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# Novel D-Galactose Isomerases from *Lactobacillus* Strains Isolated from the Sweet Sap of *Agave atrovirens*

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**Abstract:** D-tagatose is a natural monosaccharide used as a low-calorie sugar substitute in food, beverage and pharmaceutical products. Although it is a rare sugar, it can manufacture by enzymatic isomerization of D-Galactose Isomerase (D-GI). In this study, a screening was carried out to search microorganism producing D-GI in aguamiel. A rich selective medium was used in arabinose extracted from gum arabic. From 98 isolates obtained aguamiel of agave pulquero (*Agave atrovirens*), it was obtained 4 strains of *Lactobacillus* that producing D-GI and 2 strains of *Bacillus* sp. The Podi-20 strain was identified as *Lactobacillus diolivorans* based on 16S rRNA analysis, biological and biochemical characteristics. Furthermore, the gene encoding D-GI from *L. diolivorans* Podi-20 strain. Analysis of sequence revealed that the Open Reading Frame (ORF) of the *araA* gene consist of 1,428 pb that encodes a protein of 476 amino acid residues. The bioconversion yield of D-galactose to D-tagatose by the purified D-GI after 14 h at 60°C was 31.4%. D-tagatose can manufacture by enzymatic isomerization. This study contributes to new knowledge on D-GI from *Lactobacillus* strains, in particular those isolated from artisanal functional foods from agave.

**Keywords:** L-Arabinose Isomerase, D-Tagatose, Lactobacillus, Aguamiel, *Lactobacillus diolivorans*

## Introduction

Pulque is a traditional artisanal beverage originally produced in the highlands of Central Mexico. The beverage has been produced since prehispanic times by spontaneous fermentation of the agave sap of *Agave atrovirens* and *Agave salmiana*, from plant varieties known locally as “maguey”. The agave juice called aguamiel, which literally means “honey water”, is removed by extraction from the stem of 8 to 10-year old agave plants. The main sugars identified in aguamiel are glucose, sucrose, fructose and several pentoses (Escalante *et al*., 2004; Hernández-Salas *et al*., 2009; Gómez-Aldapa *et al*., 2012). Fermentation of the sap takes place due to the presence of microorganisms native of the agave sap, as well as from the environment. The final product, pulque, is a white, viscous liquid with about 45 g/L of ethanol and a pH of 3.4. Aguamiel contains a much higher abundance of acidophilic, Lactic Acid Bacteria (LAB) than fermentative yeast (Lazo, 2008). The bacterial diversity in aguamiel is mainly composed of homofermentative *Lactobacillus acidophilus* (88.1%), while major culture diversity consists of heterofermentative lactic acid bacteria such as *Leuconostoc mesenteroides* (50%) *Lactobacillus lactis* subsp. *lactis* (12.5%) and homofermentative *Lactobacillus* sp (14.8%) (Escalante *et al*., 2008). *Lactobacillus* strains have proven to be suited for foodgrade products and many lactobacilli have GRAS status (Donohue and Salminen, 1996; Morello *et al*., 2008).

L-arabinose isomerase (L-AI; EC 5.3.1.4) is an intracellular enzyme that catalyzes the reversible isomerization of L-arabinose to ribulose, an intermediary in the pentose phosphate or posphoketolase pathway (Patrick and Lee, 1968; Salonen *et al*., 2012). L-arabinose isomerase is also referred to as D-galactose isomerase (D-GI) due to its ability to isomerize D-galactose to D-tagatose.

D-tagatose is a hexoketose monosaccharide sweetener, an isomer of D-galactose, rarely found in nature. The sweetness of D-tagatose is equivalent to that of sucrose, but with only 38% of the calorie content (Levin, 2002; Kim, 2004; Salonen *et al*., 2012). In addition, D-tagatose has been shown to have numerous health and medical benefits, including its potential use in the treatment of obesity (Levin, 2002; Donner *et al*., 1999), the prevention of dental disease, the improvement of intestinal flora (Donner *et al*., 1999) and the reduction of symptoms of type 2 diabetes (Boudebbouze *et al*., 2011). Based on these properties, D-tagatose has attracted a great deal of attention in recent years as a low calorie sugar-substituting sweetener, which has reached GRAS status (FDA, 2003).

In order to find novel D-GI enzymes suitable for D-tagatose production, new microorganisms from highly diverse niches have been screened. *Lactobacillus* species are well known for their extraordinary stability at different pH ranges and variable temperatures (Roch *et al*., 2003; Hong *et al*., 2007). In this study, novel strains from *Lactobacillus* isolated from *Agave atrovirens* were selected for their ability to produce the enzyme D-GI and their suitability for the conversion of D-galactose into its isomer D-tagatose.

## Materials and Methods

### *Isolation of Bacterial Cultures*

The *Lactobacillus* strains were isolated using a liquid culture medium supplemented with arabinose as the sole carbon source. Arabinose was obtained from gum arabic using the following method (modified from Loeza-Cortez *et al*., 2007): Gum arabic (13 g) were dissolved in 75 mL of hot distilled water followed by slow addition of 18 mL HCl 1N. The solution was autoclaved for 30 min at 121°C and 15 psi. After the thermal processing, the solution was cooled down to room temperature and the pH adjusted to 7 using pure powdered calcium carbonate. The solution was centrifuged at 10,000 rpm for 10 min at 4°C and the precipitate discarded. Supernatant was added with 80 mL of warm (80°C) ethyl alcohol (96°GL) and filtered through whatman paper. The filtrate was concentrated under vacuum at 70°C and the ethyl alcohol distilled in a rotary evaporator. The concentrated extract (approximately 65 mL) was diluted to a final volume of 70 mL. The concentration of monosaccharides from the arabinose rich extract was determined by HPLC.

