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ORIGINAL ARTICLE

Modification of azo dyes by lactic acid bacteria

I.M. Pérez-Díaz and R.F. McFeeters

USDA-ARS, SAA Food Science Research Unit, North Carolina State University, Raleigh, NC, USA

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Correspondence
Pérez-Díaz, USDA-ARS, SAA Food Science Research Unit, North Carolina State University, Raleigh, NC 27695-7624, USA.
E-mail: ilenys.pérez-díaz@ars.usda.gov

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Abstract

Aim: The ability of *Lactobacillus casei* and *Lactobacillus paracasei* to modify the azo dye, tartrazine, was recently documented as the result of the investigation on red coloured spoilage in acidified cucumbers. Fourteen other lactic acid bacteria (LAB) were screened for their capability to modify the food colouring tartrazine and other azo dyes of relevance for the textile industry.
Methods and Results: Most LAB modified tartrazine under anaerobic conditions, but not under aerobic conditions in modified chemically defined media. Microbial growth was not affected by the presence of the azo dyes in the culture medium. The product of the tartrazine modification by LAB was identified as a molecule 111 daltons larger than its precursor by liquid chromatography-mass spectrometry. This product had a purple colour under aerobic conditions and was colourless under anaerobic conditions. It absorbed light at 361 and 553 nm.
Conclusion: LAB are capable of anabolizing azo dyes only under anaerobic conditions.
Impact and Significance of the Study: Although micro-organisms capable of reducing the azo bond on multiple dyes have been known for decades, this is the first report of anabolism of azo dyes by food related micro-organisms, such as LAB.

Introduction

Organisms capable of utilizing azo dyes have been the target of multiple studies due to their role in the treatment of waste waters containing azo dyes, which are primarily derived from the textile industry (Sponza and Işik 2002; Dos Santos *et al.* 2005). Although, it has been reported that several bacteria and fungi are capable of catabolizing and mineralizing azo dyes (Cheng 2006), the ability of food micro-organisms such as lactic acid bacteria (LAB) to modify azo dyes was not recognized. Recently our group documented the ability of lactobacilli, such as *Lact. casei* and *Lact. paracasei*, to utilize the azo dye FD&C yellow no. 5 also known as tartrazine. This capability resulted in the development of a red coloured spoilage in fermented vegetables (Pérez-Díaz *et al.* 2007). Understanding the ability of specific LAB to utilize azo dyes could potentially lead into the development of ways to control and prevent the undesired formation of red coloured spoilage in fermented vegetables, and in the development of novel technologies that could benefit the textile industry.

LAB are facultative anaerobic bacteria commonly found in soil, plant material, human and other animals' intestinal tracts, and foods such as milk, cheese, yogurt, wine, beer and fermented meats and vegetables. Although, LAB are commonly used industrially for fermented products due to their ability to convert a carbohydrate source into lactic acid, some species cause spoilage of selected foods. The exceptional ability of LAB to grow under somewhat extreme conditions (acidic pH, relatively high sodium chloride concentration, limiting carbohydrate sources) has allowed them to remain present in a variety of foods and environments.

Azo dyes are characterized by the presence of at least one R_1–N=N–R_2 functional group. The azo group often helps to stabilize the dyes and form a conjugated system, which very often absorb visible frequencies of light yielding coloured compounds. Aromatic azo compounds, such as tartrazine, are usually stable and have vivid and warm

colours such as red, orange and yellow. Some azo dyes are utilized as acid–base indicators, food colouring agents, for the dying and printing of textiles, and as an absorption-elution indicator for chloride estimation.

It was the objective of this research to identify members of the LAB group capable of utilizing tartrazine and other azo dyes, and identify the product(s) of their chemical/enzymatic modification.

