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ISOLATION AND IDENTIFICATION OF AN ANTIMICROBIAL SUBSTANCE PRODUCING LACTOBACILLI

ANTİMİKROBİYEL MADDE ÜRETEN BİR LACTOBACİLLİ'NİN İZOLE VE TEŞHİS EDİLMESİ

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ABSTRACT: In this study, an antimicrobial substance producing lactobacilli was isolated from a commercially fermented vegetable product and identified by using the general microbiological tests, carbohydrate fermentation (Strep-API-20, API-50-CHL and BioLog) and fatty acid profile (MIDI) identification systems. The strains were gram-positive, catalase negative, short rods with rounded ends, occuring singly and in short chains. They did not produce $\alpha^-$and β^- hemolysis on blood agar. They hydrolysed esculine and arginine, but not gelatin and starch. They grew between 15° and 40°C, at pH 4.5-9.2 and in the presence of 3-4% NaCl, but not at 45°C, pH 9.6 or 6.% NaCl. In addition, they fermented melezitose, arabinose, maltose, ribose, and melibiose whereas tehy did not ferment mannitol, mannose, and cellobiose. The major fatty acids found in the isolates were 16:0 (25.55%) and 18:1 (37:33%). Based on the results, they were identified as *Lactobacillus buchneri* LB. Furthermore, it was found that this isolate produced an antimicrobial substance which was inhibitory to some *Listeria, Bacillus, Enterococcus, Lactobacillus* and *Micrococcus* species. This antimicrobial substance was a bacteriocin and it was designated buchericin LB.

ÖZET: Bu araştırmada antimikrobiyal madde üreten bir lactobacilli ticari bir fermente sebze ürününden izole edilmiş ve genel mikrobiyolojik testler, karbonhidrat fermentasyonu (Strep-API-20, API-50-CHL ve BioLog) ve yağ asitleri (MIDI) sistemleri kullanılarak teşhis edilmiştir. Suşlar gram-pozitif, katalaz negatif, uçları yuvarlak kısa çubuklar şeklinde olup tek ve kısa zincirler şeklinde bulunmaktadırlar. Eskulin ve arjinini hidrolize etmelerine karşın jelatin ve nişastayı hidrolize edememektedirler. 15° ve 40°C'ler arasında, pH 4.5-9.2 ve %3-4 oranında NaCl varlığında gelişebilmelerine rağmen 45°C, pH 9.6 veya %6.5 NaCl'de gelişememektedirler. İlaveten, melezitoz, arabinoz, maltoz, riboz ve melibioz'u fermente edebilmelerine karşın mannitol, mannoz ve cellobioz'u fermente edememektedirler. İzolatta bulunan başlıca yağ asitleri 16:0 (%25.55) ve 18:1 (%37.33)'dır. Sonuçlara bağlı olarak bu izolat, *Lactobacillus buchneri* LB olarak tesbit edilmitir. Ayrıca bu izolatın bazı *Listeria, Bacillus, Enterococcus, Lactobacillus* ve *Micrococcus* türlerine karşı inhibitör etkiye sahip olan bir antimikrobiyal madde ürettiği belirlenmiştir. Bu antimikrobiyal madde bir bakteriyosin olup buchnericin LB olarak adlandırılmıştır.

INTRODUCTION

Lactic acid bateria (LAB) are industrally important a group of gram-positive organisms since they are recognized for their fermentative ability, their health and nutritional benefits (GILLIAND 1990, LINDGREN and DOBROGOZ 1990, SANDINE 1979, 1990). The boundries of the group have been subjected to some controversy, but there has been general aggrement that the genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* form the core of the group. Recent taxonomic revisions of these genera suggest that the LAB compromise the following: *Aerococus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus Streptococcus, Tetragenococcus* and *Vagococcus* (AXELSON 1993). The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Additional characteristics such as fatty acid composition and motility are used in classification. New tools such as nucleic acid probing techniques, partial rRNA gene sequencing using the polymerase chain reaction, and soluble protein patterns for classification and identification of LAB are underway.

Species used for food fermentations belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostocs, Lactobacillus,* and *Carnobacterium.* These organisms have been isolated from grains, green plants, dairy and meat products, fermented vegetables, and the mucosal surfaces of animals (LINDGREN and DOBROGOZ 1990).

The genus *Lactobacillus* is by far the largest of the genera included in LAB. It is very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. Lactobacilli are widespread in nature, and many species have found aplications in food industry. They are generally the most acid-tolerant of the LAB (KASHKET 1987) and will, therefore, terminate many spontaneous lactic fermentations such as silage and vegetable fermentations. Their ability to produce organic acids such as lactic and acetic acid, hydrogen peroxide, or alcohols can contribute towards the overall preservative potential of fermented food products (LINDGREN and DOBROGOZ 1990). In addition, lactobacilli can synthesize and excrete inhibitory compounds of a proteinaceous nature known as bacteriocins (TAGG et al. 1976). The objectives of this study were to isolate and identify an antimicrobial substance producing lactobacilli from a commercially fermented vegetable product.

