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RESEARCH ARTICLE

Lactic acid production by a strain of *Lactococcus lactis* subs *lactis* isolated from sugar cane plants

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Abbreviations: GC: glucose conversion
LA: lactic acid
$Y_{p/s}$: yield

A *Lactococcus lactis* subs *lactis* strain was selected from 20 lactic acid strains isolated from the leaves of sugar cane plants, variety CC 85-92. The effects of substrate concentration and pH control on lactic acid (LA) production, glucose conversion (GC) and yield ($Y_{p/s}$) were investigated using this strain. In batch fermentation at 32ºC, with 60 gl^{-1} of glucose and a pH of 6.0, concentrations of up to 35 gl^{-1} of lactic acid were obtained. Maximum production and glucose conversion was achieved at low glucose concentrations. The strain shows great potential for lactic acid production from glucose, even without using pH control during fermentation (13.7 gl^{-1}).

Lactococcus lactis, originated from the lactic Industrial products have been and are still being, extensively studied for their commercial potential, but that strain isolated from plants has been given less attention (Martínez-Cuesta et al. 1997; Van Niel and Hahn-Hagerdal, 1999); in sugar mills, *Lactococcus lactis,* like many other lactic acid bacteria, are involved in sucrose inversion, due to their potential for producing lactic acid. However, the commercial potential of these strains that are well adapted to sucrose-rich environments has not been researched, and there are no studies in the scientific literature in which lactic acid production is quantified for each of the strains involved in sucrose inversion.

Lactococcus are coccibacteria, which form chains of variable length, they have a homo-fermentative metabolism and produce exclusively L(+) lactic acid (Roissart, 1994), although Akerberg et al. (1998) report that, D(-) lactic acid can be produced at low pH. Furthermore *Lactococcus lactis* is sub-divided into other sub-species: *lactis* and *cremoris* (Schleifer and Kilpper-Balz, 1987; Kim et al. 1999).

Representatives of this genus are isolated mainly from fresh vegetables and from the skin of animals; therefore it is believed that their presence in milk is due to contamination during milking, given that fodder represents the principal contamination source. Its presence in human being or animals is accidental because of they are not normally found in a significant number in excrement or soil (Roissart, 1994). Their most important habitat is in untreated milk, fermented milk and cheeses. *Lactococcus lactis subs lactis*, either in pure form or associated with other microorganisms, is the mesophilic strain most commonly used as a starter culture for lactic products; thus they fulfill an irreplaceable role in ensuring the structure, taste, conservation and healthfulness of these products (Jensen y Hammer, 1993; Salminen, and Von Wright, 1993; Roissart, 1994; Boonmee et al. 2003; Ziadi et al. 2005; Do-Won et al. 2006); they also play an important role in aroma enhancement, the production of flavoured milks, and in milk and cheese flavourings, and recently a great deal of attention has been focused on their pro-biotic properties (Salminen, and Von Wright, 1993; Van Niel and Hahn-Hagerdal, 1999; Boonmee et al. 2003). For these reasons this microorganism has great commercial potential,

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and this is why *Lactococcus*, and more especially *Lactococcus lactis*, isolated in the lactic industry, is still being studied exhaustively. The major product of fermentation is lactic acid, a compound with a high commercial value, with applications in the food, cosmetic, medical, and pharmaceutical industries. (Boonmee et al. 2003). However, the most interesting application for the lactic acid is its potential in the production of biopolymers (Hujanen and Linko, 1996; Chang et al. 1999; Hujanen et al. 2001; Danner et al. 2002; Lee, 2005).

Production of biomass for starter cultures, and of metabolites of *Lactococcus lactis*, is carried out in batch, fedbatch, and continuous fermentations (Roukas and Kotzekidou, 1998; Ohashi et al. 1999; Akerberg and Zacchi, 2000; Boonmee et al. 2003), and membrane technologies have been used for recovery of the lactic acid (Ohashi et al. 1999; Akerberg and Zacchi, 2000; Danner et al. 2002). In these processes the microorganism can be used either in a free form, or immobilized (Roukas and Kotzekidou, 1991).