Materials and methods

Strains and growth conditions

The strains utilized in this research are listed in Table 1. The lactococci strains were transferred from frozen stocks to GM17 agar (1% glucose) (Becton, Dickinson and Co., NJ, USA). All other strains used were grown in Lactobacilli deMan Rogosa and Sharpe agar (MRSA) (Becton, Dickinson and Co.). Agar plates were incubated under aerobic conditions at 30°C for 48 h. *Lactobacillus delbrueckii* was incubated at its optimum growth temperature of 37°C instead of 30°C. Isolated colonies were transferred to either GM17 broth or MRS broth (Becton, Dickinson and Co.). Cultures were incubated at 30°C for 24 h prior to inoculation of the experimental media.

Utilization of selected azo dyes by LAB under aerobic conditions

Cultures in triplicate were transferred from either GM17 broth or MRS broth to 10 ml modified chemically defined media (mCDM). The mCDM was prepared as described by Christensen and Steele (2003) with the following modifications: (i) all individual amino acids were replaced by 10 g of Bacto Casitone (Becton Dickinson and Co., Sparks, MD, USA) and (ii) it was supplemented with 2·5 mg pyridoxamine dihydrochloride (Sigma-Aldrich Co., St Louis, MO, USA) per litre. Substitution of individual amino acids by Bacto Casitone was essential for growth of some lactobacilli. The mCDM carbohydrate content was adjusted to 10 mmol l^{-1} glucose, and was supplemented with 0·25 mmol l^{-1} of either tartrazine (Sigma-Aldrich Co.), methyl orange (Fisher Scientific, Pittsburg, PA, USA), acid orange 63 (Sigma-Aldrich Co.), or acid orange 8 (Sigma-Aldrich Co.) in triplicate tubes. Cultures were incubated at 30°C for 7 days with aeration using an Innova 3000 Platform Shaker (New Brunswick Scientific, Edison, NJ, USA) at 200 rev min^{-1}.

Growth was monitored by measuring optical density at 600 nm (OD_{600}) using a NanoDrop ND-1000 UV-Visible Spectrophotometer (Nanodrop, Wilmington, DE, USA). Culture samples were diluted when necessary before measuring OD_{600}. Disappearance of the azo dyes tartrazine, methyl orange, acid orange 63 and acid orange 8 from cultures supernatant was monitored by measuring the absorbance at 427, 450, 427 and 490 nm, respectively, using a NanoDrop ND-1000 UV-Visible Spectrophotometer. Supernatants were obtained by centrifugation of 1 ml cultures at 6000 rev min^{-1}, for 10 min at 25°C using an Eppendorf Centrifuge 5810 R (West Bury, NY, USA). Data collected using the Nanodrop ND-1000 UV-Visible Spectrophotometer was multiplied by a factor of ten to accommodate for the difference in path length of 0·10 cm to the standard 1 cm path length.

Table 1 Bacterial strains utilized in this research

Bacterium	Source or reference
Lact. plantarum	ATCC14917*
Pediococcus pentosaceous	ATCC25745*
Leuconostoc mesenteroides	ATCC8293*
Lactococcus lactis	ATCC11454*
L. cremoris	ATCC14365*
Lact. pentosaceous	ATCC8041*
Lact. brevis	ATCC14869*
Lact. casei	ATCC393*
Lact. paracasei	ATCC25302*
Lact. casei LA1133	FSRU†
Lact. paracasei LA0471	FSRU†
Lact. delbrueckii	ATCC9649
Lact. rhamnosus GG	ATCC53103*

*ATCC – American Type Culture Collection.
†FSRU – USDA-ARS, Food Science Research Unit culture collection.

