2.4.1.5), which belong to Family 70 of the glycoside-hydrolases, are cell-associated or soluble extracellular enzymes produced by the soil bacteria belonging to *Leuconostoc* genus. They catalyze the synthesis of high molecular weight glucans by utilizing D-glucose polymers from sucrose. If efficient acceptors like maltose or isomaltose are added to the reaction medium dextransucrases catalyze the synthesis of low molecular weight oligosaccharides and, to a minor extent, high molecular weight glucan polymer. Different kinds of glucans with different sizes and structures, depending on the dextransucrase-producing strain, are synthesized. Dextransucrases from *Leuconostoc mesenteroides* are widely used in the pharmaceutical and alimentary industries since the last century. Dextran produced by *L. mesenteroides* NRRL B-512F (ATCC 10830A) was one of the first biopolymers to be produced on an industrial scale, finding several applications in medicine, separation technology and biotechnology. *Leuconostoc* spp. strains are applied in fermentative processes to produce polymers of glucose (iron-dextran, and gluco-oligosaccharides). Oligosaccharides produced by *L. mesenteroides* NRRL B-1299 with one or more D-glucopyranosyl units linked through α (1-2) glucosidic bonds are highly resistant to attack by digestive enzymes and are used as prebiotics in cosmeticeutical products and human nutritional applications.
The *Leuconostoc* sp. strain B/110-1-2 was isolated from sugarcane juice and has been used for dextran production from sugarcane molasses in a large scale plant, obtaining up to 1 ton per day of dextran technical grade. It has also been applied in the production of

iron-dextran at pilot plant scale. Recently the dextran polymer produced by the B/110-1-2 dextransucrases present in the whole culture (supernatant and cells) have been used for the development of controlled release solid dosage forms (soluble drugs). In the present study the B/110-1-2 strain was taxonomically identified by 16S rRNA analysis. Activity, cellular localization, molecular weight and reaction specificity of its dextransucrase enzymes were determined. The detection of a novel dextransucrase ORF is discussed.

Taxonomic classification of L. citreum strain B/110-1-2

The strain B/110-1-2 is an aerobic, nonmotile, nonspore-forming Gram-positive bacterium. Cells are rod shaped and arranged in chains of three to four cocci. Colonies on sucrose solid media are viscous. This strain was primarily identified as *L. mesenteroides* on the basis of its morphological properties and by the API 50-CH test (Biomerieux, Marcy l'Etoile, France). However, sequence analysis of the 16S rDNA operon (GenBank accession number FJ716698) showed that strain B/110-1-2 shares a 99 % identity with the *L. citreum* strains included in the analysis (Fig. 15). It appears that taxonomy based only on phenotypic or biochemical tests is not enough to detect differences between *L. mesenteroides* and *L. citreum* because several former *L. mesenteroides* strains [CW28, NRRL B-1355, NRRL B-742 (ATCC 13145)] have been reclassified as *L. citreum* based on the sequence analysis of 16S rDNA operon.

Dextransucrase activity in cellular and supernatant fractions of L. citreum strain B/110-1-2

The strain B/110-1-2 produces both cell-associated and soluble dextransucrase enzymes. Cell-associated (insoluble) dextransucrases represent 31.54 % of the total glucosyltransferase activity. Higher levels (68.46 %) of soluble dextransucrase activity detected in the culture supernatant are in agreement with the extracellular localization of most dextransucrases produced by lactic-acid bacteria belonging to the genus *Leuconostoc*. In *L. mesenteroides* NRRL B-1299 (ATCC 11449) a dextransucrase insoluble form is responsible for the 60-95 % of the whole glucosyltransferase (dextransucrase) activity produced. The *L. citreum* NRRL B-1355 strain produces an alternansucrase (EC 2.4.140), that is predominantly cell-associated and it represents less than 10 % of the whole glucosyltransferase activity in the supernatant fraction of active young cultures. All of this is supported by the fact that the N-terminal domain from almost all sequenced dextransucrase encoding genes is preceded by a well conserved signal peptide, leading secretion of the enzymes to the extracellular medium.

The other cell-associated dextransucrase produced by the strain B/110-1-2 has an approximate molecular weight of 220-240 kDa. A similar molecular weight has been found in the *L. citreum* NRRL B-1355 (ASR alternansucrase). Kim and Robyt reported an activity band of 240 kDa in the constitutive mutant *L. citreum* B-742CB. Nevertheless, Chellapandian et al. suggested that the 240 kDa protein may represent a multimer of dextransucrase subunits. It is possible that the 220-240 kDa cell-associated dextransucrase from *L. citreum* B/110-1-2 described in this study is a similar multimer since no alternansucrase activity was detected in this strain.

Analysis of oligosaccharides and dextran produced by L. citreum B/110-1-2 dextransucrases

The acceptor reaction products formed by supernatant and cell-associated dextransucrases of *L. citreum* B/110-1-2 were compared with those produced by dextransucrase DSR-S, from *L. mesenteroides* NRRL-B-512F. In the presence of maltose as the acceptor molecule, the B/110-1-2 soluble dextransucrase synthesizes a series of isomaltodextrin analogs very similar to the ones produced by the DSR-S, these products range from the trisaccharide panose to homologous saccharides harboring 12 units of glucose. The specificity like DSR-S of the soluble dextransucrase of B/110-1-2 strain was confirmed by nuclear magnetic resonance (^{13}C-NMR) analysis of the high molecular weight (HMW) dextran produced by the supernatant fraction, which was identical to the dextran produced by the DSR-S enzyme (data not shown).

The use of high-performance anionic exchange chromatography (HPAEC-PAD) enabled to detect that the B/110-1-2 cell-associated dextransucrases synthesized malto-oligosaccharides with retention times very similar to the ones produced by the DRS-S as well, except that two additional peaks clearly appeared immediately after the products with degrees of polymerization (DP) of 7 and 8 respectively (data not shown). High-performance size-exclusion chromatography profiles (HPSEC) of the products from the polymerization reaction carried out by the cell-associated dextransucrases of the *L. citreum* strain B/110-1-2 showed that part of the oligosaccharides were not elongated and were accumulated in the reaction medium (DP< 8) whereas the others were elongated until formation of a HMW dextran polymer ($2x10^6$ Da) (Fig. 16), this is a very similar pattern to the described for the alternansucrase ASR from the *L. citreum* strain NRRL-B-1355. ^{13}C-NMR analysis (data not shown) of this dextran shown a case of intermediate branching, represented by three residues of D-glucosyl joined by α -D-(1,6) links, one 1,4,6-tri-*O*-substituted by a non reducing D-glucosyl group. A similar ^{13}C-RMN profile was previously found in the dextran present in fraction L of *Streptobacterium dextranicum* NRRL B-1254, and in the fraction L of *L. citreum* NRRL-B-742 (ATCC 13146). According to the results of the present study, it is concluded that this type of dextran polymer is produced by the cell-associated dextransucrases of the *L. citreum* strain B/110-1-2, or at least by one of them. Further characterization of the novel dextransucrase open reading frame (ORF) identified in this study will be crucial for the proper identification of the dextransucrase responsible of the synthesis of the above mentioned polymer.

