

# SCREENING AND SELECTION OF WILD STRAINS FOR L-ARABINOSE ISOMERASE PRODUCTION

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**Abstract** - The majority of L-arabinose isomerases have been isolated by recombinant techniques, but this methodology implies a reduced technological application. For this reason, 29 bacterial strains, some of them previously characterized as L-arabinose isomerase producers, were assayed as L-arabinose fermenting strains by employing conveniently designed culture media with 0.5% (w/v) L-arabinose as main carbon source. From all evaluated bacterial strains, *Enterococcus faecium* DBFIQ ID: E36, *Enterococcus faecium* DBFIQ ID: ETW4 and *Pediococcus acidilactici* ATCC ID: 8042 were, in this order, the best L-arabinose fermenting strains. Afterwards, to assay L-arabinose metabolization and L-arabinose isomerase activity, cell-free extract and saline precipitated cell-free extract of the three bacterial cultures were obtained and the production of ketoses was determined by the cysteine carbazole sulfuric acid method. Results showed that the greater the L-arabinose metabolization ability, the higher the enzymatic activity achieved, so *Enterococcus faecium* DBFIQ ID: E36 was selected to continue with production, purification and characterization studies. This work thus describes a simple microbiological method for the selection of L-arabinose fermenting bacteria for the potential production of the enzyme L-arabinose isomerase.

**Keywords:** L-arabinose isomerase; D-galactose; D-tagatose; Cheese whey; Microbiological method.

## INTRODUCTION

The dairy industry is one of the most important and relevant industries in Argentina. According to the 2009 dairy secretary report, our industries have received more than  $1.0 \times 10^{10}$  liters of raw cow milk. Approximately 75% of the total production is destined to the production of solid milk products. Cheese, in all its varieties, is the most important and popular dairy product, with more than 508,000 tons being produced, equivalent to  $5.0 \times 10^9$  liters of raw milk (Indicadores Lácteos 2009). However, industrial cheese elaboration has some economic and downstream difficulties, mainly related to cheese

whey generation. Argentina's cheese whey production in 2009 reached roughly  $4.57 \times 10^9$  liters, of which only 3-5% was used to obtain other derived products, including lactose, whey protein concentrate (WPC), whey protein isolate (WPI), casein, demineralized cheese whey, among others. The rest is converted to powder cheese whey, used both as fertilizer and as animal farm food, or finally discarded. In this way, a real need to take advantage of this unused cheese whey is present, because if discarded, its high oxygen biological demand (DBO: 40,000-50,000 ppm) turns it into a relevant water pollutant. On the other hand, due to its high protein ( $10 \text{ g l}^{-1}$ ) and carbohydrate ( $50 \text{ g l}^{-1}$ ) content, it can be used as a raw material in the food

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industry for producing food additives (Tunick 2008). Although many products are elaborated from cheese whey, none of them substantially increase the economic and technological value of the raw material (namely commodities) and advantage is not taken of a great quantity of cheese whey. Therefore, it is important to develop and apply combined research and development strategies in order to achieve value-added products, given the importance of the Argentinean dairy industry. Besides, it is relevant to highlight that whey protein exploitation has been extensively studied by the scientific community. In this sense, lactose, besides its low water solubility and intolerance to a part of world's population (Schaafsma, 2002; Gänzle *et al.*, 2008; Wilt *et al.*, 2010), is currently under an increased interest of research for achievement of value-added products, because it is only used for obtaining some foods, pharmacological-grade lactose and glucose-galactose syrup (Schaafsma, 2008).

Therefore, lactose conversion is an interesting alternative for obtaining value-added products. In fact, D-tagatose, one of the most valued and promising nutraceuticals, is a ketohexose monosaccharide sweetener, which is an isomer of D-galactose. It occurs naturally in *Sterculia setigera* gum, and is also found in small amounts in several foods such as sterilized and powdered cow milk, hot cocoa, and a variety of cheeses, yogurts, and other dairy products (Levin *et al.*, 1995; Mendoza *et al.*, 2005). D-Tagatose has unique properties as a functional sweetener because its sweetness profile is similar to that of sucrose and possesses no cooling effect or aftertaste. Its bulk value is also similar to that of sucrose, while its humecting properties are close to those of D-sorbitol. D-Tagatose, recognized as a GRAS substance by FAO/WHO since 2001, can be used as a low-calorie sweetener ( $1.5 \text{ kcal g}^{-1}$ ), as an intermediate for synthesis of other optically active compounds, and as an additive in detergent, cosmetic, and pharmaceutical formulations (Levin 2002; Kim 2004; Jørgensen *et al.* 2004).