MATERIALS and METHODS

Detection of Antagonistic Activity

Samples aseptically obtained from a commercially fermented vegetable product were put into de Mann Rogossa Sharpe (MRS) broth (Difco) and they were incubated at 30°C for 24 h. For detection of antagonistic activities, an agar spot test and the Sandwich overlay method were used (MAYR-HARTING et al. 1972). For the Sandwich overlay method, the bacterial growth in MRS broth was diluted, plated on MRS agar and grown at 30°C for 24 h. Approximatelly $5x10^8$ cells of the indicator strains were inoculated onto 5 ml of an appropriate soft agar (MRS or BHI, 0.8% w/v agar) and poured over the plate onto which producer organism had been grown. Incubation temperatures were 30° or 37°C depending on the idicator strains. After incubation for 24 h, the plates were checked for inhibitory zones. Inhibition was scored positive if the zones were wider than 2 mm.

For the aga, spot test, colonies showing inhibition zones against indicator organisms were collected and their pure culture was prepared. These pure cultures were inoculated into MRS broth and incubated overnight at 30°C. Cells were then removed by centrifugation (10, 000 x g for 15 min) and the supernatant fluid filtered through a 0.22µm pore-size filter (imlipore), adjusted to pH 6.0 with 5 mol l^{-1} NaOH, and treated with 1 mg/ml catalase at 37°C for 1 h.I The treated cell-free supernatant fluids (10 µl) were spotted onto the surface of MRS or BHI agar plates which had been overlaid with indicator organisms in 5 ml of soft agar (MRS or BHI). Afterthat, these plates were incubated for 24 h at 30° or 37°C depending on the indicator organisms. The plates were checked for inhibition zones (YILDIRIM and JOHNSON 1998a, b). The indicator organisms used were *Lactobacillus plantarum* NCDO 955, *Listeria monocytogenes* ATCC 11775, *Salmonella thyphimurium* ATCC 14028, *Enterococcus faceium* Dan Fung- *Leuconostoc oenos* UMRL and *Micrococcus luteus* ATCC 4698.

Identification of Bacteriocin Producing Strains

Colonies showing inhibitory activity against indicator organisms were collected and isolated by using general microbiological cultivation methods. For identification, these isolated strains were tested for gram-stain, their ability to grow at 15°-45°C, at 4.5-9.6 pH or at the presence of 3.0-6.5% NaCl, action on blood agar liquefication of gelatin and starch, motility, limiting pH in glucose broth, action on carbohydrates and related test substances. To determine their carbohydrate fermentation patterns, BioLog (GP microplate), Strep API-20 and API-50 CHL were used. In addition, their fatty acid patterns were determined by using MIDI fatty acid profile identification system. The isolated strains were maintained in MRS broth with 20% glycerol at-70°C.

RESULTS and DISCUSSION

Colonies showing inhibition against indicator organisms (Figure 1) were isolated from a commercially fermented vegetable product by using Sandwich overlay method (MAYR-HARTING et al. 1972). Cells of these strains were short rods with rounded ends, occuring singly and in short chains. They were gram-positive, saccharolactic, catalase negative, nonpigmented, non-sporforming, and non-motile. They did not produce α- and β-hemolysis on blood agar. The strains hydrolysed both esculine and arginine. However, they did not hydrolyse gelatin and starch, and not digest casein. They were able to grow beteen 15° and 40°C and at pH 4.59.2, but not at 45°C or pH 9.6. In addition, they grew in the presence of 3 and 4% NaCl, but not 6.5% NaCl (Table 1). Final pH in the glucose broth was between 3.9-4.0 Carbohydrate fermentation patterns of the isolates were determined using Strep-API-20, API-50CHL and BioLog. As can be seen in Table 2, they fermented arabinose, fructose, galactose, glucose, gluconate, lactose, maltose, melezitose, melibiose, raffinose, ribose, xylose, saccharose, but not amygdalin, cellobiose, mannitol, mannose, rhamnose, salicin, sorbitol, sucrose, threhalose, and inuline. Also, they formed gas from glucose and gluconate.