The data obtained in this study enhance the understanding of this industrially-significant strain and will aid in distinguishing between physiologically-similar *Leuconostoc* strains. As the cell-associated dextransucrases from *L. citreum* B/110-1-2 are able to produce branched oligosaccharides it may be easily incorporated as an immobilized biocatalyst for linear α -1,6 and α -1,4 branched isomalto-oligosaccharide production.

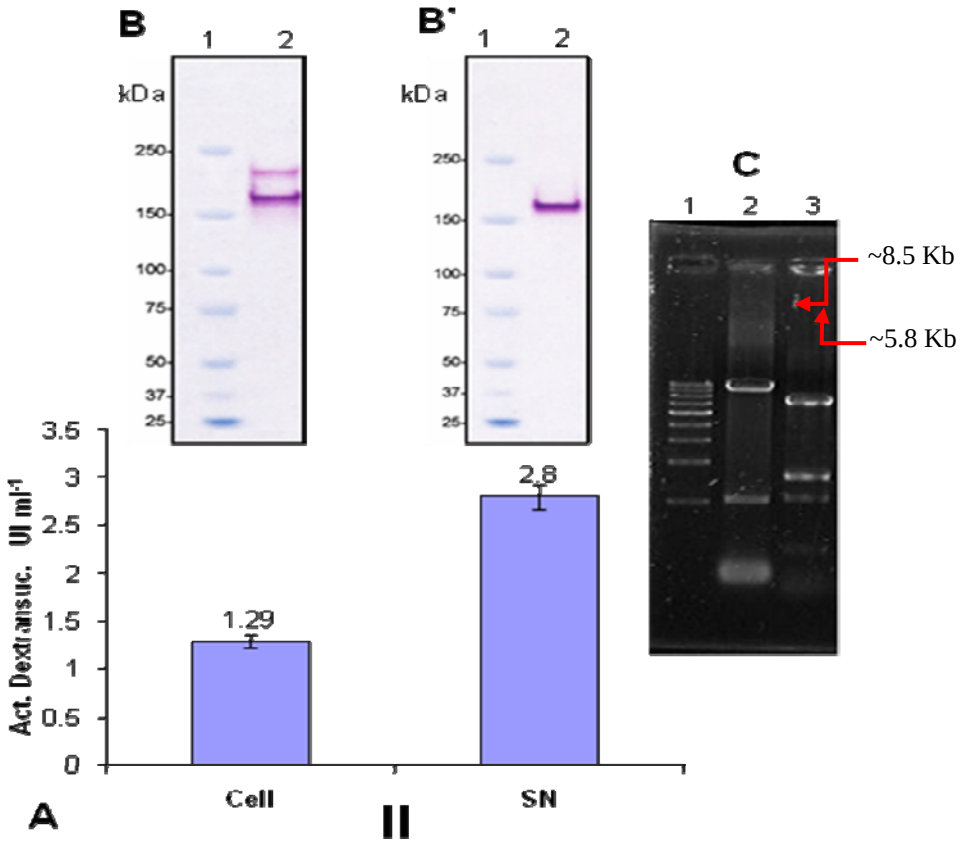


Fig. 15. Dextransucrase production and detection of dextransucrase amplicons in *L. citreum* strain B/110-1-2. Quantification of dextransucrase activity (A) and zymograms (B) of cellular (Cell) and supernatant (SN) fraction, 1B: protein marker (BioRad). (C) DNA electrophoresis of 8.5 kb (2C) and 5.8 kb (3C) amplicons encoding for dextransucrase enzymes amplified by LA PCR with primers dsrE-dir-PS, dsrE-inv-PS and asr-dir-PS, asr-inv-PS respectively. 1C: 1 Kb DNA Step Ladder (Promega)

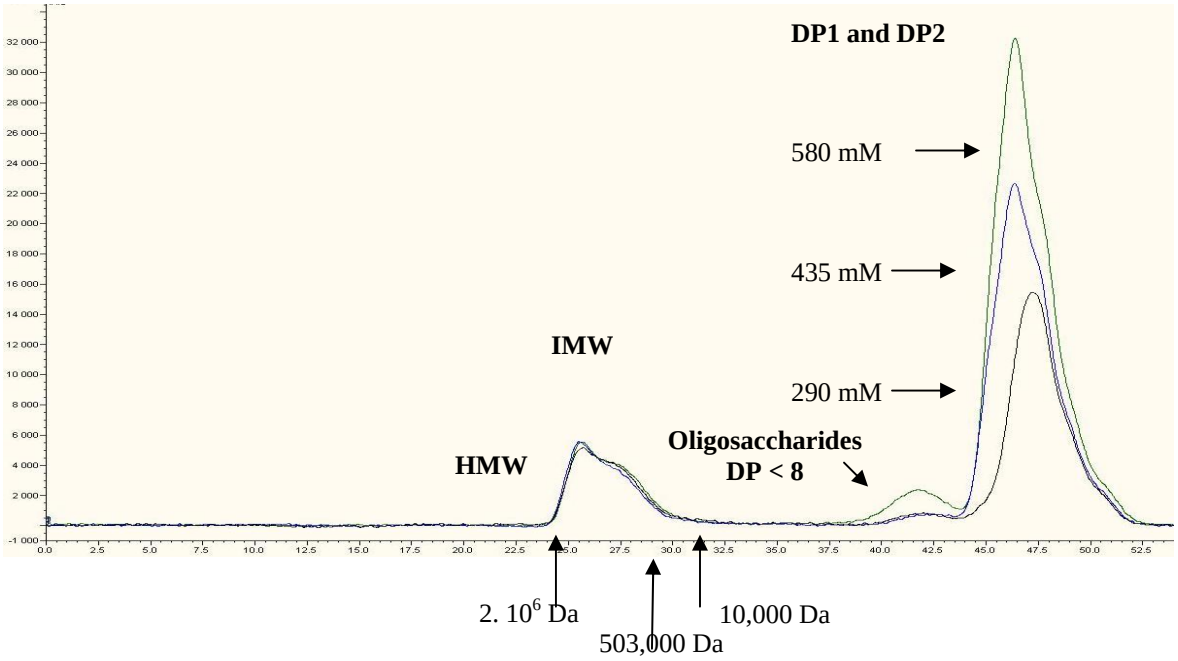


Fig. 16. HPSEC profiles of the products synthesized by *L*. *citreum* B/110-1-2 cell-associated dextransucrases during the polymerization reaction from sucrose 290 mM, 435 mM, 580 mM, respectively. HMW: high molecular weight polymers, IMW: products of intermediate molecular weight, *DP2*: Sucrose, *DP1*: monosaccharides.