Utilization of selected azo dyes by LAB under anaerobic conditions

Cultures (Table 1) in triplicate were transferred from either GM17 broth or MRS broth to 10 ml MRS broth (Becton, Dickinson and Co.), previously incubated in the anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA) for 48 h to eliminate oxygen. Cultures were incubated at 30°C for 24 h in the anaerobic chamber. Subsequently, all cultures were transferred to 10 ml mCDM containing 0·25 mmol l^{-1} of either tartrazine, methyl orange, acid orange 63, or acid orange 8. The mCDM was prepared as described previously. Oxygen was eliminated from the media by 48 h incubation in an anaerobic chamber (Coy Laboratory Products, Inc.). Anaerobic mCDM cultures were incubated at 30°C for 72 h. Growth and utilization of the azo dyes were monitored as described previously. *Lactobacillus delbrueckii* was not able to grow extensively in mCDM, and therefore was transferred to MRS broth containing 0·25 mmol l^{-1} of the azo dyes and no oxygen. The cultures were incubated at 30°C under anaerobic conditions.

Evaluation of azo dyes as an energy source for LAB

Cultures of *Lact. casei* LA1133, *Lact. paracasei* LA0471, and *Lact. plantarum* were transferred from MRS broth to 10 ml mCDM containing 2·5 mmol l^{-1} glucose and 0 mmol l^{-1} or 2·5 mmol l^{-1} of either methyl orange (Fisher Scientific, Pittsburg, PA, USA), acid orange 63, acid orange 8 or tartrazine (Sigma-Aldrich Co.) in triplicate. The mCDM with no oxygen was prepared as described previously. Cultures were incubated at 30°C for 3 days in the anaerobic chamber (Coy Laboratory Products, Inc.). Growth was monitored by measuring optical densities at 600 nm. Disappearance of the azo dyes was monitored as described previously.

Identification of end products of tartrazine modification

Lactobacillus paracasei LA0471, *Lact. casei* LA1133 and *Lact. plantarum* were transferred from frozen stocks to MRS agar (Becton, Dickinson and Co.) and incubated at 30°C for 48 h. Isolated colonies were transferred to MRS broth (Becton, Dickinson and Co.), which was incubated in the anaerobic chamber for 48 h prior to inoculation to eliminate oxygen (Anaerobic Chamber-Coy Laboratory Products, Inc.). Cultures were incubated at 30°C for 18 h under anaerobic conditions. Samples (5 ml) were spun for 10 min at 6000 rev min^{-1} under anaerobic conditions using a Costar Model 10 centrifuge (Fischer Scientific). Pellets were washed twice with saline solution and re-suspended in 1 ml of phosphate buffer, pH 7·0, supplemented with 2·5 mmol l^{-1} tartrazine and 100 μmol l^{-1} glucose (Sigma-Aldrich Co.). Cell re-suspensions were incubated at 30°C overnight under anaerobic conditions (Coy Laboratory Products, Inc.). Subsequently, cell re-suspensions were spun for 10 min at 6000 rev min^{-1} under anaerobic conditions. The supernatant was utilized to re-suspend a fresh 5 ml cell pellet prepared as described above, and incubated