Actually, D-tagatose is obtained chemically from D-galactose (Beadle *et al.*, 1992; Kim *et al.*, 2003) in an unspecific isomerization procedure that, though cheap, has many disadvantages, including sub-product formation, extremes conditions, sweetness reduction and generation of chemical wastes. Thus, an enzymatic process provides a feasible alternative for this ketohexose production. Besides, although several patented enzymatic processes exist (Ibrahim and Spradlin, 2000; Bertelsen *et al.*, 2001), to date none of them are operational. Therefore, the development of enzymatic technologies employing

the enzyme L-arabinose (D-galactose) isomerase (EC 5.3.1.4) is necessary for constructing a significant and feasible D-tagatose manufacturing process.

In this work, we report a microbiological method for the selection of wild-type L-arabinose isomerase producing strains using both the ability of bacteria to produce acids and evaluation of cell growth with L-arabinose. This will allow the identification of new sources of the enzyme L-arabinose isomerase and the establishment of a simple, reproducible and adequate process for strain selection. Finally, this methodology will permit selection of the best pentose isomerase to be studied and allow preliminary comparisons between different L-arabinose isomerases through evaluation of the enzymatic activity of cell-free extracts.

## MATERIALS AND METHODS

### Bacterial Strains

Table 1 shows the group of 29 bacterial strains used in this study. All of them were species or subspecies previously identified employing the taxonomic keys given by the 2<sup>nd</sup> Edition of Bergey's Manual of Systematic Bacteriology Volume 2 Part B (Brenner *et al.*, 2005) and Volume 3 (De Vos *et al.*, 2009) except for *Enterococcus faecium* DBFIQ ID: E36, which was also PCR-identified by partial sequencing of 16S rDNA. In the case of *E. faecium* DBFIQ ID: E36, vancomycin resistance and  $\beta$ -hemolysis tests were also performed. The collection was chosen based mainly on their previously reported ability to metabolize L-arabinose as the main carbon source, including technologically interesting bacteria that had not yet been characterized and identified as potential L-arabinose isomerase producing strains.

### Bacterial DNA Isolation, PCR Conditions, Primers and Nucleotide Sequence Analysis for *Enterococcus* Strain Identification

*E. faecium* DBFIQ ID: E36 was PCR-identified employing the 16S rRNA methodology. For this purpose, total DNA from *E. faecium* was obtained from a pure 10 mL culture in Elliker Broth (Difco Laboratories, Detroit, MI, USA) grown at 30 °C for 16 hours using the GenElute™ Bacterial Genomic DNA kit (Sigma, St Louis, MO, USA). The amplification reaction of the 1500 bp fragment belonging to 16S rDNA was made by using two universal primers, pA and pH (Edwards *et al.*, 1989), and employing 1  $\mu$ L of total DNA (dilution 1:50) as

template. The PCR reaction mixture included 2.5 U of Taq polymerase (GE Healthcare, Little Chalfont, United Kingdom), 200 nM of dNTPs and 400 nM of each primer (Sigma-Genosys, The Woodlands, TX, USA) in a 50 µL final volume. A negative reaction control without DNA was conducted. Amplification was performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at 94 °C, 36 cycles of 1 min at 94 °C, 2 min at 51 °C and 2 min at 72 °C, and a final step of 7 min at 72 °C. PCR products were visualized on 1.5% agarose gels in TBE buffer, with added GelRed (Biotium, Hayward, CA, USA), and visualized by UV light (Sambrook and Russell 2001). The PCR amplification product was purified using the GenElute™ PCR clean-up kit (Sigma,

St Louis, MO, USA) and the nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen Inc. (Seoul, Korea). Sequence data were assembled and compared using a sequence analysis software package available from the EMBL Spanish node (CNB, CSIC, Spain). The strain-identity was evaluated by nucleotide-nucleotide BLAST sequence alignment and comparison using the NCBI database (<http://www.ncbi.nlm.nih.gov/blast>).

### Culture Preservation

Long-term preservation was done by lyophilization, in order to have safe and stable stock cultures. First, cells were collected from cultures, suspended in 10% skimmed milk and frozen at -20 °C. Then,

