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Full Length Research Paper

Isolation of a *Lactobacillus plantarum* strain used for obtaining a product for the preservation of fodders

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An important problem in the field of animal nutrition is the preservation of fodder, which is done in silos for maintaining the nutritive value of the fodders. In anaerobic conditions, the lactic bacteria ferment the glucosides in the silo, leading to the formation of lactic acid and acetic acid, which cause the pH to decrease and inhibit the development of butyric bacteria. The enrichment of the ensiled fodders with probiotic products confer on them the role of maintaining a microbial flora inside the digestive tract favourable to animal health.

Key words: *Lactobacillus plantarum*, molasses, corn extract, lactic acid.

INTRODUCTION

Strains isolation from special habitats, their identification and use with a view to improving the fodder quality represent a major preoccupation for increasing farm animal performance. This includes *Lactobacillus plantarum* strains which are added into the fodder silo to lower the pH to values allowing for direct fermentation which produces lactic acid, which inhibits the development of butyric acid-producing bacteria. The importance of lactic bacteria, and the *L. plantarum* species in particular, used as silo inoculant lies in fact that the strains are a potential model for fermentation studies (de Roissart and Luquet 1994; Banu, 2000; McDonald, 1981).

The microorganisms involved in the fermentations in silos are isolated from the environment. The anaerobic bacteria, in the absence of oxygen and in the presence of glucosides (molasses) are rapidly multiplied in the whole silo mass, ensuring the silo preservation in very good conditions during the entire ensiling period. The glucosides are the source leading to lactic acid formation. In practice, the silo is supplemented with molasses for a rapid proliferation of lactic bacteria in the whole ensiled mass and for the production of lactic acid in large amounts in the shortest time possible (Goodman et al., 1995).

In all these fermenting processes there are important lactic bacteria producing as much lactic acid as possible, which consume a low amount of glucosides and which survives to high acidity (pH 3 – 4.5). Besides all these considerations, it is important to mention another aspect of the ensiling process, namely the air removal from the silo in order to hinder the development of aerobic bacteria (Scriban, 1984; Demain and Solomon, 1996; Salminen and von Wright, 1999).

The molasses is used for increasing the glucoside concentration in the ensiled fodder. Molasses is a sugar-refining by-product which provides sugar to the fodder in silo and serves as substrate for lactic bacteria development and multiplication. The addition of molasses into the silo results in improving the farm animal performances in comparison with animals to which fodder is not treated with molasses. (Asbell et al., 1991; Woolford, 1984; Pichard, 2000; Goodman et al., 1995; Olmos-Dichara et al., 1997).

The lactic bacteria as well as other types of microorganisms are also capable of producing and discharging into the environment a great variety of antimicrobial substances, which inhibit the development

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of microorganisms not suitable for ensiling. The bacteriocides form a class of antibiotic substances whose biosynthesis occurs during or at the end of the exponential stage of lactic bacteria strain growth (Enan et al., 1994; Jones, 2000; Stiles, 1996).

MATERIALS AND METHODS

Micro-organisms

Lactobacillus sp 5s was isolated from a silo, while *L. plantarum* ATCC 8014 was employed as test strain.

Media

The strains were maintained on MRS medium with the following composition: peptone 1%, yeast extract 0.5%, meat extract 0.5%, glucose 2%, dipotassium phosphate 0.2%, dibasic ammonium citrate 0.2%, sodium acetate 0.5%, magnesium sulphate 0.01%, manganese sulphate 0.005%, Tween 80, agar 2% (for solid medium) and 0.7% for semisolid medium (Tatiana Vassu et al., 2001).

Two media: GP (2% glucose, 1% peptone, 1% $CaCO_3$), GEP (2% glucose, 0.2% corn extract, 1% $CaCO_3$) were also used for tests.

Identification

The selected strain was subjected to identification by conventional taxonomic techniques specific to the bacteria (Tatiana Vassu et al., 2001; Banu, 2000). The following tests were performed with a view to pointing out the morphological-physiological and biochemical characteristics:

For establishing the minimum, optimum and maximum development temperature, the tests were carried out on MRS medium. The development was appreciated by reading the optical densities at 570 nm every 2 h.

The gas producing capacity (H_2S) was made by culturing the microorganisms on a medium containing (g/l): peptone 15, casein 5, glucose 1, Pb acetate 0.2, sodium thiosulphate 0.08, Mn sulphate 0.05, agar 15, Tween 80 1 ml, incubation for 2 weeks at 30°C.

