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NOTE

Zymographic assay to differentiate *Lactobacillus reuteri* among other *Lactobacillus*

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SUMMARY

A simple method is reported that allows the phenotypic differentiation of *Lactobacillus reuteri* strains among other *Lactobacillus*. The detection of the enzyme 1,3-propanediol:NAD oxidoreductase in non-denaturing PAGE, a constitutive enzyme of *Lb. reuteri* involved in the production of 1,3-propanediol, is used. Other member of the genus were reported as 1,3 propanediol producers (*Lactobacillus brevis and Lactobacillus buchneri*), both of their enzymes showed differential properties to that of *Lb. reuteri*. Under the conditions used in the study, the strain *Lb. reuteri* CRL1100 was found to produce 15 % more 1,3-PDL (11.5 ± 0.41 gl^{-1}) in comparison with the reference strain *Lb. reuteri* DSM 20016 (10.0 ± 0.37 gl^{-1}).

Keywords

Lactobacillus reuteri, 1,3-propanediol:NAD oxidoreductase, zymogram, 1,3-propanediol.

1 – INTRODUCTION

Besides ethanol, glycerol is the main product of yeast fermentation (LAFON-LAFOURCADE, 1983). In spoiled wines or ciders glycerol is usually degraded causing bitter components like acrolein; generated from a chemical equilibrium with 3-hydroxypropionaldehyde, the intermediary component in the production of 1,3-propanediol (1,3-PDL) (CLAISSE and LONVAUD-FUNEL, 2000). Several Lactic acid bacteria (LAB) use glycerol as an electron acceptor in an anaerobic cofermentation with a sugar, but only few of them produce 1,3-propanediol (1,3-PDL) in such conditions (SALMINEN & VON WRIGHT, 1993).

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The metabolic pathway for production of 1,3-PDL, involves two main enzymatic steps, the reductive conversion of glycerol to 3-hydroxypropionaldehyde by the enzyme glycerol dehydratase and the reduction of the aldehyde to 1,3-PDL by 1,3-propanediol:NAD oxidoreductase (EC 1.1.1.202) (TALARICO & DOBROGOSZ, 1990; TALARICO *et al.*, 1990). The three reported *Lactobacillus* producers of 1,3-PDL (*Lb. reuteri, Lb. brevis and Lb. buchneri*) showed the same metabolic pathway. Although the characteristics of the enzyme 1,3-propanediol:NAD oxidoreductase from *Lb. reuteri* are completely different from that of the other two LAB (TALARICO *et al.* 1990; VEIGA DA CUNHA and FOSTER, 1992).

Electrophoretic patterns of SDS-PAGE provided a useful tool for characterization of LAB (DE ANGELIS *et al.*, 2001); this work presents a simple phenotypic differentiation of *Lb. reuteri* among other *Lactobacillus* by a differential pattern of a zymographic technique.

2 – MATERIALS AND METHODS

The strains *Lb. brevis*, *Lb. buchneri*, *Lactobacillus plantarum* and *Lactobacillus.* spp. were obtained from PROIMI culture collection. *Lb. reuteri* strains CRL 1097, CRL1098, CRL1099 and CRL1100 from CERELA (Tucumán, Argentina). *Lb. reuteri* DSM 20016 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (Braunschweig, Germany). Glycerol dehydrogenase (E.C. 1.1.1.6) from *Bacillus megaterium* was purchased from Sigma Chemical Co. All chemicals used were of analytical grade obtained from standard sources.

2.1 Culture conditions

The strains were cultured in 125 ml flasks (hermetically closed) in a gyratory shaker (30 Hz, 37°C) containing 50 ml of culture medium g l^{-1}: glucose 10; glycerol 13; yeast extract 10; $(NH_4)_2PO_4$ 2.5; $MgSO_4.7H_2O$ 0.1 and $MnSO_4.H_2O$ 0.005. Pure N_2 was gassed across the culture medium before inoculation in order to create anaerobic conditions.

2.2 Enzyme extracts preparation

Overnight broth cultures were centrifuged at 4°C for 10 min at 1500 g, washed twice with 0.2 M potassium phosphate solution pH 9.0 (100 ml), and suspended in 5 ml of the same solution. The cell suspensions were disrupted by passing twice through a French press at 20000 psi, and centrifuging at 6200 g for 10 min, the intracellular protein extracts were stored at – 20 °C until processing.

2.3 Non-denaturing PAGE

Samples underwent electrophoresis in parallel on vertical slab gels using a MiniProteanII electrophoresis apparatus (Biorad, Laboratories). The solution 290:10 g/l acrylamide:bisacrylamide w/w, was used at final concentration of 75 g/l for the resolving gel and 45 g/l for the staking gel, containing 0.37 M Tris-HCl (pH 8.8) and 0.12 M Tris-HCl (pH 6.8) respectively. The electrode buffer was 25 mM Tris and 192 mM Glycine (pH 8.3). Samples containing 15-25 µg of protein were loaded on the gel. The electrophoresis was performed at constant 150 volts at room temperature.

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2.4 *Zymographic staining*

The gels, with the samples run in parallel, were washed twice with 0.2M potassium phosphate solution (pH 9.0) during 5 min in a rocking table at room temperature. Then were separately stained by simultaneous immersion in two different reaction conditions: one containing (g l^{-1}): 0.36 Nitro Blue Tetrazolium (NBT), 0.04 phenazine methosulfate, 0.12 NAD^+, 3.6 $(NH_4)_2SO_4$ and 2.5 glycerol in 0.2M potassium phosphate solution (pH 9.0), and the other gel in a reaction mixture containing the same reagents as the first but without glycerol. The gels were incubated at 37°C until the bands of activity were visualized. The reaction was stopped by washing and immersing the gels in 0.2M potassium phosphate solution (pH 9.0). Reproducibility of the profiles obtained on non-denaturing PAGE was estimated by comparing duplicated extracts and performing duplicate runs of a single extract in the same gel as well as on separated gels.