**Table 1: Bacterial strains used in this study.**

Strains	Source	Collection reference <sup>a</sup>
<b>Gram (+)</b>		
<i>Alicyclobacillus acidocaldarius</i> subsp. <i>acidocaldarius</i> 4328	Orange juice	CECT
<i>Bifidobacterium longum</i> 15708	Intestine of infant	ATCC
<i>Clostridium acetobutylicum</i> Ca 1	Humus	DBFIQ
<i>Clostridium sporogenes</i> Cs 1	Milk	DBFIQ
<i>Enterococcus faecium</i> E36	Raw cow milk	DBFIQ
<i>Enterococcus faecalis</i> E24	Raw cow milk	DBFIQ
<i>Enterococcus faecalis</i> E25	Raw cow milk	DBFIQ
<i>Enterococcus faecium</i> E23	Raw cow milk	DBFIQ
<i>Enterococcus faecium</i> ETW4	Artisanal ovine cheese	DBFIQ
<i>G. stearothermophilus</i> Gs 1	Double cream	CMFBCB
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ccm 403	Yogurt starter culture	DBFIQ
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> LB92	Yogurt starter culture	DBFIQ
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> 655	Artisanal starter culture	DBFIQ
<i>Lactobacillus helveticus</i> LH 303	Type-parmesan cheese	DBFIQ
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> LP28	Fermented sausage	DBFIQ
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> LP31	Fermented sausage	DBFIQ
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> LP33	Fermented sausage	DBFIQ
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> LP7	Fermented sausage	DBFIQ
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> LP9	Fermented sausage	DBFIQ
<i>Lactococcus lactis</i> subsp. <i>lactis</i> SF 1-1	Fermented milk	DBFIQ
<i>Pediococcus acidilactici</i> 8042	Silage	ATCC
<i>Pediococcus pentosaceus</i> 790	Fermented meat product	DBFIQ
<i>Streptococcus thermophilus</i> CH 3-4	Yogurt starter culture	DBFIQ
<b>Gram (-)</b>		
<i>Enterobacter aerogenes</i> EA 1	Contaminated water	ITA
<i>Enterobacter</i> sp. E7	Humus	ITA
<i>Escherichia coli</i> Ec 1	Contaminated water	DBFIQ
<i>Escherichia coli</i> Ec 8	Contaminated water	DBFIQ
<i>Escherichia coli</i> Ec 4	Spoiled potato	ITA
<i>Escherichia coli</i> Ec 1	Human faeces	CMFBCB

<sup>a</sup> ATCC: American Type Culture Collection (Manassas, VA, USA); CECT: Colección Española de Cultivos Tipos (Valencia, Spain); DBFIQ: Cátedras de Microbiología y Biotecnología, Departamento de Ingeniería en Alimentos, Facultad de Ingeniería Química (FIQ), Universidad Nacional del Litoral (UNL), Argentina collection (DBFIQ collection); ITA: Instituto de Tecnología de Alimentos (FIQ-UNL, Santa Fe, Argentina); CMFBCB: Cátedra de Microbiología General, Facultad de Bioquímica y Ciencias Biológicas, UNL (Santa Fe, Argentina).

cultures were placed in the stand and allowed to freeze at  $-30\text{ }^{\circ}\text{C}$ . After that, vacuum was applied at an inner pressure of 0.026 mbar for 12 hours, keeping the stand temperature at  $-30\text{ }^{\circ}\text{C}$ . Next, a ramp temperature from  $-30\text{ }^{\circ}\text{C}$  to  $25\text{ }^{\circ}\text{C}$  in 12 hours was applied in order to eliminate strongly-attached moisture. Finally, tubes were heat-sealed under vacuum and stored as reference cultures or until use. Besides, all bacterial strains were frozen at  $-20\text{ }^{\circ}\text{C}$  and  $-80\text{ }^{\circ}\text{C}$  in: MRS Broth (Biokar Diagnostic, Beauvais, France) for lactic acid bacteria except for enterococci, M17 Broth for *Enterococcus*, Trypticase Soy Broth for *Geobacillus stearothermophilus*, Nutrient Broth for Gram (-) bacteria (all from Difco Laboratories, Detroit, MI, USA) and *Bacillus acidocaldarius* medium (BAM; Darland and Brock 1971) for *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius*. Moreover, *Clostridium acetobutylicum* was preserved as spores at  $-20\text{ }^{\circ}\text{C}$  and  $-80\text{ }^{\circ}\text{C}$  in Thioglycollate Broth with Resazurin (Biokar Diagnostic, Beauvais, France). *Clostridium sporogenes* was also conserved as spores in UHT milk and *B. longum* was preserved in MRSC broth [MRS Broth (Biokar) supplemented with 0.1% (v/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA)] in an anaerobic jar (Oxoid, Cambridge, UK) under a 80% (v/v)  $\text{N}_2$ , 10% (v/v)  $\text{CO}_2$  and 10% (v/v)  $\text{H}_2$  atmosphere employing an anaerobic cultivation system (AnaeroPack-Anaero, MGC, Japan). All preservation media were supplemented with 20% (v/v) glycerol (Heckly 1978).