The microorganisms were isolated from aguamiel using Minimum Medium supplemented with Arabinose Extract (MMAE). MMAE had the following composition (g/L): 5.0 $K_2HPO_4$; 0.5 $NH_4NO_3$; 0.2 $MgSO_4$; 60 mL of arabinose extract and 18 g of bacteriologic agar. As selective antifungal agents, the medium also contained cycloheximide (50 mg/L) and nystatin (50 mg/L). About 400 mL of freshly collected aguamiel were used as the source of microbial inoculum and added into 200 mL liquid medium MMAE. The culture was divided in two parts. One part was incubated at 30°C and the other at 45°C. The isolation of bacteria was performed by the dilution plating technique inoculating serial dilutions on MMAE-agar followed by incubation at 30 or 45°C. Microbial colonies formed after 48-72 h were streaked on fresh agar plates, purified and preserved for further analysis.

### *Identification and Characterization of Bacterial Isolates*

The taxonomical and biological characteristics of selected D-GI producing strains were investigated using the procedures described in Bergey´s Manual of Systematic Bacteriology (Brenner *et al*., 2005). General biological and biochemical characteristics of selected bacterial strains included the determination of catalase and oxidase in pure cultures (Madigan *et al*., 2006). Physiological and biochemical characteristics were examined by the API-50 CHL kit system for *Lactobacillus* (BIOMERIEUX-Mexico), according to the manufacturer´s instructions. The bacterial metabolic profiles were determined after incubation at 37°C for 48 h. The extraction of genomic DNA, PCR amplification of 16S rDNA gene (first 500 pb) and sequencing of the purified PCR products were carried out as described previously (Baek *et al*., 2004). The 16S rRNA gene sequence was analyzed by alignment with previously described *Lactobacillus* sequences available in GenBank.

### *Microorganisms and Culture Conditions*

Selected bacterial isolates were cultured in standard MRS growth medium and incubated at 37°C for 5 days. The whole bacterial superficial cultures from 10 MRS agar plates were harvested and used as inoculum. Bacterial cultures were added to a 1 L Erlenmeyer flask containing 500 mL of a modified production medium with the following composition (g/L): 5.0 glucose, 10.0 yeast extract, 10.0 casein peptone, 10.0 sodium acetate, 0.2 $K_3PO_4$, 0.002 $MgSO_4$; 35.0 L-arabinose (Zhang *et al*., 2007). Alternatively, the medium contained either glucose (40 g/L) or arabinose (40 g/L) as a carbon source.

### *Production of D-GI*

*L. diolivorans* Podi 20 strain used the study was isolated in our laboratory from aguamiel and deposited in Agricultural Research Service (ARS) Patent Culture Collection under the accession number of NRRL Y-67346. For the production of D-GI, *L. diolivorans* Podi-20 was cultured in a medium with the following composition (g/L): 40.0 glucose; 10.0 yeast extract; 10.0 casein peptone; 10.0 sodium acetate; 0.2 $K_3PO_4$; 0.002 of $MgSO_4$. After 5 days of static cultivation at 30°C, the culture was centrifuged at 11,180 g, (4°C for 10 min). The whole cell pellet was washed twice and then suspended in one tenth of the volume of the production medium containing arabinose (40 g/L) as the sole carbon source. The concentrated bacterial culture was incubated for 5 additional days. The liquid culture was then centrifuged at 11,180 g at 4°C for 10 min and the whole cell pellet kept at -70°C until used for enzyme extraction.

### *Extraction of D-GI*

In the procedure for D-GI extraction, all steps were carried out 4°C. The frozen bacterial cell pellet was thawed and suspended in three volumes of acidified water (HCl, pH 2.5). Cells were centrifuged at 11,180 g at 4°C for 10 min followed by two additional washes with acidified water plus two final washes with distilled water. Bacterial pellet was treated with lysis buffer (50 mM phosphate buffer, pH 7.5) at a ratio of 8 mL to 2 g of wet pellet. Glass beads (10 g, 33 mm diameter) plus 1 g of small glass beads (106 μm diameter) were added to 10 g bacterial cell suspension and the mixture was vigorously vortexed for 20 min at room temperature. The mixture was passed through a steel sieve, to recover the large glass beads and the filtrate was centrifuged at 11,180 g (4°C for 10 min). Pellet was then resuspended in 8 mL of lysis buffer and the small glass beads discarded by decanting the supernatant, which contains both intracellular content and bacterial cell particles. The suspension was centrifuged at 11,180 g, (4°C for 15 min) and the pellet, containing Bacterial Cell Particles (BCP), washed with lysis buffer twice and finally recovered by the addition of 8 mL of lysis buffer (total volume ~10 mL). This BCP preparation had the highest D-GI activity when tested in a reaction mixture with D-galactose as substrate. Under standard conditions, a reaction mixture of 60 mL contained: 200 mM D-galactose; 0.5 mM $CoCl_2$; 1 mM $MnCl_2$; 10 mL of D-GI enzyme preparation of BCP at a suitable dilution; and 200 mM phosphate buffer (pH 7.5). The reaction mixture was incubated at 60°C for as long as 14 h. Aliquots from the enzyme reaction were taken at suitable intervals. The aliquots were placed in a boiling water bath (94°C) for 10 min to stop the reaction. The reaction product of the isomerization reaction (D-tagatose) was determined by colorimetric and chromatographic analysis.

### *Analytical Methods*

The arabinose rich solution from the processing of gum arabic was analyzed by HPLC with refractive Index detection (Agilent Technologies, Model 1260). An aliquot of the saccharification reaction was filtered through a 0.45 μm membrane. Arabinose was separated with an Aminex HPX-87P column (250×4 mm) (BioRad), with water as the mobile phase (flow of 0.6 mL/min; temperature set at 80°C).