For reducing the tetrazolium salts, the medium contains (g/l: peptone 15, yeast extract 1, dipotassium phosphate 2, magnesium sulphate 0.05, agar 10, Tween 80 1 ml). After been subjected to digestion 0.1% 2,3 triphenyl tetrazolium 5 was added to the medium.

Resistance to biliary salts was tested on a medium containing (g/l): tomato juice 10, peptone 1.5, glucose 2, NaCl 0.5, yeast extract 0.6, soluble starch 0.05, tauroglycocolate 2, Tween 80 0.1 ml.

For pH assay, the cultures were developed on a MRS medium at various pH ranges: 3, 3.9, 5.5, 6.5, 7, 7.5, and 8.

The fermenting capacity of various sugars was tested on a medium containing (g/l): peptone 10, NaCl 5, K_2PO_4 0.3, bromthymol blue (added as an alcoholic solution of bromthymol blue 0.06), pH 6.5.

The production of polysaccharides was performed by means of a medium having the following composition (g/l): peptone 5, meat extract 10, sodium chloride 6.5, potassium nitrate 8, sucrose 8, Tween 80 1 ml, pH 7.1.

The starch hydrolysis was performed on a medium containing (g/l): peptone 1.5, Na chloride 0.5, yeast extract 0.6, galactose 0.5, starch 0.2, agar 2, Tween 80 0.1 ml.

Determining the antibiotic resistance

The antibiotic resistance was tested on Petri plates supplemented with various antibiotics: penicillin, cephalosporine, aminoglycoside, tetracycline, quinolone, polypeptide, macrolide, rifamicine, and sulphamide. After seeding, the plates were incubated for maximum 48 h at 37°C.

Genetic analysis of the chromosomal DNA

Isolation of the chromosomal DNA was made after culturing the microbial strain on MRS for 24 h. The G + C molar percentage for the strains employed was calculated based on the melting temperature (Tm). All the Tm determinations were performed by recording the hyperchromic effect in the temperature range from 20 to 100°C, with an increment of 2°C or 3°C/min. The GC percentage was calculated by using the Owen formula: %GC = Tm 2.08 - 106.4 (Tatiana Vassu et al., 2001) .

Determining the lactic acid production

The lactic acid accumulation was detected on the three media (MRS, GP, GEP). The acidity was determined by titration with 0.1 N NaOH. 1 ml of 0.1 N NaOH corresponds to 0.009008 g of lactic acid.

RESULTS AND DISCUSSION

Identification

The preliminary investigations performed including microscopic analysis (Gram- positive bacilli), lactic acid biosynthesis and the negative catalase reaction permitted the classification of the working bacterium into the *Lactobacillus* genus (Table 1). According to the presented data, the isolated strain can be classified into the *L. plantarum* species.

Determining the molar percentage of G+ C

Determining the %GC values of the two strains employed on the basis of the melting temperature is presented in Table 2.

The data in Table 2 are within the limits stated precisely for *L. plantarum* species.. Thus, according to morphological, physiological, biochemical and genetic data, the strain could be classified as a *L. plantarum* species and it has been registered in the collection of the Centre of Applied Biochemistry and Biotechnology – Biotechnol as *L. plantarum* with the number 5s.

Obtaining the Biomass

In order to obtain the biomass of *L. plantarum* 5s economically constituted media based on glucose (2% concentration), containing either peptone or corn extract were employed. The fermentations were carried out

Table 1. Characteristics of the two strains.

Characteristics	*Lactobacillus plantarum*	
	ATCC	**5s**
I. Taxonomic classification: – family – genus - species	 *Lactobacilaceae* *Lactobacillus* *Lactobacillus plantarum*	 *Lactobacilaceae* *Lactobacillus* *Lactobacillus plantarum*
II. Morphological and culture characteristics -colour - shape - Gram coloration -development on solidified medium -development on liquid medium	 cream-coloured - beige isolated little sticks yes smooth round colonies uniform turbidity, sediment formation	 white, cream-coloured - beige isolated little sticks yes smooth round colonies uniform turbidity, sediment formation
III. Physiological characteristics - pH - temperature (°C)	 5 – 7 30-37	 4–6.5 30-42
IV. Biochemical characteristics Acid from: - ribose - galactose - D-glucose - D-fructose - D-manose - ramnose - manitol - sorbitol - maltose - lactose - sucrose Fermenting type Enzyme production: - catalase - amylase Resistance to biliary salts H_2 S formation	 Yes Yes Yes Yes No Yes Yes Yes Yes Yes Homofermentative No No Yes Yes	 Yes Yes Yes Yes No Yes Yes Yes Yes yes Homofermentative No No Yes Yes
V. Resistance to antibiotics	Resistant to cephalosporines, quinolones, tetracyclines	Resistance to cephalosporines, tetracyclines

Table 2. The Tm and %GC values of the two strains.