### Culture Medium Design and Production

In order to evaluate conveniently L-arabinose consumption, culture media were designed by modification of preexisting ones or created for this specific purpose. Therefore, for lactic acid bacteria, MRS medium without meat extract, peptone and D-glucose and with the addition of 0.002% (w/v) Chlorophenol Red (Fluka, pH range: 4.8-6.7) was prepared. For *B. longum*, the same modified medium that was used for lactic acid bacteria with extra addition of 0.1% (w/v) cysteine was employed (Kimmel *et al.* 1998). On the other hand, a medium for *Enterobacteriaceae*, identified as MPE, with the following composition ( $\text{g L}^{-1}$ ): yeast extract, 2.5, and peptone, 5.0, was created. For *Bacillus* species, a medium described in Volume Three of Bergey's Manual of Systematic Bacteriology (De Vos *et al.*, 2009) was used, whereas for clostridia, modified reinforced clostridial medium (MRC medium) without starch, meat extract and D-glucose was employed. Lastly, BAM medium without D-glucose was used for *A. acidocaldarius* subsp. *acidocaldarius*. All

basic culture media were sterilized at 1 atm for 15 minutes and L-arabinose, sterilized by filtration using  $0.22\text{ }\mu\text{m}$  pore diameter membranes (Sartorius), was added separately to all media at a 0.2% (w/v) final concentration.

### Fermentation Assay

From stock cultures, two successive culturing procedures were made in order to achieve exponential phase growth cultures. Solid-phase assay was realized by spreading each bacterium into its corresponding agar-added ( $20\text{ g L}^{-1}$ ) culture medium. Liquid-phase trials were done by transferring 0.1 mL of the previous culture to 5 ml of each designed medium (Zamudio and Zavaleta 2003). Cultures were incubated at  $37\text{ }^{\circ}\text{C}$  for 24-48 hours (lactic acid bacteria),  $37\text{ }^{\circ}\text{C}$  for 24 hours (*Enterobacteriaceae*),  $45\text{ }^{\circ}\text{C}$  during 72 hours (*A. acidocaldarius* subsp. *acidocaldarius*) and  $55\text{ }^{\circ}\text{C}$  during 4-6 days (*G. stearothermophilus*). Clostridial species and *B. longum* were grown anaerobically in a modified atmosphere at  $37\text{ }^{\circ}\text{C}$  during 24-48 hours employing an anaerobic jar (Oxoid). Lactic acid bacteria were grown in culture media with a pH indicator in their composition, so that acid production from L-arabinose metabolism could be detected by the pH indicator color change. The principal criterion related to lactic acid bacteria was thus an indicator color change; otherwise the assay was considered negative. Finally, considering Gram (-) and the rest of the Gram (+) bacterial strains, only a cell growth equal or higher than  $1 \times 10^9\text{ CFU mL}^{-1}$  in the corresponding media was utilized as a positive selection criterion.

For each experiment, control medium, which consisted of base medium without added L-arabinose was inoculated with the corresponding bacterium in order to check for unspecific cell growth or color shift.

### Cell-Free Extract Production

Bacteria that showed the best ability to metabolize L-arabinose were selected for cell-free extract production. Working cultures were prepared by adding 0.2 mL of frozen stock culture to 5 mL of MRS Broth (Biokar) and incubating for 24 hours at  $37\text{ }^{\circ}\text{C}$ . The second culture was achieved by transferring the initial culture to 200 mL of modified MRS broth [same composition except for 0.1% (w/v) D-glucose and 0.5% (w/v) L-arabinose] and incubating them at  $37\text{ }^{\circ}\text{C}$  for 24 hours. The final culture was done by adding 180 mL of the last propagation culture in the late exponential growth phase to 6000 ml of modified MRS broth and incubated at  $37\text{ }^{\circ}\text{C}$  for 24

hours (Zhang *et al.* 2007). Cells obtained were harvested by centrifugation at 5000 rpm for 15 minutes at 4 °C, resuspended in 500 mL of 50 mM phosphate buffer, pH 7.0 (activity buffer), and treated with 1 mg mL<sup>-1</sup> lysozyme for 3 hours at 37 °C. Then, the cell suspension was disrupted by pulsed sonication (20 W, pulse on, 4 s; pulse off, 3 s) for 40 min at 4 °C and, afterwards, remaining cell debris was removed by centrifugation at 20,000×g for 30 min at 4 °C. Then, cell-free extract was sterilized by filtration using 0.22 µm pore diameter membranes (Sartorius) and stored at -20 °C. Subsequently, the cell-free extract obtained was aliquoted in two halves where one was stored at -20 °C and the other was saline-fractionated by adding solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 80% saturation, followed by 24 hours of incubation at 4 °C with periodic stirring. Finally, this fraction was centrifuged at 12,000×g during 30 minutes at 4 °C and the pellet was redissolved in 100 mL of activity buffer.

