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Cheese whey: an alternative growth and protective medium for *Rhizobium loti* cells

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Abstract Cheese whey (CW)-based growth medium efficiently protects *Rhizobium loti* cells during freezing and desiccation and can maintain their growth in a manner similar to that of traditional mannitol-based medium (YEM). The cheese-whey-based medium (CW) improved viability when used to re-suspend cell pellets kept at −20 °C and −80 °C and resulted in the survival of over 90% of the cells. Moreover, bacterial pellets obtained from cells grown in CW withstand desiccation better than cells grown in YEM. Survival was over 60% after 30 days at 4 °C. No differences were observed in nodulation efficiency between YEM-grown and CW-grown cells. Fast protein liquid chromatography (FPLC) protocols are presented for total protein profile analyses of sweet and acid cheese whey.

Keywords Cheese whey · *Rhizobia* · *Lotus glaber*

In memoriam of Sylvio Cortina Vicepresident of Fundación CO-REPRO

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Introduction

Legumes are able to establish a symbiotic relationship with nitrogen-fixing bacteria present in the soil, and the potential benefit of inoculation has been known for years. All of these bacteria belong to the *Rhizobiaceae* family, which comprises three genera: *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. Bacteria from any of these genera are usually known as *Rhizobia* and their interaction with legumes induces development of specialized structures called nodules. *Rhizobia* infect and colonize nodules thereby establishing a metabolic cooperation between the two symbionts: bacteria reduce atmospheric nitrogen to ammonia and the plant provides the bacteria with sugars synthesized by carbon dioxide reduction during photosynthesis [15]. As a consequence, legume cultivation is a natural method of improving the nitrogen content of the soil, thus avoiding the use of chemical fertilizers, which are expensive and contribute to environmental pollution.

Lotus glaber (ex- *L. tenuis*) is the most important legume in the saline-alkaline lowlands of the Salado River basin. This region (approximately 9,000,000 ha), located in Buenos Aires Province (Argentina), is devoted to the breeding of beef cattle. In order to increase forage yield and improve the quality of pastures, farmers use *L. glaber*, whose adaptability to saline soils is well-known. The economic importance of this legume has led to an increasing number of studies regarding the physiological basis of its salt tolerance as well as the biodiversity of *L. glaber* symbionts (*Rhizobia* and arbuscular mycorrhizal associations). At present, "high-quality" inoculants of *L. glaber* are lacking. Therefore, numerous investigators have sought to identify an optimum growth medium for mass production of *Rhizobia*. However, excessive variations in temperature and desiccation are highly detrimental to symbiotic bacteria, as demonstrated by evaluations of legume inoculant quality. In the present study, we demonstrate that *R. loti* cells grown on a

cheese whey-based medium (CW) are better able to withstand unfavorable environmental conditions.The results described herein may be important for *L. glaber* inoculant formulation, improving the implantation of this legume in the barren lowlands of the Salado River basin of the Argentine Pampas.

Materials and methods

Bacterial strains and media

The *R loti* "INTECh" strain was selected out of ten isolates obtained simultaneously from individual farm-grown *L. glaber* plants based on their relative growth rates. While all ten strains (IIb-INTECh *Rhizobium loti* collection) are "fast growers" (specific growth rates between 0.21 and 0.15 generations/h), the fastest one was used in this study. The reference strains were *Rhizobia* strains NZP 2037 (fast grower) and NZP 2257 (slow grower), which were kindly donated by the Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand. The bacteria were grown in two different liquid media. The standard medium (YEM) contained (g/l): $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, K_2HOP_4 0.5, yeast extract (Sigma, St. Louis, Mo., USA) 1.0, and mannitol 10. The cheese-whey-based medium (CW) containing (g/l): dehydrated cheese whey (sweet or acid type; kindly donated by Gándara SA, Chascomús, Buenos Aires, Argentina) 22.0, yeast extract (Sigma) 1.0, and K_2HPO_4 0.5. In all cases, the medium was autoclaved at 121 °C for 30 min and the pH was subsequently adjusted to 7.0.

Experiments were carried out using 300-ml flasks containing 50 ml of each medium. Cultures were incubated at 28 °C on a rotary shaker (150 rpm). The flasks were inoculated with the necessary pre-inoculum volume to attain an initial absorbance < 0.05 at 620 nm. Generation times were calculated during the exponential growth phase by measuring the changes in optical density every 2 h [9] and were confirmed by cell count on YEM (see below).