Strains	Tm	%GC
L. plantarum ATCC	70.86	40.98
L. plantarum 5s	71.6	42.52

statically, at 37°C, initial pH of 6.5 to establish the growth curve (D.O. 570 nm) and the lactic acid accumulation (the determinations being performed every 2 h).

On MRS the development of *L. plantarum* 5s strain was typical; a logarithmic growth during the first 8 h, then a growth stage having a much slower speed for 2 h at the

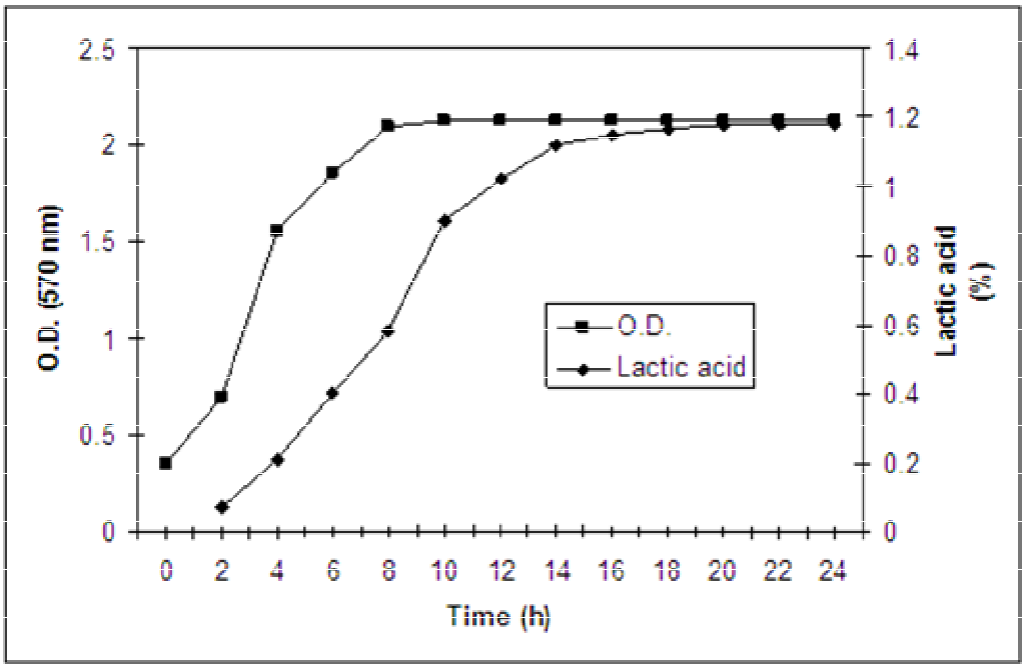

Figure 1. Lactic acid accumulation and the growth curve of *L. plantarum* 5s strain growth on MRS medium.