In a subsequent study, in order to recover more enzyme activity, a protein precipitation assay using the same conditions described above but employing different ammonium sulfate concentrations (40, 60, 80, 85, 90 and 100% saturation) was performed.

### Enzyme Assay and Protein Quantification

L-Arabinose isomerase activity was calculated by measurement of the amount of generated L-ribulose or D-tagatose. The reaction mixture contained 1mM MnCl<sub>2</sub>, 250 mM L-arabinose or D-galactose (Sigma-Aldrich; St. Louis, MO, USA), 0.1 mg of properly diluted enzyme preparation and 50 mM pH 7.0 phosphate buffer to bring the final volume to 1 mL. The assay was done by first incubating the mixture without the enzyme at the test temperature; the enzyme was then added and incubated at 50 °C for 1 hour. Subsequently, the enzymatic reaction was stopped by cooling the samples on ice. The generated L-ribulose or D-tagatose was determined by the cysteine carbazole sulfuric acid method (Dische and Borenfreund, 1951). Standard calibration curves with L-ribulose or D-tagatose (Sigma-Aldrich, St. Louis, MO, USA) were elaborated in order to quantify the ketoses produced. One unit of L-arabinose isomerase activity was defined as the amount of enzyme catalyzing the formation of 1 µmol keto-sugar per minute under above specified conditions. Protein concentration was assessed by the Bradford method employing Bovine Serum Albumin (Sigma-Aldrich; St. Louis, MO, USA) as a standard protein (Bradford, 1976).

## RESULTS

### Strain Identification and PCR Identification of the Selected *Enterococcus* Strain

PCR identification was performed in order to confirm the genotypic characteristics of *Enterococcus faecium* DBFIQ ID: E36. This strain was previously identified as *Enterococcus* sp., so full identification was performed by combining phenotypic and genotypic tests, given the importance of this strain for our study. Partial sequencing of the 16S rDNA gene revealed more than 99% homology with the strain *E. faecium* according to Genbank access number Y 18294. All results from the phenotypic tests performed match up with *E. faecium* taxonomic keys given in volume 3 of Bergey's Manual. In particular, *E. faecium* DBFIQ ID: E36 strain turned out to be both non-β-hemolytical and sensitive to the vancomycin resistance test.

All other strains were previously identified employing the taxonomic keys provided in volumes 2 and 3 of the 2<sup>nd</sup> edition of Bergey's Manual of Systematic Bacteriology and they are not shown in this work.

### Fermentation Assay

Table 2 shows L-arabinose fermentation results obtained after growing each bacterial strain in their corresponding differential culture medium. *L. plantarum* species behaved disparately, while DBFIQ ID: LP9 and DBFIQ ID: LP28 did not produce a color shift and DBFIQ ID: LP7 generated an interesting shift change. *L. delbrueckii* subsp. *bulgaricus* 92, *L. helveticus* 303 and *B. longum* ATCC ID: 15708 barely showed the ability to ferment L-arabinose, but the results were considered as positive [Fig. 1 (b)]. *Enterococcus* strains presented several disparities between each other. *E. faecalis* DBFIQ ID: E25 and *E. faecium* DBFIQ ID: E23 were negative in the microbiological test while *E. faecalis* DBFIQ ID: ETW4 gave a very positive test result, as can be seen in Figure 1 (a). In between, *E. faecalis* DBFIQ ID: E24 only covered from 25 to 50% of the total area of the Petri dish, but *E. faecium* DBFIQ ID: E36 proved to be the best L-arabinose fermenting strain, covering almost 100% of the total area, as can be seen in Fig. 1 (c).

*L. lactis* subsp. *lactis* SF DBFIQ ID: 1-1 was negative in the test because it did not experience a color change, even though it grew faintly in the corresponding liquid culture. *S. thermophilus* DBFIQ

ID: CH 3-4 and *L. delbrueckii* subsp. *bulgaricus* DBFIQ ID: ccm 403 also were negative in the fermentation test, although they experienced a slight cell growth. *P. pentosaceus* DBFIQ ID: 790 and *L. delbrueckii* subsp. *lactis* DBFIQ ID: 655 grew in both media, but no color change was evidenced, so the results were considered negative. *P. acidilactici* ATCC ID: 8042 behaved as one of the best L-arabinose metabolizing strains by color shifting up to 75% of the Petri dish area.