Protein concentration was determined by the Bradford method using bovine serum albumin as the as the standard (Protein standard II lyophilized bovine serum albumin) (Bradford, 1976; Rhimi *et al*., 2011). D-tagatose was determined by the cysteine-carbazole sulphuric-acid method and the absorbance was measured at 560 nm, using a spectrophotometer (Hach, model DR 5000) (Dische and Borenfreund, 1951). One unit of D-GI activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of ketosugar min-1 under the above-specific conditions.

For the analysis of isomerization products, Thinlayer Chromatography (TLC) of D-galactose and D-tagatose was performed in a solvent system of ethylacetate/acetic acid/water (3/3/0.5, v/v/v) by the ascending technique on 0.2 mm silica gel-coated aluminum sheets (type 60; Merck, Damastadt, Germany). The plates were sprayed with a solution containing 10% $H_2SO_4$ in methanol and then heated to visualize the spots (Yanjun *et al*., 2011).

The method for sample preparation for HPLC analysis was performed as follows: an aliquot (5 mL) of the reaction mixture was centrifuged at 2,795 x g for 10 min at 4°C in order to discard BCP; the supernatant containing the soluble reaction products was then added with 5 mL of cold (-20°C) acetone and precipitated overnight at -20°C. The protein rich precipitate was separated by centrifugation at 2,795 x g for 10 min at 4°C. The supernatant was then placed in a water bath at 60°C to evaporate the acetone and filtered through a 0.45 μm micro-filter before injection in the chromatography equipment.

The amount of tagatose formed in the reaction mixture was also measured by HPLC with a Refractive Index Detector (Agilent Technologies, model 1260). The chromatographic conditions were: Aminex HPX-87P column (250×4 mm) from BioRad; a mobile phase of water, with a flow of 0.7 mL/min. Temperature was set at 80°C. Standard solutions of D-galactose and D-tagatose (3 $gL^{-1}$) were used for calibration curves.

## *Isolation and Sequencing of the araA Gene from L. diolivorans Podi-20*

The gene encoding the D-GI *araA* from *L. diolivorans* Podi-20 was isolated by PCR amplification. In order to obtain a partial sequence of *araA*, sequences

of genes encoding the arabinose operon (*araB*, *araD* and *araA*) obtained from the GenBank database, were aligned using the Geneious program version 8.1.4 (Wanarska and Kur, 2012). On the basis of the alignment, degenerated primers (Reverse 5'GGATGCAYACVTTYTCACCDGCHAARAACTGG 3′ and Forward 5′TGGAKGAACTTCVACBCGHGGYTTRTC 3′) were designed and synthesized. The PCR reaction was performed in a mixture containing 0.1 µM of each primer, 0.1 µg of genomic DNA from *L. diolivorans* Podi-20 and 25 µL of Thermo Scientific Maxima Hot Start PCR Master Mix (2X). The reaction mixture was incubated for 4 min at 95°C, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1.5 min and a final incubation of 8 min at 72°C. The PCR product obtained was then purified.

In order to obtain sequences flanking the known amplified sequence, the Universal Genome Walking kit from Clontech (Newark, USA) was used. Four PCR products were subsequently obtained and sequenced. These sequences were assembled using the Chromas Pro software to get a final 14 bp sequence. The Open Reading Frame (ORF) was identified by using 4 peaks version 1.8 software. The ORF was deposited at the GenBank database.

## Results

Lactic Acid Bacteria (LAB), particularly Lactobacilli have been isolated from a number of habitats, such as fermented foods, plant specimens and animals (Escalante *et al*., 2008; Xu *et al*., 2012). In this study, we describe the selective enrichment of a specific habitat, the sweet sap from *Agave atrovirens*, for the selective isolation of LAB, which can produce D-GI.

### *Production of Arabinose Rich Hydrolysate*

The processing of gum arabic by the method described, allowed for the production of a concentrated solution, which contained: arabinose 46.6%; galactose 33.3%; glucose 0.02%; xylose 0.3%; rhamnose 0.19%. The main constituents, L-arabinose and D-galactose have been previously reported as the main inducers of D-GI expression in bacteria (Becker and Boles, 2003; Lee *et al*., 2005; Helanto *et al*., 2007; Zhang *et al*., 2007) and also as carbon sources which, in LAB, are converted to L-ribulose or D-tagatose, by the action of the same isomerase (Xu *et al*., 2013; Staudigl *et al*., 2014).

### *Isolation and Screening of D-GI Producing Bacteria*

Bacterial colonies were isolated and screened for D-GI activity. A total of 98 isolated bacteria isomerized D-galactose. Isolates 17, 18, 19 and 20 showed the highest activity to D-GI with D-galactose as a substrate occurred at 60°C and pH 7.5, its initial activity was retained after 14 h incubation. The conversion of D-galactose to D-tagatose in the isolate 17 showed a conversion of 1.3%, isolate 18 showed a conversion of 0.3%, isolate 19 showed a conversion of 0.005% and isolate 20 showed a conversion of 3.25%. Selected strains were tested for D-GI activity by measuring the production of D-tagatose from D-tagatose using the cysteine-carbazole-sulfuric acid spectrophotometric method.

### *Identification of Selected LAB Isolates*

Morphological, biochemical and physiological characteristics of four LAB selected strains (17, 18, 19 and 20) are shown in Table 1. In all cases, the vegetative cells were non-motile, rod-shaped structures. Under aerobic conditions, the isolates showed diverse catabolic patterns, as determined by the API 50CHL system. Isolates 17, 18 and 20 displayed similar fermentation profiles, with minor differences related to catabolism of D-galactose, D-mannose, D-mannitol, D-sorbitol, methyl-αD-glucopyranoside, D-melibiose and D-raffinose, while isolate 19 showed a very distinct carbohydrate fermentation pattern. The results of partial sequencing (500 bp) of 16S rDNA from 99.7% (isolate 17) and 99.82% identity (isolates 18 and 20) to *L. diolivorans*. Isolate 19 showed 99.8% identity to *Lactobacillus pentosus*. The phylogenetical relationship of isolates 17, 18 and 20 with closely-related species of Lactobacilli is shown in Fig. 1.