It is well-known that the quantity of nutrients required by this strain, as well as its optimal physical growth conditions, depends on the source it was isolated from, how long it has been stored and the medium used to maintain it (Van Niel and Hahn-Hagerdal, 1999). The growth of *Lactococcus lactis* is greatly influenced by the temperature, and it has only been studied in a few cases; Akerberg et al. (1998), found that 33.5ºC is the temperature which *Lactococcus lactis* produces the biggest quantity of lactic acid from glucose. The influence of pH on various characteristics has been well-studied; the optimum pH established for growth and product formation is at around 6.0 (Bibal et al. 1988; Parente et al. 1994; Akerberg et al. 1998). Mathematical models have been developed to explain the influence of the substrate concentration on microorganism growth and lactic acid production in high lactose concentrations, they include terms for the inhibition by product and by substrate (Boonmee et al. 2003); other mathematical models include also terms for the pH and temperature influence (Akerberg et al. 1998; Parente et al. 1994).

As discussed above, and bearing in mind the lack of research on *Lactococcus* that are isolated from sucrose-rich ecosystems, this paper presents the results obtained from fermentations using glucose and a strain of *Lactococcus lactis subs lactis* isolated from sugar cane plants and also the effect of substrate concentration and pH control on AL, GC and $Y_{p/s}$.

MATERIALS AND METHODS

Sucrose-rich ecosystems

Samples were taken from 12.3 month-old sugar cane plants, of the CC85-92 variety, and from sugar cane juice, at La Cabaña farm and sugar refinery (Cauca, Colombia). Samples were collected from the point of union between leaves and stem (LUS), from the surface of the leaves (SL), from exudates produced by the cane-borer insect *Diatraea saccharalis* (EX), and from the first, second and third thirds of the cane stalk (FCS, SCS, TCS). The samples taken at the sugar refinery came from sugar cane juice untreated with lime (CJ), from sugar cane juice with added lime (CJSL); from unfiltered juice (JunF), from filter residues (FR) and from the first pressing (FP). Samples were transported under refrigeration to the bioconversions laboratory at Universidad del Valle, Cali, Colombia.

Isolation of microorganisms

Each of the samples was diluted sufficiently to obtain isolated colonies, using 0.1% peptone water, and each was planted in duplicate in MRS agar (De Man et al. 1960). The medium was sterilized at 121.1ºC and its pH was adjusted to 6.2 using sulfuric acid. 2 ml/l of aniline blue was added when the culture medium was at a temperature of 50ºC. The medium was inoculated with 0.1 ml of each of the dilutions using the spread plate method and they were incubated at 36º and 45ºC for 48 hrs in anaerobic conditions. The

Table 1. Kineticparameters obtained from batch fermentation with pH control and without pH control at different glucose concentrations.

Kinetic parameters	20g/l glucose		60g/l glucose		110g/l glucose	
	+	-	+	-	+	-
Fermentation time to achieve Pmax (h)	10	48	48	48	48	24
Glucose conversion, 100*(So-S)/So (%)	92.49	73.03	90.26	23.2	57.08	16.23
Max. cellular concentration, Xmax (g/l)	2.2	1	2.57	1.33	2.7	1.13
Max. lactic acid concentration, Pmax (g/l)	10.2	13.7	32.3	10.09	30.04	6.26
Product yield, Yp/s (g/g)	0.54	0.8	0.63	0.78	0.45	0.27
Lactic acid productivity (g/lh)	1.0	0.29	0.67	0.21	0.63	0.26

+:with pH control.
-: without pH control.

medium was inoculated with 0.1 ml of each of the dilutions (on the surface) and they were incubated at 36° and 45ºC for 48 hrs in anaerobic conditions. After counting the presumed organic acid-producing colonies (which assimilated the aniline blue), pure culture was obtained from each of the grown morphologies in the medium using successive subcultures. Once the pure cultures were obtained, they were transferred to MRS liquid culture medium, and incubated under the same conditions described above. After 24 hrs the pure liquid cultures were centrifuged at 5000 g for 10 min and then filtered with Millipore HVLP02500 filters; the supernatants were then injected into an HPLC.