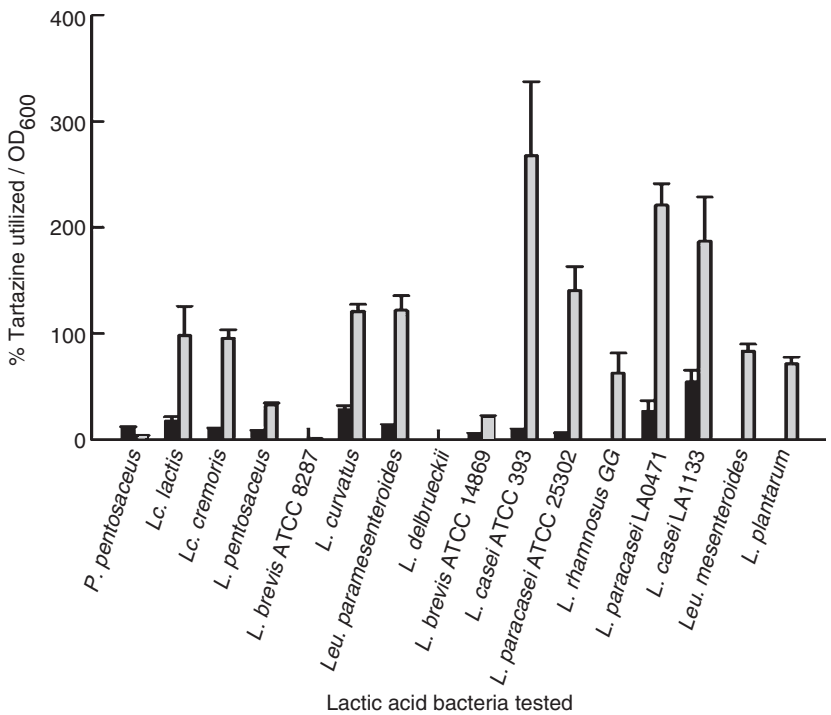

Figure 1 Ability of lactic acid bacteria (LAB) to remove tartrazine from a modified chemically defined medium (mCDM): Utilization of tartrazine by LAB was monitored in mCDM under aerobic (■), and anaerobic conditions (□). Utilization of tartrazine was monitored by measuring the reduction in absorbance at 427 nm.

Table 2 Modification of azo dyes by lactic acid bacteria (LAB) under anaerobic conditions. Results are given as the decrease in the concentration of the dyes per unit change in the OD_{600} of the bacterial cultures

	Utilization of azo dyes tested (mmol l^{-1}/OD_{600})			
LAB tested	Methyl orange	Acid orange 8	Tartrazine	Acid orange 63
Lactobacillus casei LA1133	0·65 ± 0·1	1·0 ± 0·1	0·40 ± 0·3	0·73 ± 0·1
Lact. paracasei LA0471	0·55 ± 0·1	0·54 ± 0·2	0·31 ± 0·1	0·23 ± 0·1
Lact. plantarum	0·62 ± 0·1	0·61 ± 0·1	0·49 ± 0·1	0·21 ± 0·1

overnight at 30°C under anaerobic conditions. The tartrazine containing buffer was re-used to re-suspend cells five times, until it was colourless. Aliquots of 5 μl of the colourless supernatants were utilized to obtain the absorption spectra of the products, using a NanoDrop ND-1000 UV-Visible Spectrophotometer. The colourless supernatant derived from the *Lact. paracasei* LA0471 was analysed by liquid chromatography-mass spectrometry (LC-MS) after it was expose to air and became purple in colour. Samples were analysed on a Thermo (San Jose, CA, USA) LTQ linear ion trap mass spectrometer operating under negative mode electrospray ionization. Briefly, a 5 μl sample injection was made onto a 150 × 2 mm Hypersil Gold C18 column, equilibrated in 95 : 5 A : B, where A = 0·1% formic acid, 0·08% ammonia in water, pH 3·6 and B = acetonitrile. A linear gradient of 25 min to 5 : 95, A : B at 250 μl min^{-1} was utilized to elute analytes. Column eluate was passed through a Thermo Surveyor photodiode array detector (scan range 200–800 nm). The mass spectrometer was configured for full scan MS (m/z = 115–2000) and data-dependent MS/MS on the most intense ion from the survey scan (35% normalized collision energy, NCE).

Results

Utilization of tartrazine by LAB

The ability of *Lact. casei* LA1133, *Lact. paracasei* LA0471 and other lactic acid bacteria to modify tartrazine under anaerobic conditions was confirmed