**Table 2: Results of L-arabinose bacterial fermentation assays obtained after growing each bacterial strain in their corresponding differential culture medium.**

Strains	Percentage area shifted/Cell Growth <sup>a</sup>
<b>Gram (+)</b>	
<i>A. acidocaldarius</i> subsp. <i>acidocaldarius</i> 4328	-
<i>B. longum</i> 15708	+
<i>C. acetobutylicum</i> Ca 1	+
<i>C. sporogenes</i> Cs 1	-
<i>E. faecium</i> E36	+++
<i>E. faecalis</i> E24	+
<i>E. faecalis</i> E25	-
<i>E. faecium</i> E23	-
<i>E. faecium</i> ETW4	++
<i>G. stearothermophilus</i> Gs 1	-
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ccm 403	-
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> 92	+
<i>L. delbrueckii</i> subsp. <i>lactis</i> 655	-
<i>L. helveticus</i> LH 303	+
<i>L. plantarum</i> LP28	-
<i>L. plantarum</i> LP31	+
<i>L. plantarum</i> LP33	+
<i>L. plantarum</i> LP7	++
<i>L. plantarum</i> LP9	-
<i>L. lactis</i> subsp. <i>lactis</i> SF 1-1	-
<i>P. acidilactici</i> 8042	++
<i>P. pentosaceus</i> 790	-
<i>S. thermophilus</i> CH 3-4	-
<b>Gram (-)</b>	
<i>E. aerogenes</i> EA 1	+
<i>Enterobacter</i> sp. E7	+
<i>E. coli</i> Ec 1	+
<i>E. coli</i> Ec 8	+
<i>E. coli</i> Ec 4	+
<i>E. coli</i> Ec 1	+

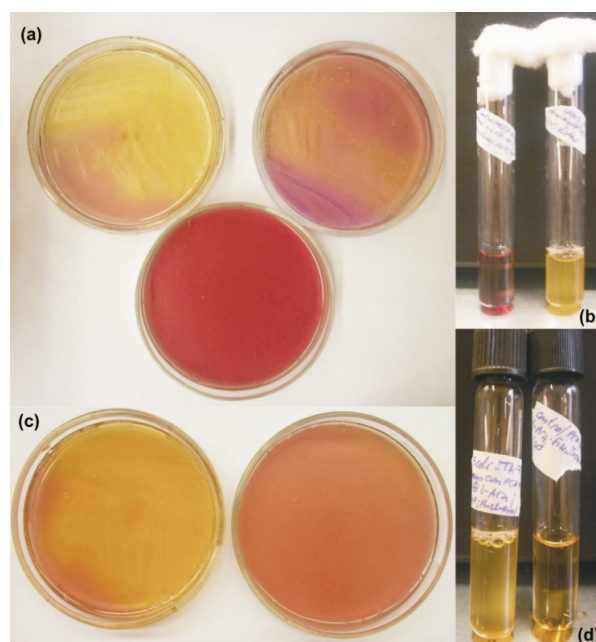
<sup>a</sup> Positivity of assay was evaluated by measuring the percentage area that shifted the indicator color relative to the total area of the Petri dish [for Gram (+) bacteria except for clostridia] or by testing cell growth [for Gram (-) bacteria and clostridia]. Color changed area (in quartiles): (-), 0-25%; (+), 25-50%; (++), 50-75%; (+++), 75-100%.

*A. acidocaldarius* CECT ID: 4328 did not grow in *Bacillus* medium because the pH was not acidic enough, which is essential for an adequate strain growth. However, employing BAM medium, it was

possible to achieve a faint and slow cell growth in liquid medium, although not in a solid culture, because acid pH caused agar hydrolysis, which made it impossible to see growth results. For these reasons, results were ascribed as negative. Another *Bacillus* species, *G. stearothermophilus* DBFIQ ID: Cs 1, was assayed. This strain, in relation to *A. acidocaldarius*, showed a vague color change due to pH in solid medium and turbidity in liquid medium; nevertheless, the results were considered negative.

Finally, *C. sporogenes* was negative in the fermentation test, which coincides with volume 3 of Bergey's Manual, whereas for *C. acetobutylicum* L-arabinose turned out to be a good substrate for fermentation (from 25 to 50% of the total Petri dish area covered) and the results were taken as positive.

Gram (-) bacteria were assessed according to their growth ability in the corresponding culture media. Due to the slow acid production rate, pH variation from sugar fermentation was not be evaluated and only cell growth was assessed. As shown in Table 2, all enterobacterial strains developed in solid and liquid culture media with added L-arabinose. Fig. 1 (d) shows *E. coli* ITA ID: Ec 4 cell growth in the above specified L-arabinose modified medium.



**Figure 1:** Solid and liquid culture fermentation assay results. (a) Upper Left: *E. faecium* DBFIQ ID: ETW4, Upper Right: *E. faecalis* DBFIQ ID: E24, Centre: negative control; (b) Left: negative control, Right: *L. helveticus* DBFIQ ID: LH 303 (c) Right: *E. faecium* DBFIQ ID: E36, Left: negative control (d) Left: *E. coli* ITA ID: Ec 4; Right: negative control.