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EVIPOL effect on mill station disinfection and the purity on cane juice.

For this experiment, 10 liters of jasmonic acid copper salt, dilute 1:1 with water were applied to the first and sixth mill, repeating the treatment after 30 minutes.

The mill station had the processing capacity of 260 T/h. This allowed for the application of a treatment dose of EVIPOL of approximately 0.007%. Samples were collected before treatment, immediately after treatment, 5 min. after treatment, 5 min. after the second treatment, and 30 min. after the second treatment.
Microorganism counts were determined (yeasts, bacteria and *Leuconostoc*). pH, pool, purity were measured to determinate the stability of the juice.

Contact time is defined as the time blended juice can be retained before heat treatment in the clarification process. This parameter varies according to the operative characteristics of the each processing plant. In thias study, contact times of 1, 5 and 30 minutes were studied.

The number of viable cells (UFC/ml) was determined using selective media to establish the antimicrobial effect of each one of the products in study on the total microorganisms (nutritiente Agar), polysaccharide- producing bacteria (Yeast extract - Glucose-Agar) and eukaryotes (Malt agar). Samples were incubated at 37 and 30 ^{o}C for 48 h. The experiences were done in triplicate. The inhibition % was defined as % Inhibition = (UFC/ml inicial - UFC/ml final) / UFC/ml inicial x 100

Results and Discussion

Evipol effect in the mill station of three sugar factories.

From results to laboratory studies that evidenced the inhibitory effect of the jasmoinic acid on the Leuconostoc mesenteroides, we proceeded to the product application in industry.

A microbiologic diagnosis of the mills area was done in order to know the disinfection and hygiene state. Table 8 shows the microbial state in the extraction process of cane juice, in the three sugar companies.

Table 8. Microbial count in the mill station of three sugar factories.

	Mesofilic bacteria, UFC/ml	Polysachharides bacteria, UFC/ml	Yeast, UFC/ml
Sugar cane juice in the basculater, CAI "España Republicana"	1.10^6	4.10^7	$1{,}1.\ 10^7$
Primary juice CAI "España Republicana" Argentinean enterprise CAI "Héctor Molina"	$3.\ 10^7$ $1.\ 10^8$ $1{,}4.\ 10^7$	2.10^7 $5.\ 10^7$ $3{,}1.\ 10^7$	$1.\ 10^7$ 3.10^6 $1{,}8.\ 10^7$
Blended juice CAI "España Republicana" CAI "Héctor Molina"	$7.\ 10^6$ $1{,}9.\ 10^7$	$1{,}2.10^7$ $2.\ 10^7$	$8{,}3.\ 10^6$ $1{,}7.\ 10^7$

Although the values are not markedly inferior that the historical stockings, there is a slight tendency toward smaller contaminations in the Cuban cane. This fact can be due to the little time of demurrage after cutting and before the industrial process, influenced by shortage that impact favourably on their smallest deterioration.

Nevertheless, it is beneficial to adopt a system of microbial control in the factory that allows detecting problems like:

- deteriorated sugar cane
- microbial situation of the cane juices in the mill station
- microbial quality of the cane sugar

The product was dosed by graveness with easy manipulation, in the mills area and in industry juice tank, with the objective of decreasing the microbial infection. Thirty minutes as maximum contact time was studied.

Table 9. Microbicide effect of the EVIPOL 0.007 % on microbial content in the primary and blended juice of the CAI "España Republicana".

	Mesofilic bacteria, UFC/ml	Polysachharides bacteria, UFC/ml	Yeast, UFC/ml
Primary Juice 0	$3.\ 10^7$	$2.\ 10^7$	1.10^7
1er treatment 1 min 5 min	$1{,}5.\ 10^6$ $8{,}9.\ 10^5$	$2{,}7.\ 10^6$ $1{,}2.\ 10^6$	$5.\ 10^5$ $3{,}9.\ 10^5$
2do. treatment 5 min 30 min	$1.\ 10^5$ $1{,}1.\ 10^5$	$6{,}1.\ 10^5$ $2{,}9.\ 10^5$	$2{,}4.\ 10^5$ $4{,}0.\ 10^5$

% Inhibition	99,6	98,5	96,0
Blended Juice			
0	7. 10^6	1,2. 10^7	8,3.10^6
1er treatment			
1 min	2,6. 10^5	3,8. 10^6	6,2. 10^5
5 min	9,3. 10^5	1,3. 10^6	1,4. 10^6
2do. treatment	7,5. 10^5	3,2. 10^5	1. 10^6
5 min	4,5. 10^5	4,9. 10^5	7,3. 10^5
30 min			
% Inhibition	93,6	95,9	94,2

Table 10. Microbicide effect of the EVIPOL 0.007 % on microbial content in the primary and blended juice of the CAI "Héctor Molina".

	Mesofilic bacteria, UFC/ml	Polysachharides bacteria, UFC/ml	Yeast, UFC/ml
Primary juice			
0	1,4. 10^7	3,1. 10^7	1,8.10^7
1 min	6,2. 10^6	3,8. 10^5	4,7. 10^5
10 min	2,0. 10^5	1,8. 10^5	1,4. 10^5
30 min	3,8. 10^6	2,8.10^6	2,0. 10^6
% Inhibition	72,8	90,9	88,9
Industry juice			
0	1,5. 10^7	1,9. 10^7	9,8. 10^6
1 min	1,1 .10^6	1,2 .10^6	2,7 .10^6
10 min	6,3.10^5	5,4 .10^5	1,5 .10^6
30 min	1,9.10^6	3,1.10^6	2,6.10^6
% Inhibition	87,3	83,7	73,5

It could be observed that in "Héctor Molina" factory bigger effectiveness was obtained to the 10 min. nevertheless, the obtained data allow to conclude that dose of 0.007% with contacting of the 30 min is effective to inhibit above 87% and until the initial bacteria population in 99,6% and above 83% of the microorganisms polysaccharides producer and able to degrade sucrose, as well as above 73% of the present yeasts. The results indicated that still with 1 min. of contact the product is effective.