The strains that produced more than 12 gl^{-1} of lactic acid under the described conditions were stored in MRS culture medium with glycerol, and they were frozen for preservation and later use.

Selection of the strain

The homo-fermentative strain which produced the highest lactic acid concentration was selected for this study.

Fermentation

Previous studies showed that the optimal temperature for lactic acid production of the selected strain was 32ºC (results not shown); thus, for this investigation, 18 fermentation trials were carried out at this temperature, using MRS broth, in 500 ml conical flasks, with a working volume of 250 ml. The strain was adapted to the cultivation temperature for three generations.

In order to study the effect of pH control, fermentations were carried out without pH control, and with pH controlled at 6.0, through the manual addition of 4 M sodium hydroxide.

The effect of substrate concentration was studied by varying the glucose concentration in MRS broth, between 20, 60 and 110 grams per litter.

In all cases: 10% inoculate with respect to volume of substrate was used, a fermentation time of 48 hrs at 120 rpm.

Analytical method

The strain was biochemical identified by duplicate, using API 50 CHL. The isomeric identification of the produced acid was made by enzymatic analysis D-lactic acid / L-lactic acid.

The identification of the glucose metabolism by homofermentative or heterofermentative pathway was made using HPLC, quantifying the concentration of alcohol and acetate in MRS broth after the fermentative process.

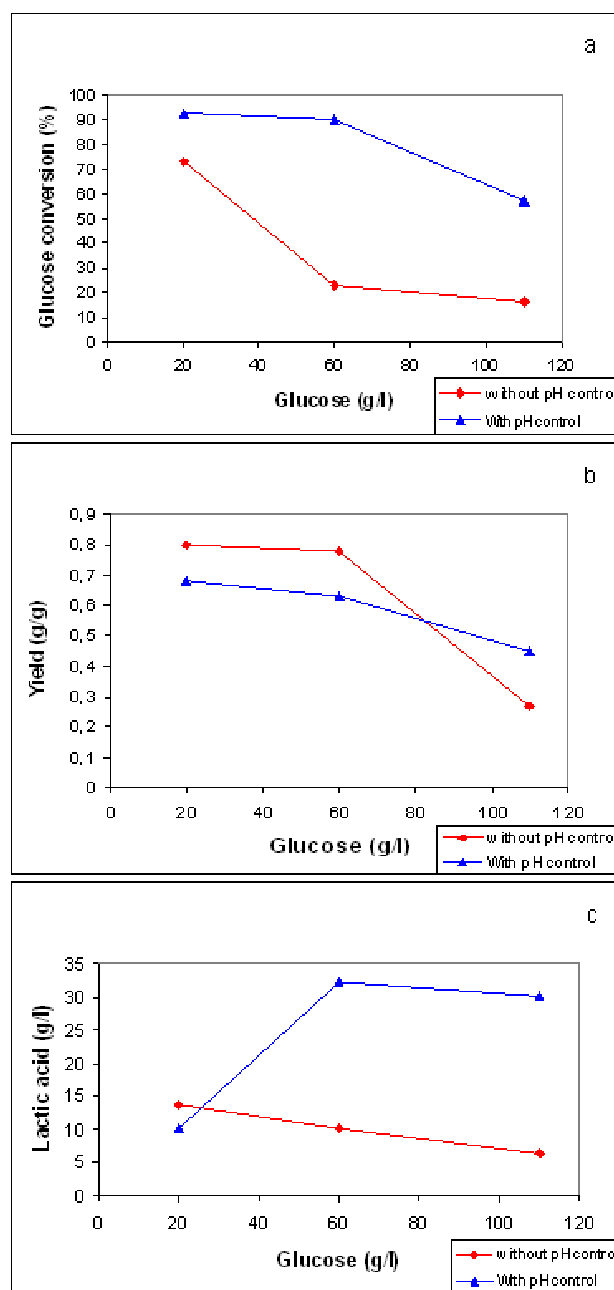

Figure 1. **Interaction graphics between glucose concentration and pH control.**
a. Glucose conversion, b. Yield, c. Lactic acid concentration