Each result showed in Table 2 is a combination of three independent tests. In all tests, the controls turned out to be negative.

### Cell-Free Extract Enzyme Assay

The best four L-arabinose fermenting strains, *E. faecium* DBFIQ ID: E36, *E. faecium* DBFIQ ID: ETW4, *P. acidilactici* ATCC ID: 8042 and *L. plantarum* DBFIQ ID: LP7 were selected for cell-free extract production. Table 3 shows the enzymatic activity results after assaying the isomerization ability of each cell-free extract employing L-arabinose and D-galactose as substrates. It can be seen that the cell-free extract from both *Enterococcus* strains produced L-ribulose as the ketoheptose product from L-arabinose, but D-tagatose production could not be detected in the three cell-free extracts assayed. However, saline precipitated cell-free extract produced D-tagatose from D-galactose after enzymatic assay of the three strains. In addition, the selected *L. plantarum* strain did not produce D-tagatose from D-galactose, but showed a reasonable production of L-ribulose from L-arabinose, indicating that the enzyme produced by this bacterium would not be suitable for D-galactose exploitation. These results demonstrate that *E. faecium* DBFIQ ID: E36 is one of the most active strains in L-arabinose fermentation and L-arabinose isomerase production, showing proportionality between the fermentation assay and enzymatic activity results. Therefore, *E. faecium* DBFIQ ID: E36 was selected as the first producer strain, followed by *E. faecium* DBFIQ ID: ETW4 and *P. acidilactici* ATCC ID: 8042, respectively, in concordance with the two experiments. The other strain was discarded for this study, but could be interesting for its potential use as L-ribulose producer, a precursor for the synthesis of antiviral drugs (Cho *et al.*, 2005).

### DISCUSSION

Over the last ten years, many authors have described the biological production of D-tagatose from D-galactose employing the enzyme L-arabinose isomerase isolated from several bacterial sources. Different technological criteria concerning a higher isomerization rate and conversion yield and decreased viscosity of the substrate in the product stream, have highlighted enzymes with increased thermal stability and optimum temperature (Prabhu *et al.* 2008; Lee *et al.* 2005). However, this does not benefit other important technological aspects related to the process such as the low-growth kinetics of the isolated thermophilic or hyperthermophilic organisms (such as *A. acidocaldarius* CECT ID: 4328 and *G. stearothermophilus* CMFBCB ID: Gs1), the unsafe and harmful environments where they were obtained (Lee *et al.* 2004; Kim *et al.* 2002) and the already mentioned undesirable effects, such as browning and unwanted by-product formation (Kim, 2004; Liu *et al.*, 1996). The absence of these characteristics makes it very difficult to obtain, in a non-recombinant way, enough quantity of the target enzyme. This compels, as described by many authors, the use of molecular biology techniques for recombinant enzyme expression, purification and characterization, such as gene extraction, fitting-out, cloning and protein expression in heterologous vectors. This means that all hygienic and sanitary consequences of manipulation of DNA sequences and utilization of non-GRAS host microorganisms such as *E. coli* must be considered, so a direct technological applicability remains uncertain when a food product for human use is to be designed (Burdock and Carabin, 2004). These limitations can be avoided by producing L-arabinose isomerase in a non-recombinant mode using generally regarded as safe (GRAS) wild-type organisms. For this aim, we

**Table 3: Enzymatic activity results after assaying the isomerization ability of each cell-free extract employing L-arabinose and D-galactose as substrates. Specific enzymatic activity expressed as  $\mu\text{mol}$  of D-tagatose produced  $\text{min}^{-1}\mu\text{g protein}^{-1}$ .**

Strains	<i>E. faecium</i> DBFIQ ID: E36	<i>E. faecium</i> DBFIQ ID: ETW4	<i>P. acidilactici</i> ATCC ID: 8042	<i>L. plantarum</i> DBFIQ ID: LP7
<b>Cell-free extract</b>				
L-ribulose	51.8	15.2	0.0	8.9
D-tagatose	0.0	0.0	0.0	0.0
<b>80% saline precipitated cell-free extract</b>				
L-ribulose	131.6	97.7	10.0	65.2
D-tagatose	7.2	6.9	4.9	0.0

Substrates employed: L-arabinose and D-galactose

have focused on lactic acid bacteria because they are GRAS bacteria, possess a high cell growth rate, are employed in numerous food products and are mesophile organisms, so culture conditions are cheaper, more reproducible and feasible to be carried out in food industries. In this sense, our L-arabinose isomerase does not possess the highest conversion yield but, as indicated by Hugenholtz and Smid (2002) and Hugenholtz *et al.* (2002), may permit a more viable technological process for obtaining a human food product or additive.