The figure 17 shows the microbial inhibition in the cane juice due to the EVIPOL application.

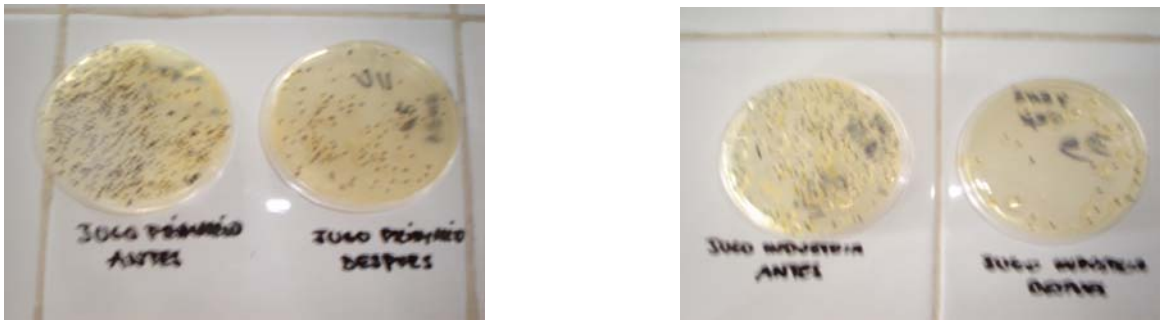

Fig. 17. Inhibitory effect on Leuconostoc mesenteroides indicated by the difference among before and after the 0,007% EVIPOL application on primary juice.

Evaluation of the sugar cane deterioration. Effect of the EVIPOL addition on the cane juice purity.

Numerous products produced by the Leuconostoc metabolism can take as indicators of the deterioration of the sugar cane: sugar free reducers is proportional to the fructose formation; pH decrease indicates formation of acetic and lactic acid, invertase and dextransucrase enzyme like induced enzymes of the sucrose, and directly the concentration of polysaccharides and dextrans as a result of the microbial action that increase the viscosity of the juices.

EVIPOL was applied to two concentrations looking for effect stabilizer in the purity of the juice. The results in "España Republicana¨ factory indicated that 4.8 units decrease the purity to the 12 hours, while with the addition of the inhibitor only 2 units decrease the purity and the stabilization of the juice was obtained almost completely in the variant 3 of the study. The juice tried with biocide retained its dark brown, fresh scent, and pH without big variations to the initial. On the contrary and with a strong contrast, the juice non treaty became light brown, with a strong scent to alcohol and pH and purity markedly smaller. This stabilized effect has not been reported for other disinfection agents commonly used in the sugar industry.

In the CAI “Héctor Molina" EVIPOL was applied to three concentrations looking for effect stabilizer in the purity of the juice. In the sample witness the purity decrease 29.08 units to the 24 hours, what shows a total deterioration of the juice, while with the addition of the inhibitor an inferior decrease took place to the 11 units and the stabilization of the juice took place almost completely in the variant 3 of the study. These results agree with "España Republicana"

Table 11. EVIPOL effect on sugar cane purity in the CAI “Héctor Molina”

	pH	Purity
Industry juice		
Inicial time	4.9	82.85
Witness (non traeted)		
12 hours after	3.9	73.4
24 hours after	3.7	53.77
After of the treatment 0.005 %		
12 hours after	4.1	74.7
24 hours after	4.7	71.04
After of the treatment 0.01 %		
12 hours after	4.5	77.6
24 hours after	4.2	74.3
After of the treatment 0.02 %		
12 hours after	4.7	81.1
24 hours after	4.6	79.6
Clarified juice		
Initial time	6.3	84.41
Witness (non traeted)		
12 hours after	6.3	83.68
24 hours after	6.2	81.35

After of the treatment		
0.005 %		
12 hours after	6.3	82.84
24 hours after	6.3	83.21
After of the treatment		
0.01 %		
12 hours after	6.3	84.43
24 hours after	6.3	83.21
After of the treatment		
0.02 %		
12 hours after	6.3	82.84
24 hours after	6.3	82.13

The figure 18 shows the visual differences among the treated juice and witness after 4 hours of the treatment, where the coloration change is observed due to the deterioration.

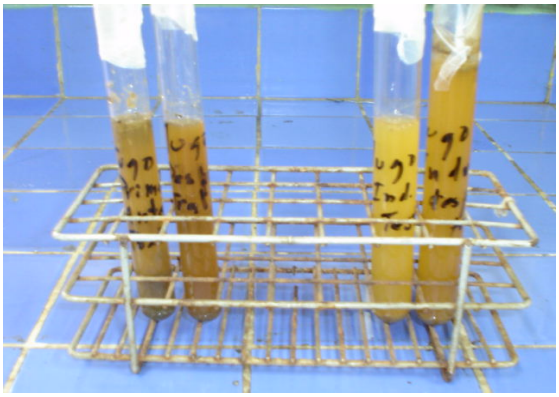

Fig. 18. Effect on the stabilization of the cane juice indicated by the color among the witness and 4 hours after the EVIPOL application with dose of 0,007%.

The Fig. 19 shows the viscosity, the pH and the free sugar reducers' behaviour in treaty and non treaty juice, at the 24 hours after application.

The increment of the free sugar reducers and of the viscosity in the juices non treaties at the 24 hours is a measure of the dextran formation and sucrose losses, as consequence pH decreases for formation of organic acids in the juice non treaty better than with the treaty.

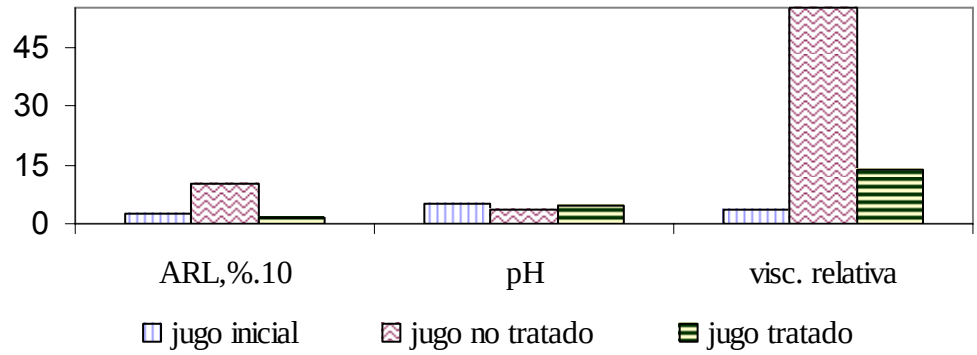

Fig. 3 Indicadores del deterioro del jugo de caña a las 24 horas.