2.5 Analytical assays

Protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard. The concentration of 1,3-PDL was determined by HPLC using a Rezex Organic Acid column (300 x 7.8 mm) fitted with a precolumn and a Gilson R32 refractive index detector (Gilson, Villers le Bel, France), 0.025 mol l^{-1} H_2SO_4 at 55°C was used as the mobile phase.

3 - RESULTS AND DISCUSSION

Intracellular protein extracts of nine strains of *Lactobacillus* developed by the zymographic analysis with and without glycerol were compared. The assay performed without glycerol showed bands of activity corresponding to the strains *Lb. brevis*, *Lb. buchneri* and *Lb. plantarum* (figure 1A). The same samples stained with glycerol showed a different pattern (figure 1B). The bands of activity appearing in presence of glycerol, all corresponded to the strains of *Lb. reuteri.* The extract from *Lactobacillus spp.,* did not show bands of activity in any of the two staining procedures.

The method is based in the differential pattern of the zymographic staining with and without glycerol, although, the origin of the bands of activity in absence of glycerol in figure 1A remains unknown. A zymographic analysis of the commercially available glycerol dehydrogenase from *Bacillus megaterium* showed a clear band of activity in presence of glycerol and no bands in its absence (Data not shown), indicating that the glycerol used in the electrophoresis sample buffer did not interfere in the results of this method.

Lb.reuteri, *Lb. brevis* and *Lb. buchneri* are the *Lactobacillus* species reported as producers of 1,3-PDL. Although the enzyme 1,3-PDL NAD: oxidoreductase of these strains showed different catalytic properties (TALARICO *et al.* 1990; VEIGA DA CUNHA and FOSTER, 1992). The enzymes from *Lb. brevis* and *Lb. buchneri* were similar to that of *Klebsiella pneumoniae* including low activity (<10%) displayed with the substrate glycerol. They also need a previous treatment with EDTA and Mn^{2+} salts before running non-denaturing-PAGE in order to detect them in a zymographic assay (Veiga Da Cunha and Foster, 1992). The enzyme of *Lb. reuteri*, however, has wide substrate specificity, displaying the highest activity "in vitro" with glycerol, showing similar properties to the glycerol dehydrogenase of *K. pneumoniae* (VEIGA DA CUNHA and FOSTER, 1992).

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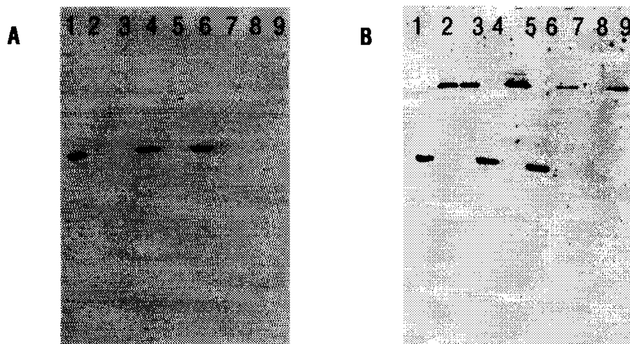

Figure 1

Zymographic patterns of intracellular extract of *Lactobacillus* run in parallel non-denaturing-PAGE: **A**. Stained without glycerol. **B.** Stained with glycerol (See details in materials and methods). Lanes: 1, *Lb. brevis*; 2, *Lb. reuteri* DSM 20016; 3, *Lb. reuteri* CRL 1100; 4, *Lb. buchneri*; 5, *Lb. reuteri* CRl 1099; 6, *Lb. plantarum*; 7, *Lb. reuteri* CRL 1098; 8, *Lactobacillus spp.*; 9, *Lb. reuteri* CRL 1097.

In order to establish a correlation between the zymographic assay and the 1,3 PDL produced during growth, this was quantified in the supernatant by HPLC. The five strains of *Lb. reuteri* produced 1,3 PDL ranging between 8 - 11.9 g l^{-1}; while *Lb. brevis* and *Lb. buchneri* produced lower amounts of 1,3 PDL (1 - 2.5 g l^{-1}) and it was not detected in supernatants of *Lactobacillus spp.* and *Lb. plantarum,* which is not reported in the literature as producer of 1,3 PDL, but it has a sequence of the enzyme 1,3-PDL NAD: oxidoreductase inferred from electronic annotation (Expasy, accession number: Q88TC1).

The strains of *Lb. reuteri* used were isolated from different sources in Argentina. Even though the production range of 1,3-PDL among them was narrow, the *Lb. reuteri* CRL 1100 (11.5 ± 0.41 gl^{-1}) produced 15% more than the reference strain *Lb. reuteri* DSM 20016 (10.0 ± 0.37 gl^{-1}), and it was selected for further studies of 1,3-PDL production in bench scale (Ledesma *et al.*, 2001)

A recently reported method proposed denaturing SDS-PAGE whole cell protein fingerprinting for differentiation of LAB (Sánchez *et al.*, 2003). Previous studies showed difficulties for phenotypic differentiation of *Lb. reuteri* (Hayford *et al.,* 1999). The present work developed a simple and reliable zymographic method for phenotypic differentiation of *Lb. reuteri* among other *Lactobacillus*, taking advantage of its "in vitro" glycerol dehydrogenase activity. It is quick and simple and it can be used as support technique to other molecular or biochemical methods. An important advantage is that it avoids the need of special culture conditions or media, due to the constitutive character of the enzyme 1,3-PDL NAD: oxidoreductase (Talarico & Dobrogosz, 1990).

4 – ACKNOWLEDGMENTS

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