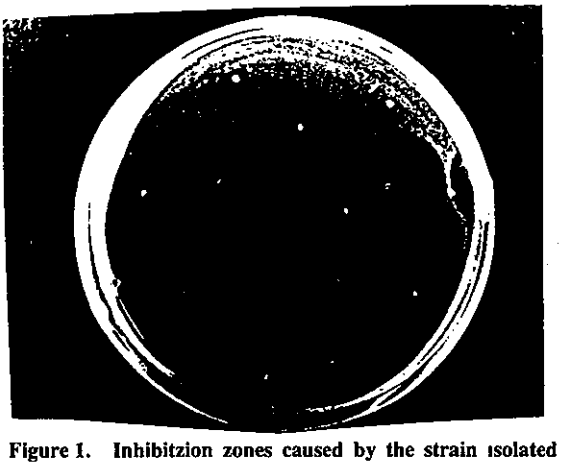

Figure 1. Inhibitzion zones caused by the strain isolated from a Commercial Fermented Vegetable Product against *Lactobacillus plantarum*

Table 1. General Characteristics of the Strain Isolated from a Commercially Fermented Vegetable Product

Condition	Isolate	Condition	Isolate
15°C	+	pH 4.5	+
30°C	+	pH 6.0	+
40°C	+	pH 7.0	+
45°C	–	pH 8.0	+
50°C	–	pH 9.2	+
3% NaCl	+	pH 9.6	–
4% NaCl	+		
6.5 NaCl	–		

Symbols; +, positive; -, negative

Table 2. Patern of Fermented Carbohydrates of the Strain Isolated from a Commercially Fermented Vegetable Product

Carbohydrate	Isolate	Carbohydrate	Isolate
Amygdalin	–	Glycogen	–
D-arabinose	–	Inositol	–
L-arabinose	+	Inuline	–
Arbutine	–	Lactose	+
Adonitol	–	Maltose	+
Amidon	–	Mannitol	–
D-arabitol	–	Mannose	–
L-arabitol	–	α-methyl-mannoside	–
Cellobiose	–	Melezitose	+
Dulcitol	–	Melibiose	+
Esculine	+	D-raffinose	+
Erythritol	–	Rhamnose	–
D-fructose	+	Ribose	+
D-fucose	–	Salicin	–
L-fucose	–	Sorbitol	–
Galactose	+	L-sorbose	–
β-gentiobiose	–	Sucrose	+
D-glucose	+	D-tagatose	–
α-methyl-glucoside	+	Threhalose	–
Gluconate	+	D-trunose	–
2 ceto-gluconate	–	D-xylose	+
5 cote-gluconate	+	L-xylose	–
Glycerol	–	Xylitol	–

Sybols: +, positive; –, negative

Fatty acid profiles of the strains was shown in Table 3. The major fatty acids found in the isolate were straight chain 16:0 (25.55%), monounsaturated 18:1 (37.33%) and 15:0 iso 2OH (5.5%). Based on the results obtained from the general microbiologiöcal tests, Strep-API-20, API-50-CHL, BioLog and fatty acid profiles (MIDI) identification systems, the isolate was identified as *Lactobacillus buchneri* LB. *Lb. buchneri* is identical in almost all characteristics with *Lb. brevis,* except *Lb. buchneri* ferments melezitose,

Table 3. Fatty Acid Profile (MIDI) of the Strain Isolated from a Commercially Fermented Vegetable Product

Fatty acid	%
12:0	1.37
14:0	1.56
15:0	1.13
15:0 iso 2OH	5.49
16:0	25.55
16:1 w7c	2.08
17:0	1.22
18:0	1.27
18:1 w9c[a]	37.33
18:2 w6, 9c[b]	3.30
19:0 cyclo w 10c	3.00
20:1 w9c	2.08

[a]w7c refers to the single bond in 16:1 between 7 and 8 carbons

[b]w6,9c refers to the double bond in 18:2 between 6 and 7, and 9 and 10 carbons.

Differential chracteristics of the obligately heterofermentative *Lb. buchneri* are given as follows: it ferments melezitose, arabinose, maltose, ribose and melibiose, but not mannitol, mannose and cellobiose. It, also, grows at 15°C, but not at 45°C (KANDLER and WEIS 1986). It was reported that the major fatty acids in lactobacillus were straight chain saturated, monounsaturated and sometimes cyclo propane ring fatty acids (HOLT et al. 1994).

Cell-free-supernatant of *Lb. buchneri* LB was inhibitory to *some Lactobacillus, Listeria, Bacillus, Enterococus, Micrococcus* and *Leuconostoc* species, but not *E. coli,* and *Salmonella* species. This inhibitory activity was not due to acidity or H_2O_2 since treatment of cell-free supernatant with catalase or adjusment of pH to 6.0-7.0 did not resulted in any loss in the inhibitory activity. The antimicrobial substance, bacteriocin, was designated buchnericin LB. The physico-chemical characteristics, molecular weight, stability and purification of buchnericin LB have been written and will be published soon.

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