Starch, wax, soluble phosphates, dextrans and other polysaccharides difficult the juices filtration. The filterability of the blended juice was calculated as delays to filter 100 mL of juice in filter paper Whatman nr. 4. The results indicated abrupt diminutions in the velocity of filtration in treated samples and showed a tendency to the decrease in the daily samples taken in the 6to. mill during the assay. (Fig. 20).

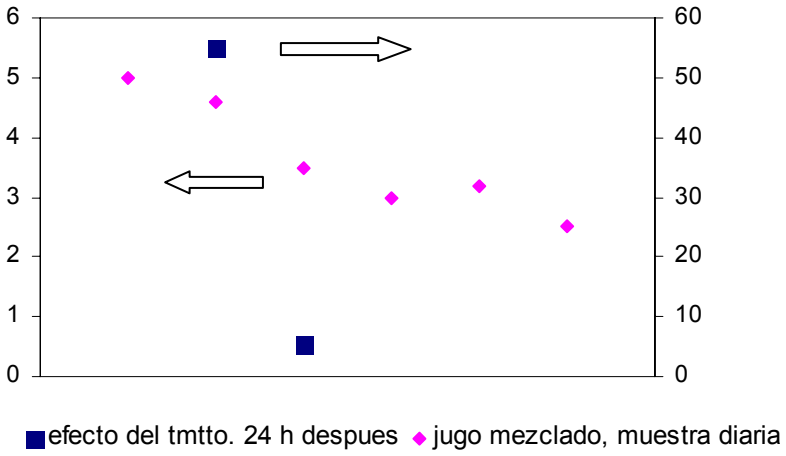

Fig. 4. Comportamiento de la velocidad de filtración del jugo de caña durante el ensayo industrial en centrales cubanos.

Behavior of the blended juice stability (of the strain CO 65 - 357) with the addition of EVIPOL in an Argentinean sugar station.

In the Fig. 21 are shown the results of the EVIPOL (0.005%) application on the blended juice (strain CO 65 - 357) in an Argentinean sugar station

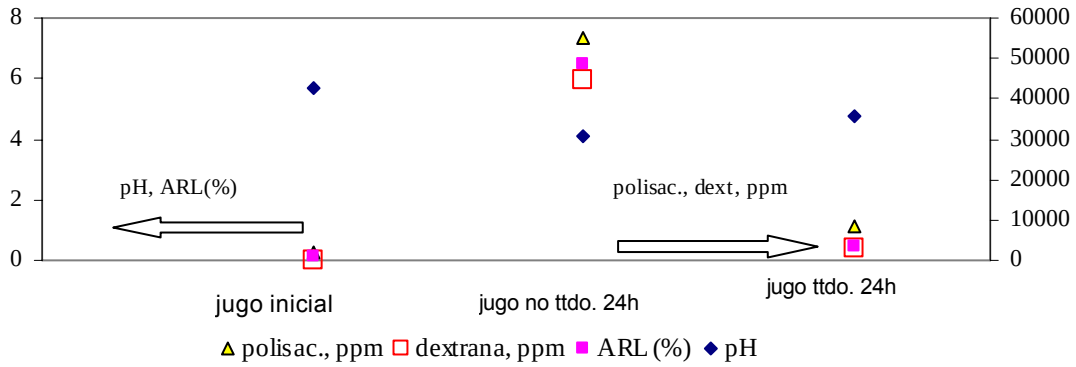

Fig. 5 Efecto del EVIPOL (0.005%) sobre la estabilidad del jugo mezclado argentino (cepa CO65-357)

According to these results the indicators decreases between 14 and 20 times with relationship to the witness non treaty in free sugar reducers (0.46%/6.46%), dextrans (2756ppm/55101ppm) and polysaccharides (2756ppm/44793ppm). This fact indicates an almost total preservation of the juice. This result has a sensitive economic importance when diminishing the sugar losses for formation of polysaccharides in the cutting cane or during the industrial process stops of the industrial process.

Correlation between some analyses about the prediction of sugar cane juice deterioration.

A prediction of some parameters can serve in the determination of the deterioration of sugar cane juice. In the sugar laboratories the pool and purity allow to decide about the feasibility of the industrial material use. However, the pol can hide dextrans formation because this has an angle of optic rotation of the polarized light approximately three times to the sucrose angle, for what the sucrose determination can be affected by dextran presence. On the other hand, the ^{o}Brix doesn't vary notably for formation of polysaccharides; although it can have remarkable variations for formation of ethanol, acetic and carbon dioxide that vary the result of the analysis.
The incidence in the viscosity by dextran molecular weight will be in dependence of Leuconostoc, the sugar cane variety, and the humidity and temperature conditions. It should be considered the possible formation of ethanol by the presence of yeasts in anaerobiosis.

Eggleston (10) studied the sugar cane deterioration from mannitol concentration, leucrose, and other products of the degradation of sugar.

The correlations among pH, viscosity, filterability vs. dextran were studied and other combinations could be of utility in the mensuration of the juice deterioration.