Samples of the liquid products from fermentation were treated as described above before HPLC analysis. Sugars and lactic acid concentrations were measured using high-efficiency liquid chromatography, HPLC (Hitachi L-6000A, integrator D-2500, equipped with an Aminex HPX 87H, 300 mm column), working temperature for the column was 60ºC, mobile phase was sulfuric acid 0.005 M and flow rate was of 0.6 ml/second. Biomass was calculated as 540 nm from optical density data, using a spectrum-photometer (Milton Roy 401). The pH was measured with an pH meter (ORION 710A).

The percentages of substrate conversion (GC) were calculated using the following expression:

$$GC = \frac{100 * (S_0 - S)}{S_0}$$

S_0: initial glucose concentration (gl^{-1})

S: Final glucose concentration (gl^{-1}), to time when P is maximum

The yields ($Y_{p/s}$) in grams per gram, were calculated as the gradient of the curve obtained by plotting product concentration against substrate concentration.

Reactives

Reactive grade products were used (Sigma Chemical Co.).

Statistical design

A factor design of 2 x 3, with three replicates, was used to analyze the effect of glucose concentration and pH control on AL, GC and $Y_{p/s}$. The pH was evaluated with two levels, using pH control and the other without pH control, and glucose concentration was evaluated at three levels: 20, 60 and 110 gl^{-1}. The data obtained was analyzed by Analysis of Variance (ANOVA) using MINITAB version 14 statistical software.

RESULTS

Selection of the strain

20 strains isolated from LUS, SL, EX, FCS, SCS, CJ and CJSL were selected for production of lactic acid, of which only one homo-fermentative strain produced significant quantities (12.4 g l^{-1} of lactic acid at 36ºC and 13.7 g l^{-1} a 32ºC). None of the strains produced significant quantities of lactic acid at 45ºC. The strain was biochemical identified as *Lactococcus lactis subs lactis.* The strain produced lactic acid with the isomeric configuration L(+).

Effect of pH control and substrate concentration

The variance analysis showed that glucose concentration and pH control have a highly significant effect on lactic acid concentration, glucose conversion and product yield ($P < 0.005$) with S values of 0.4457, 0.7696 and 0.02321 respectively. The interaction graphics (Figure 1) show the combined effects of glucose concentration and pH control when they change simultaneously. The graphic shows a considerable interaction between pH and glucose concentration upon lactic acid concentration and yield, as it was already seen in the ANOVA, and less marked interaction effect occurs for the glucose conversion.

The kinetic parameters, calculated from the experimental data, can be seen in Table 1, while the kinetics of lactic acid production and of substrate consumption can be seen in Figure 2 and Figures 3 respectively.

The highest glucose concentration was achieved with 60 g l^{-1} of glucose and with pH control (Figure 2); Ackerberg et al. (1998) found that the maximum glucose concentration for lactic acid production by *Lactococcus lactis ssp lactis* is 80 gl^{-1}. It is also important to highlight the good performance of the strain at low glucose concentrations (20 gl^{-1}) without pH control, in terms of lactic acid production and yield; in this case product generation was associated with growth, with an r_p of 0.29 $gl^{-1}h^{-1}$. In other cases, lactic acid was associated, and non-associated, with growth; Boonmee et al. (2003) using lactose and *Lactococcus lactis*, report this same behaviour. Working with concentrations of 20 to 40 gl^{-1}, they found that the production of AL is associated, and non-associated, with growth.

The highest $Y_{p/s}$ were obtained at low glucose concentrations and without pH control, while the largest percentages of *GC* were obtained with low glucose concentrations, but with pH control.