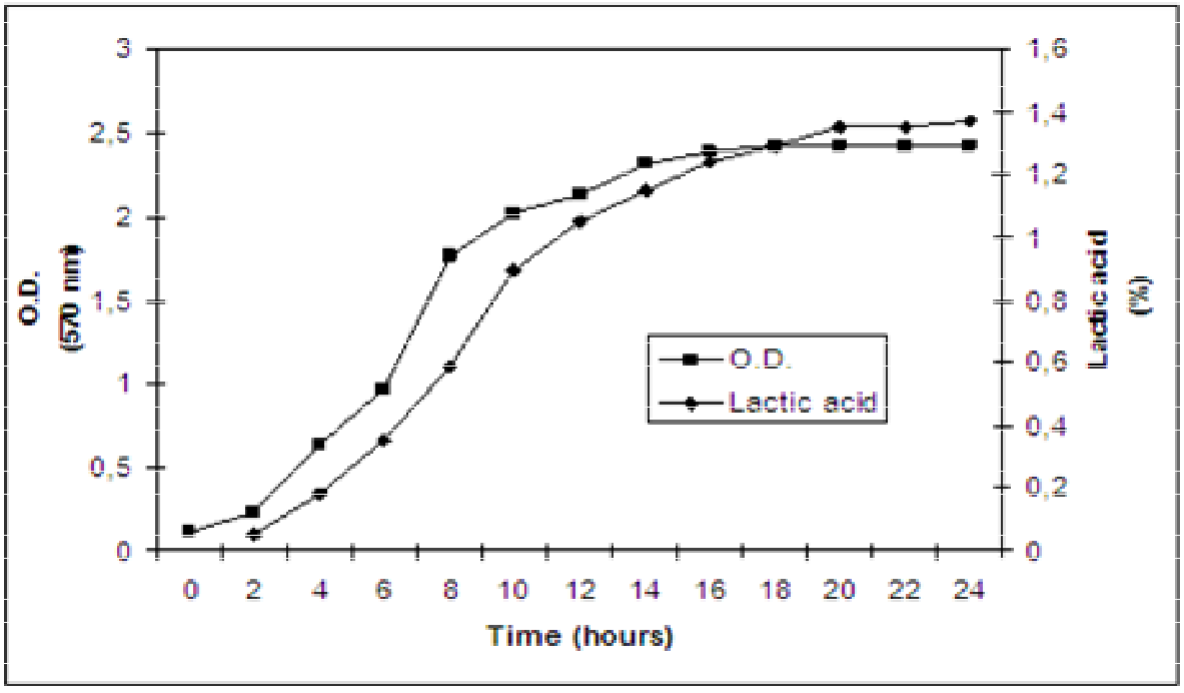

Figure 2. Lactic acid accumulation and growth curve of *Lactobacillus plantarum 5s* strain on GP medium.

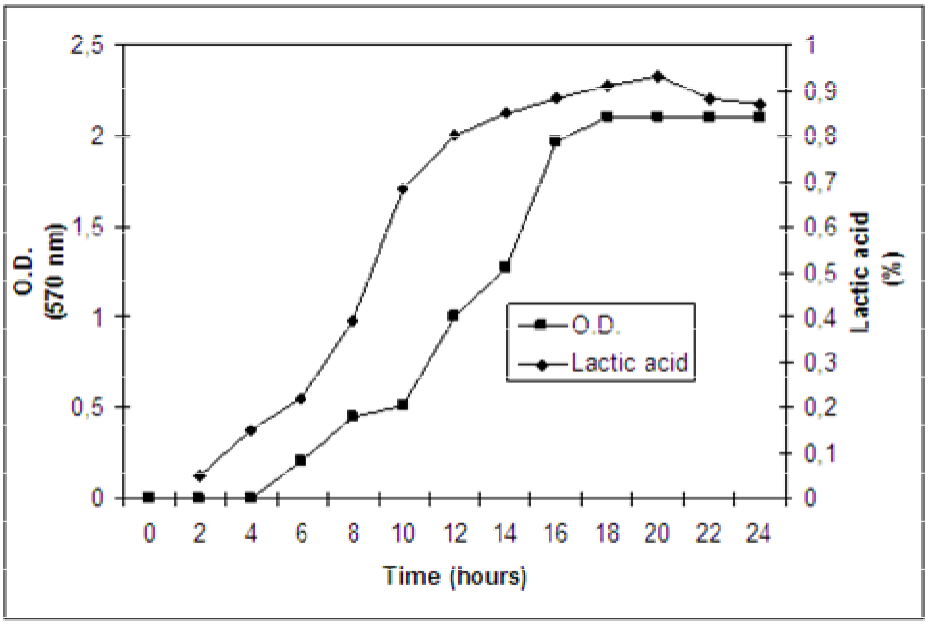

Figure 3. Lactic acid accumulation and the growth curve of *L. plantarum* 5s strain on GEP medium

most, followed by the strain entering the stationary stage. The lactic acid accumulation had a significant increase at the middle of the logarithmic stage. During the stationary stage the accumulation continues, but at a slower rate (Figure 1).

On GP based on glucose and peptone, accumulation of the biomass is similar to the MRS medium. The logarithmic growth stage extends for a period of 10 h, being followed by a slowed down growth stage. The lactic acid accumulation was observed during the whole period, with an increased accumulation during the logarithmic growth stage (Figure 2).

On GEP based on glucose and corn extract the maximum values of the lactic acid concentration are less than the values obtained on GP medium. The lag phase lasts for 4 h, and the logarithmic growth stage has a duration of 12 h. The lactic acid accumulation reached its peak after 20 h of fermentation (Figure 3).

The biochemical and molecular analysis tests allowed the classification of this isolate as a *L. plantarum* strain. A pH of 4 of the silo ensures the proteolysis and harmful microbial species inhibition. The use of the inoculant is the best method for improving fodder quality. The efficiency of a biological preservative depends on the selected strain and the culture medium. In order to ensure a better distribution of the inoculant, the addition of enzymes that degrade the polyglucosides is preferred. In the present case, the reducing glucoside concentration did not exceed 3% which is a sufficient concentration to develop the *L. plantarum* strains. A lactic acid concentration of approximately 2% is sufficient to preserve the fodders.

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