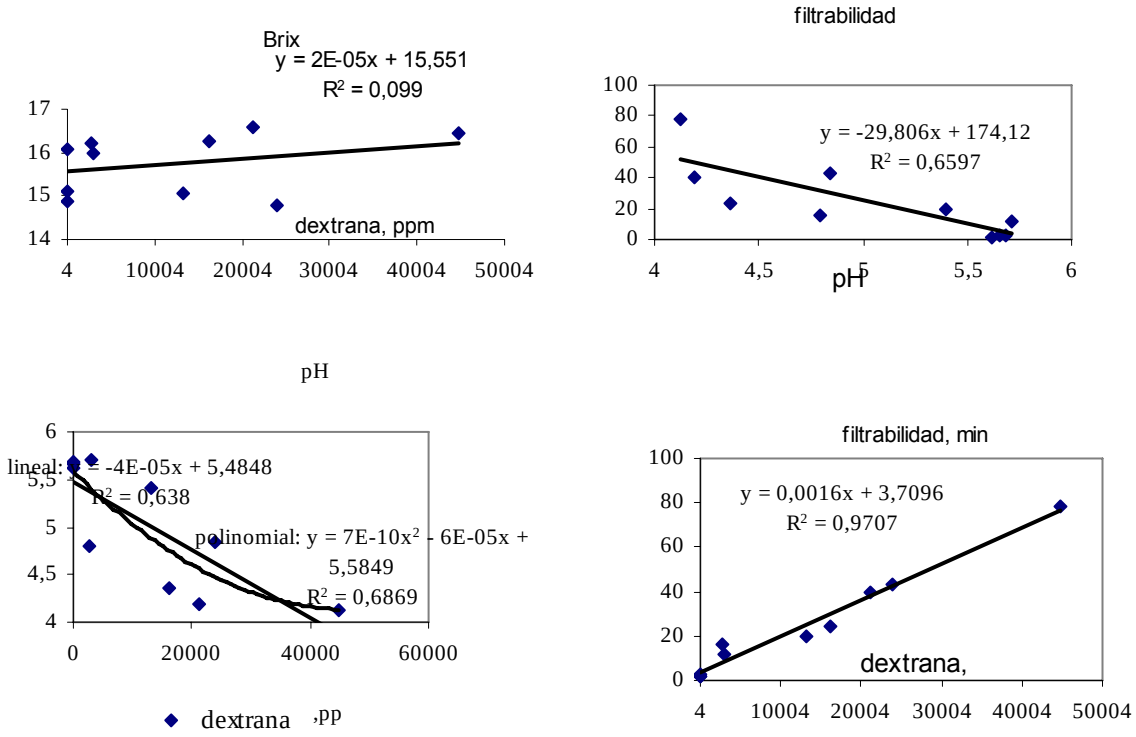

Fig. 22 Correlations as prediction of the juice cane deterioration.

The filterability was strongly correlated with the dextran content (r2 = 0,9707, P <0,005), while a poor correlation was observed between the pH and the dextran (r2 = 0,638, P <0,005), improving lightly when it was adjusted with quadratic polynomial (r2 = 0,687, P <0,005). Eggleston studies found better correlations with these models (10). Equally, the filterability and the pH had a smaller correlation (r2 = 0,6597, P <0,005) and it was null among Brix and dextran formation.

The strong correlation between filterability and dextran is given by the gummy formation associated to the dextran. It increases the permeation times. On the other hand, the dextrans formation is associated with the decrease of the pH, due to dextransucrase enzyme and lactic and acetic acid formation that causes pH decreases. There are not correlations between Brix and dextran by what it should not be analyzed as indicator of the juice deterioration.

DETERMINATION OF SUCROSE LOSSES.

Stequiometrically 342 g of sucrose produces 162 g dextrana and 180 g of fructose. In the synthesis, dextrans can be achieved between 40 – 70 kDa until l several millions in the molecular weight. Considering this fact, the lost of sucrose for dextrans concept are near at 2 % and considering other metabolites could be at 4 %, that's mean the sucrose losses will be 90 g/kg juice due to the action microbial.
If it these losses are calculated for half of the volume of the tank of industry juice (20 m3) that could be retained in the process by some cause, the lost sucrose is of 1800 kg (356,4 USD taking the price from the sugar to $0.09/lb = $0.198/kg)
If the deterioration of the sugar cane juice happens in a Cuban typical clarifier (300 m3), it could be the complete loss of the clarifier, that which 27 T of sugar (5346 USD).
For the clarifier treatment would be needed according to the doses of this study 21 liters of the product with a cost of 1,2 USD/L, for a total of 25 USD.
The balance cost - benefit gives highly favourable, for what it is feasibility the use of the product to avoid the juice deterioration during the stops process.

If it is considered the decrease of the sugar price by dextran in the international market, this analysis is favoured.

CONCLUSIONS

1. Dose of 0.007% of EVIPOL with 30 min. of contact was effective to inhibit above 87% bacteria, above 83% the microorganisms producing of polysaccharides, as well as above 73% yeasts in the primary and blended juice.
1. EVIPOL is able to stabilize the present sucrose in the juices, to prevent the sucrose degradation during the mill, to diminish the dextrans and other polysaccharides formation, to improve the viscosity of the juices, besides its effect microbial inhibition.
2. A strong lineal correlation exists between the filterability and the dextran content, as prediction of the juice deterioration.
3. The balance cost - benefit favours the EVIPOL application during the stops sugar process.

III. Impact of the achieved result

For diminishing the effect of the microorganisms on sucrose, it was developed the EVIPOL product that acts in the mill station and on sugar cane juice.

Inside the described result, are outstanding contributions as:

1. EVIPOL was developed, introduce and validate to industrial level with the objective of decreasing the deterioration of the cane juices for microbial action.
2. The toxicological studies were done. The toxical assays show that the product was innocuous for animals and human people.
3. EVIPOL as commercial brand of the product was obtained.
4. A patent of a biocide from jasmonate salt with microbicide effect was achieved.
5. The balance cost - benefit favours the EVIPOL application during the stops sugar process.

The accreditations related with this document are those that next are related:

Industrial introduction accreditation of the result.

1. Accreditation of the result by the ICIDCA scientific organ
2. Accreditation of the result for the CAI “España Republicana”
3. Accreditation of the result for the CAI “Hector Molina”
4. Accreditation of the result for the Argentinean enterprise, Tucumán, Argentina

Accreditation of contribution to the knowledge.

1. Patent. "Biocide composed by a jasmonate salt and other additives for sugar industry application" No. : 23555. Resolution: 1428/2010, 14 th June, 2010.
2. Certificate of the Register of the Commercial Brand: EVIPOL. Resolution: 1243/2005.

IV. Dissemination activities

a) Web site

The information for web page of the ISPLI Project have been given and hosted by the web site www.icidca.cu

b) Seminars and events

Two conferences about thematic were dictated in the UNT (Argentina). 43 students participated.

Two conferences about thematic were dictated in the UNAERP (Brazil). 25 students participated.

A doctoral thesis in this topic is being executed by brazilean student (UNAERP, Universidad de Riberao Preto, Brazil) and co- tuthoreaded by Cuban part.

c) Publications

- Michelena G., Martinez A., Cerutti G., Coronel M., Carrera E., Lopez- Murguia A., Bell A. y col. "Inhibitory effect on Leuconostoc and other baacteria" International Sugar Journal. 2006, 108, 1285: 44- 48.