Determination of living cells

Cells plated on YEM agar supplemented with Congo Red were incubated for 72 h, diluted in peptone-phosphate buffer (pH 7.0) [containing (g/l): peptone 1.0, KH_2OP_4 0.34 and K_2HOP_4 1.21], and colony-forming units (CFU) were then counted.

Evaluation of carbon source utilization and antibiotic resistance

Carbon source utilization by strain INTECh and *Rhizobia* reference strains was evaluated by replacing the mannitol with glucose, galactose, lactose, succinate, sucrose, or citrate at the same concentration used in YEM (10 mg/ml) [9]. Results were expressed as positive (+) or negative (−) according to the absorbance reached after 2 days. Values < 0.1 were considered negative and those < 0.5 were expressed as (±).

Routinely, strains were identified by analyzing tetracycline, ampicillin, and kanamycin resistance. Concentrations ranged from 0.1 to 100 μg/ml. As controls, the same strains were plated in a YEM without antibiotic addition.

Plant nodulation test

The time course of root-nodule formation was analyzed in *L. glaber* seedlings inoculated with *Rhizobia* cell suspensions grown and/or conserved in cheese-whey- and mannitol-based media. The *L. glaber* variety (La Plata University, Argentina) used in this work was obtained by recurrent selection for high yield under saline conditions.

The procedure for growing seedlings under aseptic conditions was similar to that described by Pankhurst [12]. Seeds were surface-sterilized by immersion in an ethanol/H_2O_2 (1:1) solution for 3–5 min followed by several rinsing steps with sterile water. The seeds were then germinated on sterile water agar. After germination, seedlings were sown into 210-ml glass jars containing sterile vermiculite. Three to 4 days later, bacterial suspensions were added to the jars to reach a final concentration of 10^6 cells/ml. Plants vigor was estimated visually after 4 weeks. Each strain assay was run in triplicate.

Survival of *R. loti* cells in different growth media

Ten ml of homogenized bacterial medium was aseptically removed 7 days after the stationary growth phase had been reached. The sample was centrifuged at 6,000 *g* for 10 min at 4 °C; the pellets were washed thrice in peptone-phosphate buffer and suspended in fresh medium for counting.

To study the freezing effect on cell survival, the pellets of unwashed 48-h cells grown on YEM and CW were suspended in the same volume of fresh medium, and then frozen immediately at −20 or −80 °C. Cell suspensions set up in parallel were aseptically kept at 4 °C. Thirty days later, frozen samples were rapidly thawed in a stream of hot water and together with the 4 °C samples were analyzed for CFU.

To evaluate the desiccation effect on the survival of *R. loti* cells, unwashed cell pellets grown either on YEM or CW for 48 h were placed in a drying chamber (LYOVAC GT2, Leybold, Cologne, Germany) and a mild vacuum (ca. 30 torr) was established until constant weight was obtained. These samples were stored for 30 days at 4 °C, after which they were suspended in peptone-phosphate buffer for CFU determinations.

Analysis of whey

The protein pattern from each type of cheese whey was analyzed by fast protein liquid chromatography (FPLC) using ion-exchange (Mono Q and Mono S columns) and molecular filtration (Superose 12 column) criteria.

The concentrations of proteins analyzed by cation-exchange chromatography ranged from 5 to 10 mg/ml and the injection volume varied between 250 and 500 µl. The elution program started with the passage of 3 ml salt-free buffer and was followed by 20 ml 0.30 M NaCl solution and 5 ml high-salt buffer containing 0.35–0.5 M NaCl. Finally, 5 ml buffer without salt was added in order to re-equilibrate the column. Routinely, a flow of 1 ml/min was used.

Anion-exchange chromatography was done with 500 µl whey sample dissolved in 50 mM sodium formate buffer, pH 3.5, containing 8 M urea and 20 mM 2-mercaptoethanol. The column was eluted at 1 ml/min using 10 ml of a 0–0.5 mM NaCl gradient.