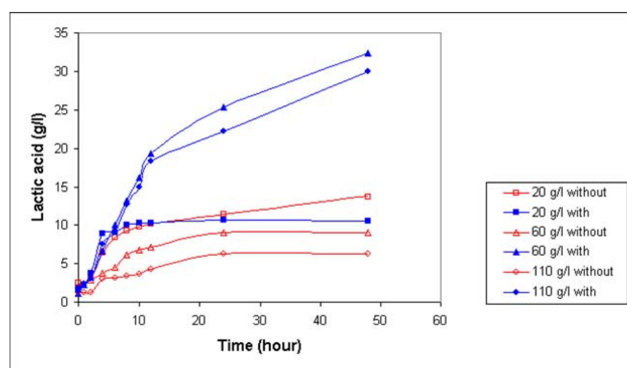

Figure 2. Kinetics of lactic acid production at different glucose concentration, with pH control and without pH control.

DISCUSSION

As can be seen in Figure 2, the fermentations with controlled pH using 60 and 110 g l^{-1} of glucose showed the same kinetic behaviour for lactic acid production. However, glucose conversion when 110 gl^{-1} was used is only 57%. This value is far lower than the obtained value when using 60 gl^{-1}, when glucose conversion is 90.2% (Table 1); this last situation is considered ideal in batch type fermentations, due to the product purification costs are lower. The significant concentration of lactic acid obtained in 48 hrs of fermentation with 20 gl^{-1} of glucose, and without pH control (13.7 gl^{-1}) can be explained by the adaptation of the strain to environments with high sucrose concentrations and besides because it has been demonstrated that *Lactococcus lactis* ssp. *lactis* is capable of surviving and replicating in more extreme levels of stress, compared to other lactic acid strains (Kim et al. 1999). This is why the microorganism has an enzymatic

system that hydrolyzes the disaccharide and then metabolizes the glucose using the glycolytic pathway; the behaviour of this isolation in substrates rich in sucrose was studied by Serna-Cock and Rodriguez-de Stouvenel (2004). The lower lactic acid concentration obtained using 20 gl^{-1} and pH control is due to an evident limitation of substrate.

In both the trials using pH control and those without pH control, it was observed that, as the glucose concentration is increased, the GC and $Y_{p/s}$ decrease, phenomenon that can occur by substrate inhibition, product inhibition or exhaustion of one restricting nutrient or their combined effect, however, concentrations of lactic acid obtained using 60 and 110 gl^{-1} of glucose do not differ significantly. This behaviour is also reported by Kious, 2000, in batch type lactic fermentations without pH control and with varying glucose concentrations from 20 gl^{-1} to 100 gl^{-1}; the results for lactic acid concentrations, yields and glucose conversion obtained by this author are similar to those reported in this study. Hujanen, 2001, also reports inhibition by substrate in fermentations with pH controlled at 6.3, using *Lactobacillus casei* NRRL B-441, and varying the glucose concentration between 80 and 160 gl^{-1}.

The strain of *Lactococcus lactis subs lactis* isolated from the leaves of variety CC-8592 sugar cane shows great potential for lactic acid production using glucose as substrate, even without using pH control during fermentation. Since there has been little studies

Lactococcus isolated from plant material, the results presented here suggest the importance of evaluating the genetic and commercial potential of this microorganism isolated from sugar cane, and also of other lactic acid bacteria adapted to substrates different to the lactose.

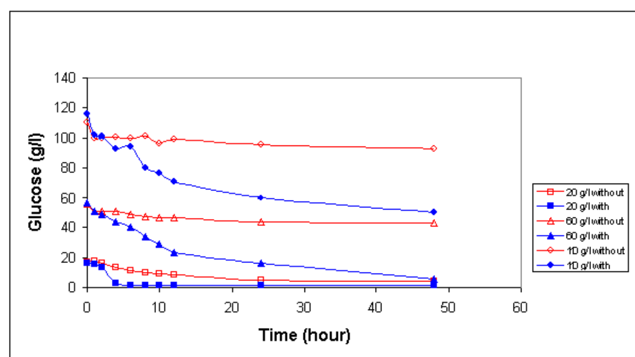

Figure 3. Kinetics of substrate consumption at different glucose concentration, with pH control and without pH control

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