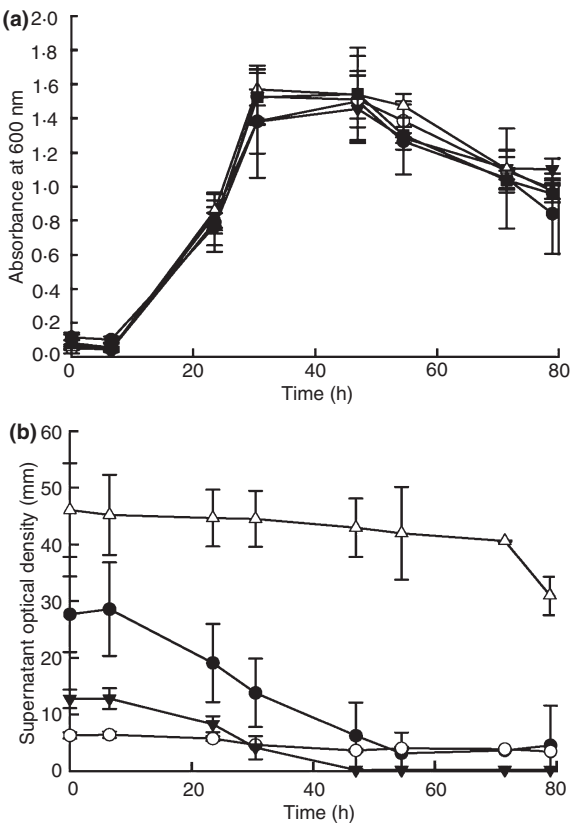

Figure 2 (a) Evaluation of the ability of azo dyes to serve as energy sources for *Lactobacillus paracasei*: Growth of *Lactobacillus paracasei* LA0471 (a), and disappearance of azo dyes (b) from modified chemically defined medium at 30°C. Azo dyes monitored include acid orange 8 at 427 nm (●), acid orange 63 at 490 nm (○), methyl orange at 450 nm (▼), tartrazine at 427 nm (Δ), and no dyes (■). Similar results were obtained for *Lactobacillus casei* LA1133, and *Lactobacillus plantarum* (data not shown).

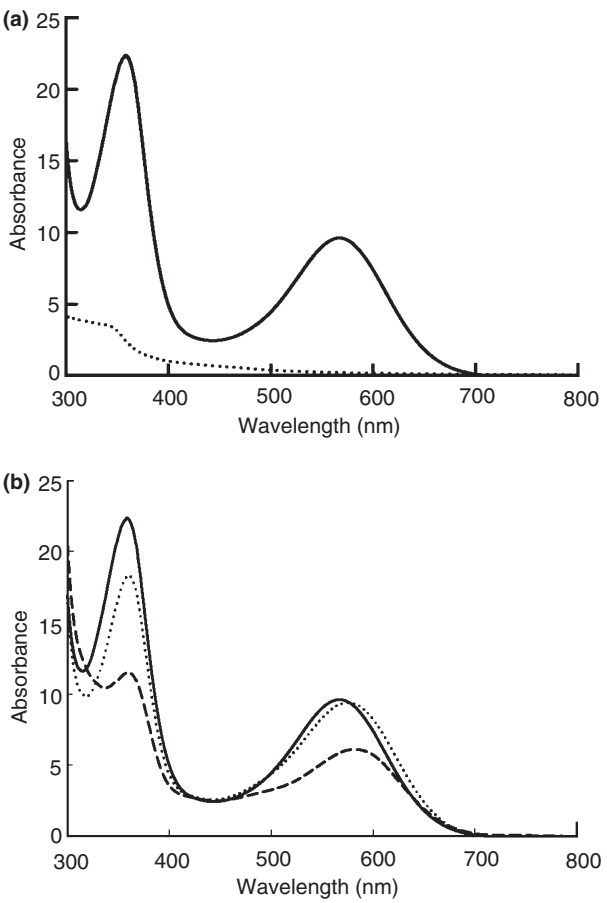

Figure 3 Absorption spectra of the products from tartrazine metabolism by LAB: (a) Absorption spectra of the product of tartrazine metabolism by *Lact. paracasei* LA0471 before (···) and after (–) exposure to air. (b) Absorption spectra of the air-exposed-product after tartrazine metabolism by *Lact. paracasei* LA0471 (–), *Lact. casei* LA1133 (– – –), and *Lact. plantarum* (·····).