### *Bioconversion of Galactose Into Tagatose*

Isolate 20 (*L. diolivorans*, denominated strain Podi-20) was studied in depth for its ability to produce D-GI, strain selected with highest production of tagatose. For that purpose, the strain was cultivated in medium of production (PM) containing glucose 40 g/L. After 5 days incubation at 30°C, a biomass yield of 18 g/L was obtained. In order to study the effect of different carbon source as inducers of D-GI, the biomass was concentrated to one tenth of the original volume in PM medium, only this time containing either L-arabinose or glucose as the sole carbon source (40 g/L). After 5 additional days of cultivation, the cells were recovered and lysed to obtained two fractions: the BCP and the culture Supernatant (SN) and analyze their D-GI, both by TLC and by HPLC. After the enzyme reaction, a small aliquot of reaction mixture was analyzed by HPLC and the produced D-tagatose concentration determined by using an authentic D-tagatose standard. After 6 or 14 h of isomerization reaction at 60°C, the tagatose peak was detected without any byproducts at 11 min, using a flow rate of 0.7 mL/min. Both fractions (SN and BCP) showed D-GI activity, but the highest activity was detected when the BCP fraction was used as the enzyme source (Fig. 2).

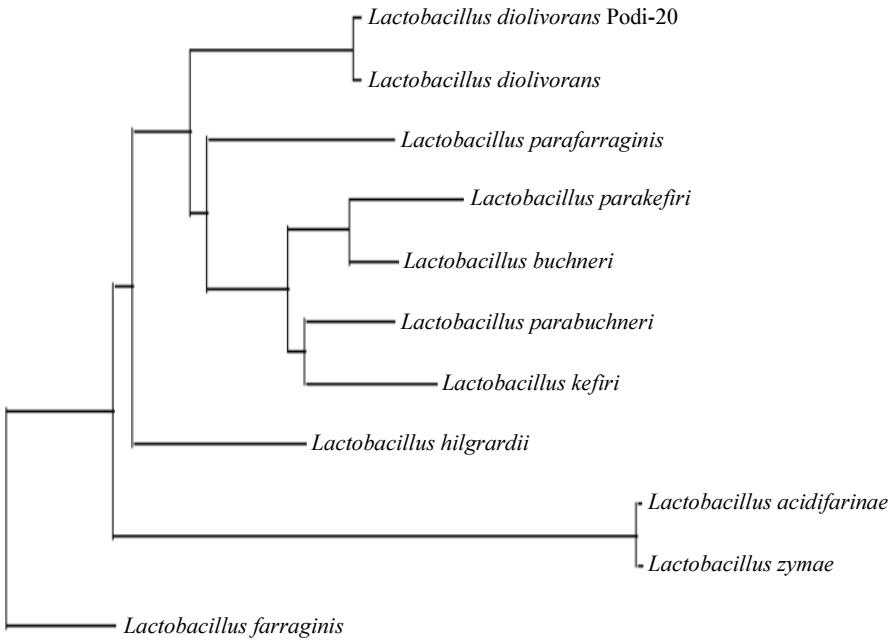
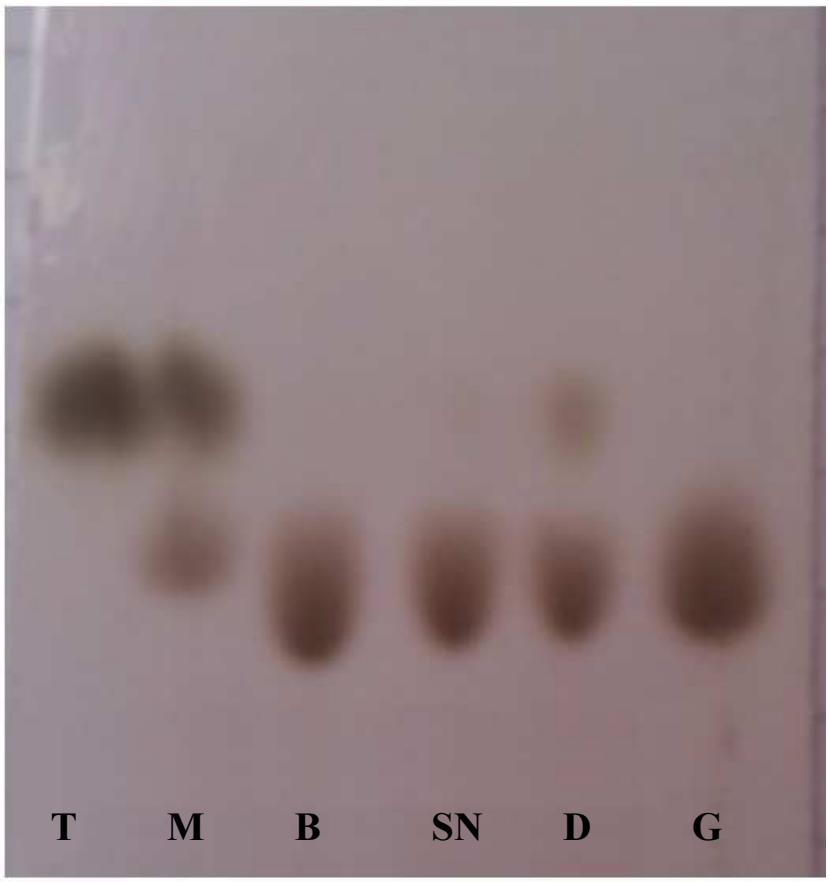
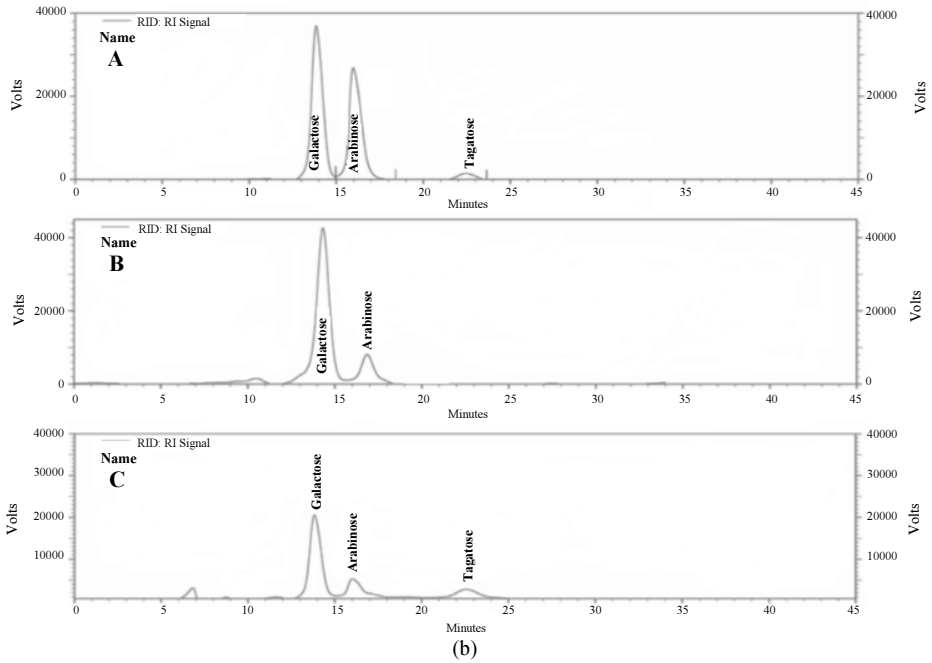