Havana, August 30th, 2010

V. Anexs

- Patent. "Biocide composed by a jasmonate salt and other additives for sugar industry application" No. : 23555. Resolution: 1428/2010, 14 th June, 2010.
- Certificate of the Register of the Commercial brand: EVIPOL. Resolution: 1243/2005
- Michelena G., Martinez A., Cerutti G., Coronel M., Carrera E., Lopez- Murguia A., Bell A. y col. "Inhibitory effect on Leuconostoc and other baacteria" International Sugar Journal. 2006, 108, 1285: 44- 48.
- Accreditation of the result by the ICIDCA scientific organ
- Accreditation of the result for the CAI "España Republicana"
- Accreditation of the result for the CAI "Hector Molina"
- Accreditation of the result for the Argentinean enterprise, Tucumán, Argentina

REPÚBLICA DE CUBA

La Directora General de la Oficina Cubana de la Propiedad Industrial en uso de sus facultades y de acuerdo con lo establecido en las disposiciones legales vigentes, concede el presente:

CERTIFICADO DE AUTOR DE INVENCION

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INSTITUTO CUBANO DE INVESTIGACIONES DE LOS DERIVADOS DE LA CAÑA DE AZÚCAR, con domicilio legal en calle Vía Blanca, número 804, esquina a carretera Central, 11000, San Miguel del Padrón, Ciudad de La Habana, República de Cuba.

(11) Certificado Nro. : **23555** Concedido por Resolución No.1428/2010

(54) Título: **BIOCIDA COMPUESTO POR UNA SAL DE JASMONATO Y OTROS ADITIVOS DE USO EN LA INDUSTRIA AZUCARERA**

Dado en La Habana, a 14 de junio de 2010.

M.Sc. María de los Angeles Sánchez Torres
Directora General
Oficina Cubana de la Propiedad Industrial

REPÚBLICA DE CUBA

El Director de la Oficina Cubana de la Propiedad Industrial,
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otorga el presente:

Certificado de Registro de Marca.

A favor de: INSTITUTO CUBANO DE INVESTIGACIONES DE LOS DERIVADOS DE LA CAÑA DE AZÚCAR (ICIDCA)

Con domicilio en: Vía Blanca, número 804, esquina Carretera Central, San Miguel del Padrón, Ciudad de La Habana, República de Cuba

CERTIFICADO NÚMERO: 2004-0470 Concedido por RESOLUCIÓN: 1243/2005

Válido por diez años y vigente hasta el: 19 de julio de 2014
Consistente en: la denominación EVIPOL, con grafismo especial

Para distinguir productos solicitados en la clase 1
de la Clasificación Internacional de Productos y Servicios para el Registro de las Marcas.

Dado en La Habana, a 31 de agosto de 2005

Ing. María de los Angeles Sánchez Torres
Directora General
Oficina Cubana de la Propiedad Industrial

Inhibitory effect on *Leuconostoc* and other bacteria

By **Georgina Michelena*, Aidín Martínez*, Graciela Cerutti**, Mónica Coronel **, Antonio Bell*, Emilia Carrera*, Agustín López- Murguía***, Roberto Portuondo*, Lázaro Mergarejo* and Frost M Steele******

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Abstract

In the production of the sugar from the mill station until the clarification of the juice, an important cause of the sucrose destruction it is the action of microorganisms brought in with the cane. Sucrose loss and dextran formation are often associated with the microbial deterioration of sugar cane. Throughout the years, this has been a problem the industry has faced as it tries to improve the quality of sugar. In this research, the effect of different disinfection methods on cane juice is presented, with the ensuing microbial decrease and respective purity indices, which directly impact sugar yield. The antimicrobial effect of Inhibitor S at 0.007% on the microbial flora present in the primary and blended juice produced at a sugar mill was demonstrated. In the control sample, a decrease in purity of 4.8 units was observed after 12 hours. With the addition of Inhibitor S, a lesser decrease of 2 units, and stabilization of the juice took place almost completely in variant 3 of the experiment. The juice treated with Inhibitor S retained its dark brown colour, fresh scent, and pH without significant changes with respect to the initial. On the other hand, the untreated juice turned clear brown, had a strong scent of alcohol, and its pH and purity were markedly lower. This stabilization effect has not been reported for other disinfecting agents commonly used in the sugar industry. Also, this paper demonstrates it is possible to use Inhibitor S for the stabilization of blended cane juice. After treatment with Inhibitor S, free reducers and polysaccharides were six times lower than in the untreated samples. These results indicate a decrease in formed dextrans and therefore in free fructose.

Efectos inhibidores sobre Leuconostoc y otras bacterias

En la producción de azúcar, desde el ingenio hasta la clarificación del jugo, una causa importante de la destrucción de sacarosa es la acción de microorganismos que llegan con la caña. La pérdida de sacarosa y la formación de dextranas se asocian frecuentemente con la deterioración microbiana de la caña de azúcar. A través de los años esto ha constituido un problema que la industria ha tenido que hacer frente al tratar de mejorar la calidad del azúcar. En éste trabajo se analiza el efecto de diferentes métodos de desinfección del jugo de caña con la consecuente disminución de los microbios y los respectivos índices de pureza que, directamente, inciden sobre el rendimiento del azúcar. Se demostró el efecto inhibidor del Inhibitor S a 0,007% sobre la flora microbiana presente en el jugo primario y el mezclado que se producen en el ingenio. En la muestra control se observó una disminución de 4,8 unidades en la pureza después de 12 horas. Al añadir el Inhibitor S, se produjo una disminución menor, de 2 unidades, y la casi completa estabilización del jugo en la variante 3 del experimento. El jugo tratado con el Inhibitor S retuvo su color marrón oscuro, aroma fresco, y un pH sin cambios significativos con respecto al inicial. Por el otro lado, el color del jugo sin tratar cambió a marrón claro, tenía un fuerte olor a alcohol, y tanto el pH como la pureza eran significativamente más bajos. Este efecto estabilizador no ha sido descripto como resultado del uso de otros desinfectantes usados comúnmente en la industria azucarera. En este artículo se demuestra también que es posible utilizar el Inhibitor S para la estabilización del jugo de caña mezclado. Luego del tratamiento con el Inhibitor S, los reductores libres y los