Whey gel filtration was carried out using 100-µl sample solution (or, for best resolution, 50 µl) and flow-rates between 0.25 (for highest resolution) and 1 ml/min. Usually, 100 mM Tris-ClH buffer, pH 7.0, containing 0.500 M NaCl was used to provide a high ionic strength environment and to prevent possible ionic interaction between proteins and gel matrix. Ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine albumin (66 kDa) ovoalbumine (54 kDa), carbonic anhydrase (29 kDa) and cytocrome c (12,4 kDa) served as reference standards.

Fat concentration was determined by the Gerber test [13], soluble protein by Lowry et al. [6], lactose by the DNSA method described by Robyt and Whelan [14] and minerals [ash] by gravimetric analysis.

Results

Cheese whey composition

The composition of cheese whey depends on that of the milk, the cheese variety, the type of casein, and the processing conditions. The cheese whey by-products utilized in this work were the acid and sweet types. The compositions are listed in Table 1. The differences are basically the pH value, mineral concentration, and lactose level.

Milk protein that remains dissolved when casein precipitates by addition of rennet is the most valuable component of whey. In our sample, the protein content was lower than that of other whey products typically obtained in the dairy industry by ultrafiltration and diafiltration processes.

Protein separation in whey samples using a Mono S column yielded only two large peaks; the first at 19 min corresponding to α-lactalbumin, and the second at 22 min, corresponding to β-lactoglobulin. The two variants described for β-lactoglobulin were not separated, and bovine serum albumin gave a very broad peak. Under the conditions used in this study, IgG was not retained. Retention times were confirmed by spiking the whey samples with standard mixtures. Cation-exchange chromatography on a Mono S column was not judged suitable for an appropriate and rapid analysis of whey proteins under the study conditions. In contrast, whey proteins were well separated by anion-exchange chromatography on a Mono Q column and Superose 12 gel filtration, even though minor peaks, as yet unidentified, were obtained. The major components (including both variants of β-lactoglobulin) were fully separated. The principal Ig peak did not adsorb to the Mono Q column and eluted in the void volume. The main proteins observed in whey samples were β-lactoglobuline and α-lactalbumin. The presence of these proteins was confirmed in both types of whey. The ratio between β-lactoglobulin and α-lactalbumin varied from 2.7 to 2.4, and the typical composition of the main proteins groups in sweet whey was 52, 23, 10, and 6% for β-lactoglobulin, α-lactalbumin, IgG, and serum albumin, respectively. This relative composition is quite similar to those previously reported by others [1, 3]. The elution profile obtained on Superose 12 was essentially the same when acid whey was analyzed (data no shown).

Table 1 Composition of cheese wheys used in this work. The composition is expressed as average values of analytical measurements carried out in triplicate on three independent samples of each cheese type

Component	Sweet whey	Acid whey
True protein (%)	0.54	0.57
Non-protein nitrogen (%)	0.18	0.22
Lactose (%)	4.32	4.05
Lactic acid (%)	0.14	0.55
Mineral (%)	0.48	0.70
Fat (%)	0.05	0.05
Total solid (%)	5.71	6.14
pH	6.0	4.45

Analysis of antibiotic resistance and carbon feeding source

The antibiotic sensitivity of the INTECH and reference strains is shown in Table 2. Carbon source utilization, including disaccharides such as lactose (the most important sugar component in whey), monosaccharides, and carboxylic acids, was also analyzed. Table 3, shows that the mono- and disaccharides were good substrates, except for glucose utilization by strain NZP 2257, whereas citrate was not a good carbon source for *Rhizobium* cell growth.

Protective properties and relative growth rates on mannitol vs. cheese-whey medium

In order to determine the behavior of *R. loti* cells growing YEM and CW , the CFUs of an culture vol-

Table 2 Specific growth rates and comparative responses to antibiotics of the *Rhizobium* strains used in this work. Specific growth rates (generations per hour in the exponential phase) were calculated from triplicate optical density measurements in cheese whey medium and were calibrated for CFU on yeast-mannitol agar after serial dilution in peptone-phosphate. Data are means ($n=3$) of a single representative assay from 3 experiments

Rhizobium strain	Specific growth rate	Tetracycline[a]	Ampicillin[a]	Kanamycin[a]
INTECH	0,21	0,1	100	25
NZP 2037	0,28	0,1	25	100
NZP 2257	0,08	0,1	100	0