**Fig. 1:** Phylogenetic tree showing the relationship among *L. diolivorans* Podi-20, related species of the *L. buchneri* group and species representative of different lineages within the genus *Lactobacillus*




(a)




(b)

**Fig. 2:** Tagatose production by D-galactose isomerase from *L. diolivorans* Podi-20. In (a): Thin-layer chromatography analysis of isomerization reactions using fractions from Podi-20 cultures (14 h, 60°C). Lane T: Pure tagatose standard; M: Mixture of D-tagatose and D-galactose pure standards; B: Enzyme reaction (14 h), with no enzyme; SN: Enzyme reaction (14 h), with Podi-20 lysis supernatant as source of enzyme; D: Enzyme reaction (14 h), with Podi-20 lysed Bacterial Cell Particles (BCP), as source of enzyme; and G: Pure galactose standard. In (b): HPLC analysis of D-galactose isomerization reactions by Bacterial Cell Particles (BCP) of *L. diolivorans* Podi-20. A: Pure standards of galactose, arabinose and tagatose. B: No enzyme control C: Enzyme reaction (14 h, 60°C), with Podi-20 lysed Bacterial Cell Particles (BCP), as source of enzyme

**Table 1:** Microbiological and biochemical characteristics of selected D-GI producing *Lactobacillus* strains, when grown at 30-37°C and pH = 7.0, media MRS and MMAE

| Characteristics | Strain 17<br>*L. diolivorans* | Strain 18<br>*L. diolivorans* | Strain 19<br>*L. pentosus* | Strain 20<br>*L. diolivorans* |
|---|---|---|---|---|
| Shape | Rod | Rod | Rod | Rod |
| Size (width x length, μm) | 0.6 to 1×1.8 to 2 μm | 0.6 to 1×1.8 to 2 μm | 0.6 to 1.8 μm to 2 μm | 0.6 to 1×1.8 to 2 μm |
| Motility | - | - | - | - |
| Gram strain | + | + | + | + |
| Spore | - | - | - | - |
| Production of indol | - | - | - | - |
| Production $H_2S$ | - | - | - | - |
| Urease | - | - | - | - |
| Oxidase | + | + | + | + |
| Catalase | - | - | + | - |
| Citrate | - | - | - | - |
| D-Glucose | + | + | + | + |
| Lactose | - | + | - | - |
| Sucrose | - | - | + | + |
| L-Arabinose | + | + | + | + |
| D-Galactose | w | + | + | - |
| D-Arabitol | w | w | w | w |
| D-Fructose | - | - | - | - |
| D-Raffinose | w | + | + | W |
| D-Mannose | - | w | + | - |
| D-Mannitol | w | w | + | - |
| D-Sorbitol | w | w | + | - |
| D-Lactose | w | + | + | w |
| D-Melibiose | + | + | + | w |
| Methyl-αD-glucopyranoside | w | + | + | w |
| Anaerobic grown | - | - | - | - |