Hemmwirkung auf Leuconostoc und andere Bakterien

Eine wichtige Ursache der Saccharosezerstörung in der Zuckerproduktion von der Mahlstation bis zur Klärung des Safts ist die Aktivität von Mikroorganismen, die mit dem Rohr hereingebracht werden. Saccharoseverluste und Dextran-Bildung stehen oft mit der mikrobischen Zersetzung von Zuckerrohr in Verbindung. Dies ist seit vielen Jahren ein Problem, dem sich die Zuckerindustrie bei ihren Bemühungen um Verbesserung der Zuckerqualität gegenübersieht. In der vorliegenden Forschungsarbeit präsentiert wird die Wirkung unterschiedlicher Desinfizierungsmethoden für den Zuckerrohrsaft, zusammen mit der anschließenden Abnahme mikrobischer Organismen und den damit einhergehenden Reinheitsindizes, die einen direkten Einfluss auf den Zuckerertrag haben. Demonstriert wurde die antimikrobische Wirkung von Inhibitor S bei 0,007% auf die mikrobische Flora, die im primären und vermischten Saft, der in einer Zuckerfabrik produziert wurde, präsent war. In der Kontrollstichprobe wurde nach 12 Stunden eine Abnahme der Reinheit um 4,8 Einheiten festgestellt. Bei Beigabe von Inhibitor S erfolgte eine geringere Abnahme um 2 Einheiten, und in der Variante 3 des Versuchs wurde der Saft nahezu völlig stabilisiert. Der mit Inhibitor S behandelte Saft behielt seine dunkelbraune Farbe, seinen frischen Geruch und seinen pH ohne signifikante Änderungen gegenüber dem Anfangszustand. Der unbehandelte Saft hingegen wurde hellbraun, er hatte einen starken Alkoholgeruch, und sein pH und seine Reinheit waren deutlich niedriger. Dieser Stabilisierungseffekt ist für keine anderen Desinfektionsmittel, die üblicherweise in der Zuckerindustrie eingesetzt werden, berichtet worden. Dieses Referat demonstriert darüber hinaus, dass es

Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar

ICIDCA

AVAL DEL CONSEJO CIENTIFICO

Proyecto "Increase in sugar production by microbial inhibition of *Leuconostoc* sp. and other bacteria (Phase II)" ISPLI- II

El proyecto presenta el efecto del producto EVIPOL en la desinfección de la estación de molinos y en la estabilización de los jugos de la caña, produciendo la disminución del tenor microbiano y la preservación de los índices de pureza, lo cual incide directamente en un mayor rendimiento de azúcar.

Se demostró el efecto microbicida del EVIPOL a 0.007 % sobre la flora microbiana del jugo primario y mezclado del CAI "España Republicana" y "Héctor Molina".

Igualmente se demostró en la EEAOC, de Tucumán, Argentina, la posibilidad de usar el EVIPOL para la estabilización del jugo de caña mezclado (cepa CP 65- 357). Según estos resultados se observaron disminuciones de 6 veces con relación al testigo no tratado en reductores libres y polisacáridos lo cual indica disminución en las dextranas formadas y por tanto en la fructosa liberada

La aplicación del producto en un clarificador durante una parada larga del ingenio- imprevista- trae ahorros económicos valorados en la decena de miles de USD. El balance costo- beneficio da altamente favorable, por lo que aboga por el uso del producto para evitar las inversiones del jugo durante las paradas.

En el marco del proyecto se realizaron los estudios ecotoxicológicos que permitieron el registro de la marca del producto y se obtuvo una patente que involucra a los grupos de trabajo de la región que participaron en el proyecto.

Por los elementos anteriormente expuestos el Consejo Científico del ICIDCA acordó por unanimidad extender su **aval** a los 13 días del mes de Julio de 2010

Lic. Miguel A. Otero Rambla
Secretario del CCC

ICIDCA

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CAI España Repúblicana

Perico, Matanzas, Cuba

Perico, 7 de julio de 2003
"Año de Gloriosos Aniversarios de Martí y el Moncada"

A quien corresponda:

Por este medio certificamos que a finales de la zafra 2003 fue realizado en el CAI España República la introducción del producto EVIPOL para su aplicación en la desinfección del área de molinos por el ICIDCA, lográndose inhibiciones de la población de bacterias y levaduras, pero especialmente de los microorganismos productores de polisacáridos en niveles por encima al 90 %.

Por otra parte, se realizó un ensayo del efecto del producto sobre la estabilidad del jugo clarificado, encontrándose a las 12 horas efectos de conservación del color, el olor y el pH con relación a un testigo no tratado.

El efecto estabilizante del jugo tiene una sensible importancia económica al evitar las pérdidas de grandes volúmenes del dulce durante las paradas del ingenio que pueden invertirse por la acción de microorganismos y no hacerlo factible para la continuación del proceso de fabricación.

Director del CAI España República

CAI Héctor Molina

San Nicolás de Bari, La Habana, Cuba

San Nicolás de Bari, 27 de abril de 2005
"Año de la Alternativa Bolivariana de las Américas"

A quien corresponda:

Por este medio certificamos que a finales de la zafra 2005 fue realizado en el CAI Héctor Molina la prueba industrial del producto Evipol para su aplicación en la desinfección del área de molinos y estabilizador del jugo de caña por el ICIDCA, lográndose inhibiciones de la población de bacterias y levaduras, pero especialmente de los microorganismos productores de polisacáridos en niveles por encima al 70 %.

Por otra parte, la aplicación del producto sobre el jugo de industria, durante 24 horas demostró los efectos estabilizadores del producto permitiendo la conservación del color, el olor, el pH y la pureza con relación a un testigo no tratado.

El efecto estabilizante del jugo es de importancia económica al evitar las pérdidas de grandes volúmenes durante las paradas del ingenio que pueden invertirse por la acción de microorganismos y no hacerlo factible para la continuación del proceso de fabricación.

CAI Héctor Molina

ESTACION EXPERIMENTAL AGROINDUSTRIAL
OBISPO COLOMBRES (EEAOC)

Av. William Cross 3150 – Las Talitas
4101. Tucumán. Argentina

San Miguel de Tucumán, Argentina, 13 de junio de 2003

A quien corresponda:

Por la presente tengo el agrado de avalar los resultados obtenidos según ensayo realizado con el inhibidor EVIPOL (obtenido en el Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar, ICIDCA) sobre jugo mezclado obtenido de caña de azúcar, cepa CP65-357 cultivada en Tucumán, Argentina, a concentraciones de 0.006 % donde se logró evitar el deterioro del jugo a las 24 horas evidenciado por la disminución de 6 veces la formación de azúcares reductores libres e igual número de veces la formación de dextranas, comparado con un testigo no tratado.

Este resultado es de una incuestionable importancia económica, pues abre las posibilidades de desarrollo de un producto comercial con perspectivas de uso industrial en los ingenios azucareros donde la formación de dextranas y las pérdidas de azúcar por este concepto constituyen un serio problema.

Graciela I. Corallí
DOCTOR EN BIOQUIMICA

Ing. Geronimo Cárdenas
Director Asistente en
Investigación y Tecnologia Industrial