[a]Maximum concentration (μg/ml) of an antibiotic at which normal growth on yeast–mannitol agar occurred. Measurements were made in triplicate using 0.1; 1; 10; 25; 50 and 100 μg/ml

Table 3 Carbon source for *Rhizobia* strains used in this work. Results are expressed as positive (+) or negative (−) according to the absorbance after 2 days. Values <0.1 were considered negative and those <0.5 were expressed as (±)

Strain	Glucose	Galactose	Lactose	Sucrose	Citrate	Succinate
INTECh	+	±	+	+	−	+
NZP 2037	+	+	+	+	−	+
NZP 2257	−	+	±	+	−	+

umes were determined at different times. Generation times of the INTECH and NZP 2037 strains on CW (Table 2) were similar to those previously described for YEM [4]. In addition, the survival of INTECh *Rhizobia* cells grown in CW was longer. Data showed that a 54% viability was found 7 days after stationary phase had been reached. Only 19% of INTECh cells were viable when YEM was used. The number of viable INTECh cells in the stationary phase was approximately the same (2×10^9 cells/ml) on the two growth media. Moreover, the viability of unwashed cell suspensions grown on YEM and resuspended in the same medium was less than that of cells grown in CW and subjected to refrigeration and freeze-thaw conditions. When pellets were suspended in CW and immediately frozen at −20 or −80 C, the survival after 30 days was over 90%, independent of the medium used (Fig. 1B). By contrast, less than 9% of YEM-grown cells survived after being resuspended in the same medium and frozen at −20 °C or −80 °C, indicating that YEM did not provide the cells with the necessary protection for freezing. Lower values were observed with samples kept at 4 °C (Fig. 1A). Furthermore, once the whey was washed off and the CW-grown cells were suspended in YEM, 72 and 81% still survived at −20 or −80 °C, respectively, and 17% survived when the culture was kept at 4 °C. (Fig. 1 A.).

As reported in an earlier study [2], the supernatant of CW was more fluid than that of YEM. Moreover, the pellets obtained in YEM were completely dry in 48 h, while those of whey-grown cells remained wet longer and showed a higher level of survival, reaching 1.3 and 0.42×10^9 cells/ml, respectively.

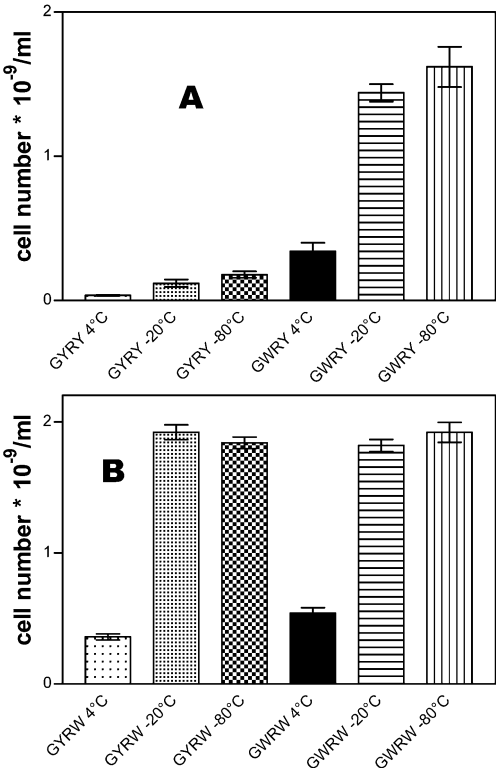

Fig. 1A, B Survival of *Rhizobium loti* cells at different temperatures after being resuspended in mannitol-based medium (YEM) or cheese-whey-based medium (CW). **A** GY (grown) and RY (resuspended) in YEM. **B** GW (grown) and RW (resuspended) in a cheese-whey-based medium. *Bars* Mean ± SE of n≥3 independent samples, each determination assayed at least 3 times

To evaluate the effectiveness of the symbiosis capacity of re-isolated *Rhizobia*, plant vigor and the time-course of nodule appearance were compared. No differences were observed between YEM- and CW-grown cells.