+, positive; -, negative; w, weakly positive

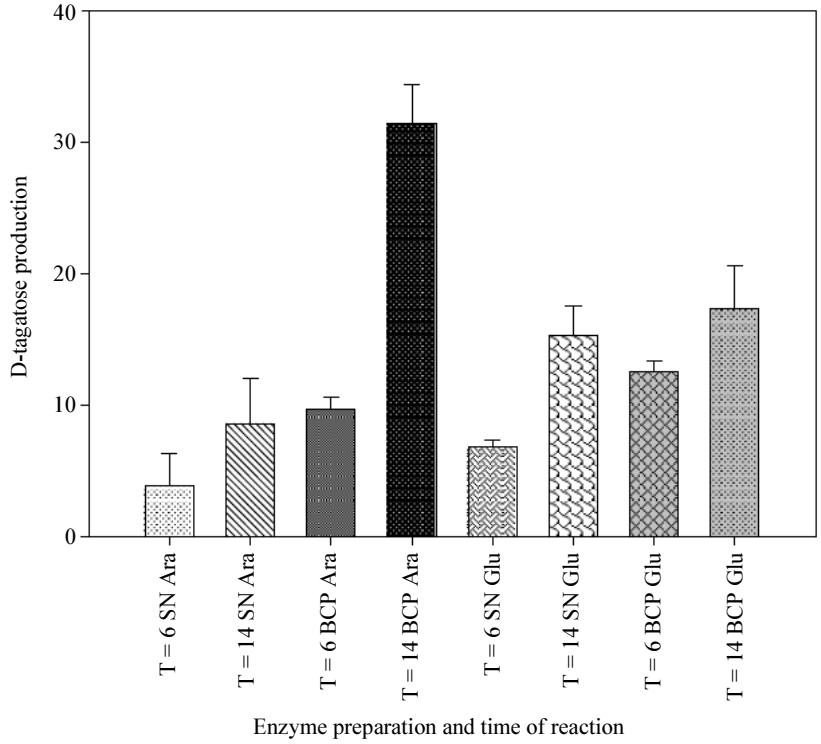



**Fig. 3:** Effect of enzyme reaction time and carbon source of induction medium, on the extent of conversion of D-galactose into D-tagatose, by culture fractions of *L. diolivorans* Podi-20. T: Time points of isomerization reaction at 60°C (6 and 14 h); the enzyme source from Podi-20 cultures was either SN: Lysis Supernatants; or BCP: Bacterial Cell Particles. *L. diolivorans* Podi-20 was grown in production medium containing glucose for 5 days. The cultures were concentrated to one tenth of the volume of the original growth medium, in an induction medium containing as the sole carbon source either arabinose (Ara), or glucose (Glu). After 5 additional days of cultivation, the bacterial cells were lysed and both lysis supernatants and Bacterial Cell Fractions were recovered and tested as enzyme source for D-galactose isomerase

In Fig. 3, results are shown on the effect of the carbon source of the induction medium (PM in one tenth of the original culture medium), on the extent of conversion of D-galactose into D-tagatose, by culture fractions of *L. diolivorans* Podi-20, at 6 and 14 h of reaction time at 60°C.

When *L. diolivorans* Podi-20 cultures were induced in glucose, the bioconversion of D-galactose into D-tagatose, was much lower when SN of cell lysis were used as enzyme source. The yield of D-tagatose based on D-galactose was 7.8% at 6 h and 15.3% at 14 h. When the BCP fraction was used as the enzyme source, the D-tagatose yield was 12.5% at 6 h and 17.3% at 14 h.

When *L. diolivorans* Podi-20 cultures were induced with arabinose, the bioconversion of D-galactose into D-tagatose, was also much lower when SN of cell lysis were used as enzyme source. The yield of D-tagatose based on D-galactose was 5.6% at 6 h and 8.6% at 14 h. When the BCP fraction was used as the enzyme source, the D-tagatose yield was 9.7% at 6 h and 31.4% at 14 h.

Another set of tests was performed with a crude enzyme (whole lysate) from *L. diolivorans* Podi-20 cultures in PM medium containing arabinose as the sole carbon source. The reaction gave a maximum of 13.6% of D-galactose converted into D-tagatose after 14 h of reaction at 60°C (data not shown).

### *D-GI Gene from L. diolivorans Podi-20*

In order to amplify by PCR the *araA* gene from *L. diolivorans* Podi-20, degenerate primers reverse and forward were designed. The sequences were derived from consensus sequences of 2 *araA* structural genes from *Lactobacillus* species found in GenBank, one from *L. plantarum* and one from *L. pentosus*. A PCR product (900 bp) was obtained by amplification of a genome DNA template from a pure culture of *L. diolivorans* Podi-20. Based on the amplicon nucleotide sequence, new primers were designed and used to obtain additional PCR amplified sequences by the use of the Kit Genome Walker (Clontech, USA). The amplicons (between 500 and 1000 bp) were obtained and used to complete the whole sequence of the *araA* gene. A complete sequence of 1,428 bp, corresponding to the *araA* putative gene, has a predicted D-GI protein of 476 amino acids (53.696 kDa), as well as a theoretical isoelectric point of 4.98.

```
atgttacaagttcctgattatgaattttggtttgttactggtagt
 M  L  Q  V  P  D  Y  E  F  W  F  V  T  G  S
caacacctatatggtgaagaacaacttaaatcagttgaaaaagat
 Q  H  L  Y  G  E  E  Q  L  K  S  V  E  K  D
gcccgtgacatcgttgacaaactgaatgctactggtacattacct
 A  R  D  I  V  D  K  L  N  A  T  G  T  L  P
tacccaattaagtttaagatggttgcaacaaccgctgacagcatc
 Y  P  I  K  F  K  M  V  A  T  T  A  D  S  I
actcaattaatgaaagatgttaactataatgataaggtagccggt
 T  Q  L  M  K  D  V  N  Y  N  D  K  V  A  G
gtcattacttggatgcacacattctcacctgctaagaactggatc
 V  I  T  W  M  H  T  F  S  P  A  K  N  W  I
agaggtactaagttacttcaaaagccattacttcatttagctact
 R  G  T  K  L  L  Q  K  P  L  L  H  L  A  T
caatacttggatcatattccatatgacaccattgattttgactac
 Q  Y  L  D  H  I  P  Y  D  T  I  D  F  D  Y
atgaacttaaaccaaagtgctcatggtgaccgtgaatacggcttt
 M  N  L  N  Q  S  A  H  G  D  R  E  Y  G  F
atcaatgcgcgtcttcaaaaacacaataagattgtttatggctac
 I  N  A  R  L  Q  K  H  N  K  I  V  Y  G  Y
tggggcgatcccgaagttcaacaagaaattgctgactgggaagac
 W  G  D  P  E  V  Q  Q  E  I  A  D  W  E  D
gttgctgttgcttacgacgaaagctttaagatcaaagttgcccgt
 V  A  V  A  Y  D  E  S  F  K  I  K  V  A  R
tttggtgacaacatgcgtaatgttgctgttactgaaggtgacaaa
 F  G  D  N  M  R  N  V  A  V  T  E  G  D  K
gttgaagctcaaattcaatttggctggaccgttgattactatgcg
 V  E  A  Q  I  Q  F  G  W  T  V  D  Y  Y  A
ttaggtgacttggttgaaactgttaacgcagttcctgaaagtgat
 L  G  D  L  V  E  T  V  N  A  V  P  E  S  D
attgacgctaagtacaaggaattacaagataaatacgaatttgtt
 I  D  A  K  Y  K  E  L  Q  D  K  Y  E  F  V
caaggcgataacgacaaagataaatatgaacattcagttcgttac
 Q  G  D  N  D  K  D  K  Y  E  H  S  V  R  Y
cctgttgaaggacacaaagcgccaaagccaacgccacatctacca
 P  V  E  G  H  K  A  P  K  P  T  P  H  L  P
gttgcaaaacaaatgtggacaccaaaggttggtttgaagcatggt
 V  A  K  Q  M  W  T  P  K  V  G  L  K  H  G
gctactcaatggattcaaaatggtggtggccatcacactgttttg
 A  T  Q  W  I  Q  N  G  G  G  H  H  T  V  L
acattcgctgctactgagactcaaattcaagaccttgcaacaatg
 T  F  A  A  T  E  T  Q  I  Q  D  L  A  T  M
tttggtctcccatttgctgatatcaacgaataattgttgtttata
 F  G  L  P  F  A  D  I  N  E  -  L  L  F  I
aatcatatttaaagcacacaaaagtaaggttggggcggctgcggc
 N  H  I  -  S  T  Q  K  -  G  W  G  G  C  G
tgtcgcaaccttctttcgtacataaaatgaatccgttatctttga
 C  R  N  L  L  S  Y  I  K  -  I  R  Y  L  -
tataataaagtaatcaaatggttggaggttgcttttatggc
 Y  N  K  V  I  K  W  L  E  V  A  F  M
```