This is the first scientific work that describes a simple, fast and reliable methodology to identify fast-growing L-arabinose fermenting bacteria. This assay was designed by assuming the following considerations: a) that the expression of the enzyme L-arabinose isomerase and the entire L-arabinose metabolic pathway is induced by the addition to cultures of the pentose L-arabinose (Dobrogosz and DeMoss, 1963); b) that bacteria which can metabolize L-arabinose, isomerize it to L-ribulose because they possess the enzyme L-arabinose isomerase as the first enzyme in the L-arabinose metabolic pathway (Heath *et al.* 1958). This methodology probably has an important disadvantage of low sensibility because a negative result does not mean the absence of L-arabinose fermenting activity. However, with the development of the assay, only bacteria with a good ability of cell-growing and L-arabinose metabolization stand out. Besides, it could be used as an initial step for L-arabinose fermenting strain selection before applying recombinant DNA techniques.

Non-lactic acid bacteria were employed in order to study a wide spectrum of bacteria, analyze the behavior of the corresponding methodology in the presence of previously characterized L-arabinose isomerase producing bacteria and compare them with lactic acid bacteria. In this sense, all Gram (-) bacteria grew in their corresponding modified L-arabinose fermenting medium, which matches up with the information given in volume 2 of Bergey's Manual of Systematic Bacteriology and with scientific publications (Yamanaka and Wood, 1966; Patrick and Lee, 1975; Yoon *et al.*, 2003). However, growth in modified medium was not as good as lactic acid bacteria performance and, furthermore, all the desired requirements were not fulfilled, so they were not considered as potential L-arabinose isomerase producers. Finally, *A. acidocaldarius* CECT ID: 4328 and *G. stearothermophilus* CMFBCB ID: Gs1 also did not show the preferred technological characteristics and they were not selected as potential producers of the mentioned enzyme.

However, studies were focused on lactic acid bacterial strains, considering both the technological characteristics above and results obtained with the acid-carbohydrate fermentation technique. From the results, it can be said that the L-arabinose fermentation ability is strain-dependent, as shown by the results obtained with several *L. plantarum* strains tested, which must be understood as a variable phenotypic characteristic of each strain, depending primarily on its isolation source. Between different enterococci, we have also evidenced the same behavior as lactobacilli. In fact, *E. faecium* DBFIQ ID: E23 and DBFIQ ID: E24 strains did not show a considerable L-arabinose fermenting ability, while DBFIQ ID: ETW4 and DBFIQ ID: E36 strains were evaluated and selected as two of the best L-arabinose fermenting bacteria in this assay.

From the results, it can be affirmed that there exists a correlation between the microbiological and the enzymatic activity assays. In that way, *E. faecium* DBFIQ ID: E36, *E. faecium* DBFIQ ID: ETW4 and *P. acidilactici* ATCC ID: 8042 were, in this order, the best L-arabinose fermenting strains, a connection that was still maintained in the cell-free extract and redissolved saline precipitated enzymatic assays. Furthermore, this means that L-arabinose fermenting ability is directly related to L-arabinose isomerase produced by each bacterium, so the greater the L-arabinose fermenting ability, the more L-arabinose isomerase produced.

After analyzing Table 3, cell-free extract concentration by saline precipitation was necessary in order to detect D-tagatose production. Although D-tagatose biosynthesis remained low, it was always above the colorimetric assay detection limit. Besides, even if D-tagatose production was higher in *E. faecium* DBFIQ ID: E36 than in *E. faecium* DBFIQ ID: ETW4, both quantities remained close to each other, so it was not possible to categorically select only one strain according to this criterion. D-Tagatose production could be increased by optimization of cell-free extract production and enzymatic assay.

Therefore, aside from the criterion mentioned above, *E. faecium* DBFIQ ID: E36 was selected as the L-arabinose isomerase producing bacteria because it presented the best L-arabinose fermenting ability, was the fastest growing bacterium in modified MRS medium containing L-arabinose, and showed the highest detected L-ribulose output after enzymatic assay in either crude or saline-precipitated cell-free extracts. Furthermore, it is a bacterium strain that is sensitive to vancomycin and it is not  $\beta$ -hemolytical, which increase even more its techno-



logical potential if used in fermented foods or as an additive.

Thus, from this study, *E. faecium* DBFIQ ID: E36 strain was selected to produce the enzyme L-arabinose isomerase, which had not yet been studied in this genus. This strain belongs to the lactic acid bacteria which are considered by FAO and WHO as GRAS organisms. Consequently, it can be directly employed in industrial bioprocesses for production of value-added products from cheese whey or other industrial by-products (Rhim *et al.*, 2007). Finally, current studies are centered on non-recombinant L-arabinose isomerase production, purification and characterization from chosen wild-type strains.

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