Discussion

The Instituto de Investigaciones Biotecnológicas- Instituto Tecnológico de Chascomús (IIB-INTECh) in Chascomús is located in a vast area of about 50,000 km^2, called "Pampa Deprimida del Río Salado" or the Salado River basin, where cattle and dairy farms are based mainly on the native grassland. The naturalized *Lotus* found in the alkaline-saline lowland soils of this region is the accession *glaber* (ex-*tenuis*). There is agreement that any future improvement of animal breeding requires an increase in the amount and quality of the forage supply. Because a given legume growing in saline soils can be nodulated only by certain bacterial species, all legume breeding programs must be carried out together with an inoculant research program.

Cheese whey has traditionally been regarded as an unwanted by-product of cheese or casein production. While in some countries, whey has been used as pig or calf feed or for the production of whey powder,

demineralized whey powder, and lactose, in other countries, it has simply been disposed off in the dairy-plant wastewater, thereby causing significant environmental problems [5]. The results obtained in this work demonstrate that cheese whey by-products with a composition similar to that described herein can be used as a good and inexpensive growth media for *Rhizobium loti* cells, allowing the harvested cells to better withstand freezing and desiccation. Based on these properties, cheese whey provides an interesting alternative as a protective agent suitable for use in inoculant production of the most important forage legume adapted to marginal soils in the Argentine Pampas. In this work, we have optimized the separation of bovine whey proteins by anion-exchange chromatography and gel filtration. Whey proteins were not well separated using Mono S but were well resolved on Mono Q and Superose 12 columns. The reported composition obtained is similar to those described by other authors [1, 3, 10, 11], suggesting that the optimized protocols can be used for rapid analysis of bovine whey.

This work is part of an extensive study of the use of CW-based media for culturing liquid inoculants of *Rhizobia* species. Based on the results of this study, such media could be of particular interest in the production of *Bradyrhizobium japonicum* inoculants, taking into account the enormous economic importance of soybeans (16,000,000 ha sown last year) in our country.

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References

1. Andrews AT, Taylor MD, Owen AJ (1985) Rapid analysis of bovine milk proteins by fast protein liquid chromatography. J Chromatogr 348:177–185
2. 2- Bissonnette N, Lalande (1988) High survivability of cheese whey-*grown Rhizobium meliloti* cells upon exposure to physical stress. Appl Environ Microbiol 54:183–187
3. Chaplin LC (1986) Hydrophobic interaction fast protein liquid chromatography of milk proteins. J. Chromatogr 363:329–335
4. Fulchieri MM, Estrella MJ, Iglesias AA (2001). 1–4. Characterization of *Rhizobium loti* strains from the Salado River Basin. Anton Leeuw Int J Gen and Mol Microbiol 79:119–125
5. Gonzalez Siso MI 1(996) The biotechnological utilisation of cheese whey: a review. Biores Technol 57:1–11
6. Lowry OH, Rosebrough NJ, Farr AL, RJ Randall (1951) Protein measurement with Folin-phenol reagent. J Biol Chem 193:265–275
7. Martinez de Drets G, Arias A, Rovira de Cutinella M (1974) Fast and slow-growing *rhizobia*: differences in sucrose utilisation and invertasa activity. Can J Microbiol 20:605–609
8. Montes L (1987) Current research on *Lotus tenuis* in Balcarce (Argentina). Lotus Newslett 18:13–15
9. Monza J, Fabiano E, Arias A (1992) Characterization of an indigenous population of rhizobia nodulating *Lotus corniculatus*. Soil Biol Biochem 24:241–247
10. Musale DA, Kulkarni SS (1998) Effect of whey composition on ultrafiltration perfomence. J Agric Food Chem 46:4717–4722
11. Nielsen S (1996) Introduction to the chemical analysis of foods. Jones and Barlett, Boston
12. Pankhurst CE (1977) Symbiotic effectiveness of antibiotic-resistant mutants of fast- and slow-growing strains of *Rhizobium*- nodulating *Lotus* species. Can J Microbiol 23:1026–1033
13. Pearson D (1970) The chemical analysis of foods, 6th edn. Churchill, London
14. Robyt JF, Whelan. WJ (1972) Reducing value methods for maltodextrins: I Chain-length dependence of alkaline 3,5-dinitrosalicylate and chain-length independence of alkaline copper. Anal Biochem 45:510–516
15. Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. I.B.P. Handbook. no. 15. Blackwell, Oxford