**Fig. 4:** DNA sequence of the putative structural gene *araA* of D-galactose isomerase from *L. diolivorans* Podi-20. And deduced amino acid sequence. The stop codon is marked in a box. The sequence data reported here has been submitted to the GenBank database and have been assigned the accession number: KX365247

The sequence data the *araA* gene from *L. diolivorans* Podi-20 have been submitted to the GenBank database under accession number KX365247 (Fig. 4). The *araA* gene was sequenced, the deduced primary structure of the D-GI. The predicted amino acid sequence of *araA* from *L. diolivorans* Podi-20, exhibited high homology (76-88%) to that of other D-GI, which suggests that these genes might have evolved from a common ancestor.

The *araA* gene products from related LAB strains previously sequenced, including *L. buchneri*, *Pediococcus pentosaceus*, *L. koreensis* and *L. brevis* (Zhang *et al*., 2007; Chouayekh *et al*., 2007; Zheng *et al*., 2013). The *araA* from *L. diolivorans* also showed high similarity to other isomerases from several species of the genus *Bacillus*. The process described in this work for D-tagatose production was compared with others *Lactobacillus* where *L. diolivorans* Podi-20 offers an advantage as a generally recognized as safe organisms, it is attractive to produce D-GI for the industrial production of food-grade D-tagatose.

## Discussion

The aim of the present study was to isolate, select and characterize novel LAB, capable of D-GI (also known as L-arabinose isomerase) production and its conversion into D-tagatose. The selected niche of study, the *Agave atrovirens* sap (aguamiel), is a traditional medicinal juice from the central highlands of Mexico, known for its effect in stabilizing microbial flora because of its high diversity and abundance of beneficial acidophilic bacteria and yeast (Trejo, 2005). Aguamiel contains oligofructans, glucose, fructose and sucrose and is locally consumed as a healthy artisanal beverage (Trejo, 2005). Several authors have described the microbial production of D-GI (Kim *et al*., 2001; 2002; Jorgensen *et al*., 2004; Kim, 2004; Deok-Kun, 2007; Patel *et al*., 2012). L-arabinose is catabolized in gram-positive bacteria through isomerization and converted into L-ribulose, which constitutes the first step in the catabolic pathway of L-arabinose (Lee *et al*., 2004; Xu *et al*., 2012; 2013; 2015). The same enzyme catalyzes the isomerization of D-galactose into D-tagatose (Zhang *et al*., 2007; Cheng *et al*., 2009; Mei *et al*., 2016). L-arabinose has been previously reported as the main inducer for the expression of the *araA* gene (Yanjun *et al*., 2011; Rhimi *et al*., 2011; Xu *et al*., 2013) and thus, the preferred carbon source for enrichment and selective media, to be used in a large scale screening for the detection and selection of novel bacterial isolates producers of D-GI. Due to the high cost of pure L-arabinose, in the present study a process to obtain L-arabinose from arabic gum was adapted and improved. The hydrolytic and fractionation process allowed for the recovery of 46.6% of L-arabinose as a free monosaccharide. In previous reports, hydrolysis and saccharification yields of free arabinose, ranged from 27 to 46%, from polysaccharides of different species of *Acacia* (Islam *et al*., 1997; Loeza-Cotez *et al*., 2007); and up to 40% when obtained from mesquite gum (Qi *et al*., 1991). Using exclusively aguamiel as the source of LAB, 20% of the bacterial isolates were positive to the cistein-carbazol reaction and could be cultivated in D-galactose as the sole carbon source. All of the isolates were able to partially convert D-galactose into D-tagatose, which confirms the hypothesis of aguamiel as a good, partially unexplored source of bacterial biodiversity for biotechnological applications. Other groups have explored similar habitats for the same purpose (Cheetham and Wootton, 1993; Zhang *et al*., 2007; Xu *et al*., 2011; Yanjun *et al*., 2011; Linang *et al*., 2012). The most relevant D-GI producing strains, isolated from aguamiel, were identified as *Bacillus* sp, *L. pentosus* and *L. diolivorans*. Strains from Bacillus and from *L. pentosus* have been previously reported as D-GI producers (Oh, 2007; Seo, 2013), whereas no previous report exists for *L. diolivorans* as capable of isomerization of D-galactose. For the induction of D-GI production, a culture medium reported by Zhang *et al*. (2007), was modified. First, a growing medium containing 4% (w/v) glucose allowed for the production of bacterial viable biomass. Then, the concentrated culture is induced in a medium containing L-arabinose (3% w/v). The use of media reduces the time for maximum enzyme production; and the cost, mainly due to the high commercial price of L-arabinose. Yanjun *et al*. (2011) reported the wide diversity of isomerization conditions under which different bacterial enzymes perform the isomerization of D-galactose. The main focus of the present study was *L. diolivorans* Podi-20, a selected strain capable of a high rate of D-galactose isomerization using 200 mM of D-galactose; and a reaction performed at pH 7.5 and 60°C. Under those conditions, whole cells of Podi-20 convert from 5 to 8% of D-galactose into D-tagatose in a 14 h reaction. BCP from the same culture, obtained by the controlled lysis and fractionation of *L. diolivorans* Podi-20, converted up to 31% of D-galactose into D-tagatose under the same isomerization conditions. Previous studies using *Geobacillus thermodenitrificans* (Linang *et al*., 2012; Zhou and Wu, 2012) reported the use of D-galactose at 100 mM and a preferred pH 7, for the same reaction. With a different *Lactobacillus* species Zhang *et al*. (2007) obtained 39% conversion after a 96 h reaction, using a purified enzyme obtained from *L. plantarum*, Baek *et al*. (2004) used *G. thermodenitrificans* as the source of enzyme, obtaining 15% of D-tagatose based on D-galactose, after an isomerization reaction of 3 h. A disadvantage using *E. coli* as the host may bring about potential poisoning problems (Xu *et al*., 2012). An additional advantage of Podi-20 is that Lactobacilli are regarded as safe sources of enzymes applied to food processing, whereas microorganisms belonging to