(Fig. 1). Among the bacteria tested are those which predominate in fermented vegetables such as *Lact. plantarum* and *Leuconostoc mesenteroides*. However, the red coloured spoilage isolates, *Lact. casei* LA1133 and *Lact. paracasei* LA0471 modified tartrazine more efficiently under anaerobic conditions as compared to the other LAB. Additionally, *Lact. casei* LA1133 and *Lact. paracasei* LA0471 were among the few LAB capable of modifying the dye to a significant extent under aerobic conditions (Fig. 1).

Although, other LAB, commonly associated with fermented vegetables such as *Lact. brevis* and *P. pentosaceous*, were capable of growing in mCDM under anaerobic conditions, they were not capable of modifying tartrazine to a significant extent. *Lactobacillus delbrueckii* was incapable of growing in mCDM under aerobic and anaerobic conditions. Therefore, the ability of this strain to utilize tartrazine was evaluated in MRS broth under aerobic and anaerobic conditions. Tartrazine was not utilized in MRS broth in either aerobic or anaerobic conditions by *Lact. delbrueckii* (Fig. 1).

Utilization of other selected azo dyes by LAB

The ability of some LAB to modify other azo dyes such as methyl orange, acid orange 8 and acid orange 63 was tested. Similar to tartrazine, the three azo dyes were modified by the LAB tested (*Lact. plantarum*, *Lact. casei* LA1133 and *Lact. paracasei* LA0471) under anaerobic conditions (Table 2). No utilization of azo dyes was observed under aerobic conditions (data not shown).

Evaluation of azo dyes as an energy source for LAB

Even though, *Lact. plantarum*, *Lact. casei* LA1133 and *Lact. paracasei* LA0471 were capable of utilizing the azo dyes tested, maximum cell numbers were not influenced by their presence in the culture medium (Fig. 2). Together these observations suggest that LAB modify azo dyes by a mechanism that does not generate metabolic energy for growth.

Identification of end products of tartrazine modification

Incubation of a buffer solution containing LAB and tartrazine under anaerobic conditions yielded colourless solutions. The colourless solutions turned to an intense purple colour when exposed to air. Two absorption peaks were observed in the oxidized product sample at 361 and 553 nm. These peaks were absent in the initial solutions containing either tartrazine or the colourless product (Fig. 3). Additionally, the absorption peak at 427 nm from tartrazine was missing after incubation with LAB. The product from tartrazine after incubation with *Lact. paracasei* LA0471 with absorption peaks at 361 and 553 nm was found to be 111 daltons larger than tartrazine. The MS-MS results suggested that (i) the modification is not simply an addition of an alkyl, aryl, or a glucosyl moiety, (ii) at least one of the sulfate moieties remains, likely both and (ii) the carboxylic acid group is still present in the product. However, it has not yet been possible to determine the structure of the product derived from tartrazine.

Discussion

Degradation of azo dyes by micro-organisms has been extensively documented. Azo dyes can be degraded by lignin-degrading fungi, white rot fungi, bacterial peroxidases, aerobic and anaerobic bacteria possessing azoreductases, and anaerobic bacteria capable of producing reduced flavins and hydroquinones (Stolz 2001; Cheng 2006). Bacterial biodegradation of azo dyes is often initiated by cleavage of azo bonds by azoreductases which are followed by the aerobic degradation of the resulting amines (Stolz 2001). Fungal degradation of azo dyes mainly occurs from the lignin peroxidase activity under aerobic conditions (Cheng 2006). Some of the amines produced from these types of reaction are potentially carcinogenic. However, this is the first report that shows formation of a product from the microbial metabolism of an azo dye with a molecular mass greater than that of the parent compound. Anabolism of azo dyes by LAB does not appear to be an energy deriving mechanism and primarily occurs under anaerobic conditions.

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