*Geobacillus* and other thermophilic genera, may not have been studied enough to ensure safety.

In the present study, the enzyme activity was detected and assayed in different fractions: the supernatant of lytic treatment of the whole cell pellet; and the bacterial cell particles derived from the lytic treatment. The enzyme activity was barely detected in the cell pellet and more significantly in the BCP derived from a chemical and mechanical treatment. The high isomerization D-GI activity from the BCP fraction is strongly bound, in such a degree, that an "immobilized" kind of function is evident, even comparable to Wang *et al*. (2010), where a recombinant L-arabinose isomerase from *Bacillus licheniformis* was immobilized in alginate, providing stability and high stability.

A great disadvantage of the production of D-GI from Podi-20 is the strong need of an enzyme expression inducer. As reported by many authors (Oh, 2007; Chouayekh *et al*., 2007; Rhimi *et al*., 2011; Xu *et al*., 2011; 2012), arabinose is the strongest inducer of D-GI (L-arabinose isomerase). The high cost of pure L-arabinose was partially circumvented by the production of Podi-20 in a medium containing glucose (4% w/v), which allowed for a much cheaper process and the 10 to 20-fold concentration of the whole cell pellet for the subsequent suspension in an induction medium containing L-arabinose as the sole carbon source. The best L-arabinose concentration for the induction stage was 3.5% (w/v).

The novel *Lactobacillus* strains, in particular Podi-20, described in the present study, are candidates for the production of D-GI and for its heterologous expression in suitable bacterial systems. Moreover, lactobacilli have been approved as GRAS, which makes this *L. diolivorans* strain an interesting substitute for recombinant D-tagatose from *E. coli*. The use of *E. coli* as the host may bring about potential poisoning problems (Xu *et al*., 2012).

The use of the free or immobilized enzyme for the production of D-tagatose is feasible in the short term. The bioconversion of D-galactose into D-tagatose is an industrial need in Mexico and elsewhere.

## Conclusion

We found aguamiel to be an excellent source of microorganisms for food and nutrition applications and biotechnology relevant catalysts. The novel D-galactose isomerase (D-GI) from *L. diolivorans* Podi-20 is highly active and has potential applications in the commercial production of D-tagatose. D-tagatose is a highly functional and rare ketohexose. Many attempts have been made to convert D-galactose into its valuable isomer D-tagatose, using D-GI. D-tagatose is a natural monosaccharide, which can be used as a low-calorie sugar substitute in processed foods, beverages and pharmaceutical products. It is currently being tested as an anti-diabetic and obesity control additive. D-tagatose can manufacture by enzymatic isomerization. This study contributes to new knowledge on D-galactose isomerases from *Lactobacillus* strains, in particular those isolated from artisanal functional foods from Agave

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## Author's Contributions

**María Miriam Hernández-Arroyo:** Worked on enzymatic isomerization and molecular identification of bacterial isolate, gene amplification and sequencing as well as writing of the document.

**Miguel Ángel Plascencia-Espinosa:** Advised the team on molecular identification of the selected strain. Supervised amplification of encoding gene of the enzyme.

**María Eugenia Hidalgo-Lara:** Developed the manuscript reviewing.

**Mariana Tinajero-Trejo:** Supervised amplification of encoding gene of the enzyme and manuscript reviewing.

**Emilio Méndez-Merino:** Developed the manuscript reviewing.

**Sergio Rubén Trejo-Estrada:** Designed the study strategy and objectives, worked on reviewing and writing of the manuscript as well as analysis and interpretation of data.

## Ethics

There are not any ethical issues to declare that could arise after the publication of this manuscript.

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