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ORIGINAL PAPER

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Studies on an inhibitor produced by lactic acid bacteria of wines on the control of malolactic fermentation

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Abstract Malolactic fermentation is the microbiological process in wines, where lactic acid bacteria (LAB) govern the process of converting L-malic acid into L-lactic acid. During this process a high microbial load of LAB may lead to an unwanted spoilage phenomena by formation of excessive amounts of undesirable flavor compounds. This study is mainly focused on the isolation of LAB from the native flora of the wine, which has an inhibitory potential against malolactic activity of LAB inherent in wines. An isolate of *Leuconostoc mesenteroides* subsp. *cremoris* was found to produce an inhibitory compound against the LAB of wines. This compound was found to be a bacteriocin-like inhibitory substance (BLIS), which has a molecular weight of 32,000 Da, and it was shown that this BLIS was effective in the control of malolactic fermentation.

Keywords Malolactic fermentation · Bacteriocin · Lactic acid bacteria · Wine · *Leuconostocs*

Introduction

Malolactic fermentation, which is a deacidification process achieved by the conversion of dicarboxylic malic acid to monocarboxylic lactic acid and operated by the lactic acid bacteria (LAB), represented by *Lactococci, Lactobacilli, Pediococcus* and *Leuconostocs*. These bacteria play an important role in governing the acidity and sensory characteristic of certain wines and are of paramount importance in winemaking, because of the influence of malolactic fermentation on the taste of wine. The malolactic fermentation may be advantageous for some wines but detrimental for others, depending on the style and composition of the wine. In some cases, malolactic fermentation is regarded as a spoilage activity, but under proper circumstances, either naturally expected or artificially encouraged, it is a normal part of good winemaking practice to be encouraged or desired. For high quality wines, it can give positive effects, like deacidification, bacteriological stabilization, and increased flavor and aroma complexity. If malolactic fermentation is not controlled, the concentration of compounds such as diacetyl and acetoin increase to a high extent in wine [1]. The same phenomena are even valid for histamine and tyramine [2, 3].

Until today, the control process of malolactic fermentation has been achieved by several methods, including SO_2 application, low temperatures, high concentrations of ethanol, sterile filtration, bacteriophages, lysozyme, heating of the wine and chemical inhibitors such as fumaric acid and dimethyl dicarbonate [4, 5, 6, 7] All of these methods are considered to be either harmful to consumers' health, ineffective or even to cause elimination of the native microflora of wine. One method of controlling malolactic fermentation is carried out by bacteriocins, which are small polypeptides produced by certain LAB inhibitory to other bacteria. Nisin is an example of these compounds and was used to inhibit malolactic fermentation completely and to eliminate LAB in wine [8, 9]. Although a variety of LAB have been reported to produce bacteriocins, to date few studies have been carried out either on bacteriocins produced by LAB of oenological origin, or on bacteriocins present in a finished wine [10, 11]

This study was mainly focused on the control of malolactic fermentation by carrying out a screening procedure for the potential inhibitory isolates from LAB of several Turkish wines. The highest inhibition-showing isolate was further investigated for inhibition activity and its active bacteriocin-like inhibitory substance (BLIS) was characterized and studied for its activity on malolactic fermentation.

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Materials and method

The survey was designed to detect the inhibitory potential inherent in LAB, isolated from two types of red and white wines, one belonging to wines produced in a 1998 lot (Type A) and the other produced in a 1999 lot (Type B), all obtained from Kavaklidere Wines and Vinteries Co. (Akyurt-Ankara Turkey). These lots of wines were obtained having had an incomplete alcoholic fermentation.

The isolation and characterization of LAB

A cross-inhibition procedure, by sterile toothpicks, was carried out in petri plates containing MRS agar (Oxoid) for 120 isolates. All incubation procedures were carried out at 30 °C for 24 h. Sixteen presumptive LAB isolates having inhibition zones were subjected to Gram staining, endospore staining, catalase reaction, heterofermentative/homofermentative distinguishing, temperature dependence and API CHL 50 kit identification tests. Any H_20_2-dependent inhibitory effect was ruled out by anaerobic incubation in an anaerobic jar equipped with a BBL Gas-Pak anaerobic system (Oxoid).

The preparation of the crude BLIS

A clear and potent inhibition zone former isolate W3 (*Leuconostoc mesenteroides* subsp. *cremoris*) was selected due to its highest inhibitory activity. The isolate was subcultured twice in MRS broth (Oxoid) (pH=6). The culture was incubated for up to 24 h at 30 °C and samples were taken at regular intervals in order to observe the maximum synthesis time of BLIS and to obtain the growth curve. Aliquots (50 ml) of subcultured W3 (*Leuconostoc mesenteroides* subsp. *cremoris*) were inoculated into 2 l MRS broth, grown at 30 °C, and pH=2 (where the production of BLIS was maximum) for 8 h in 5 l Erlenmeyer flasks and then subjected to centrifugation at 4,000 rpm for 20 min. The supernatant was filtered through 0.45 µm pore-size filters (MFS) to remove the remaining cells. The activity of the crude extract of BLIS was detected by transferring an aliquot of 5 µl crude BLIS onto the sterile filter paper discs placed on the seeded indicator lawn isolates of W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) and W7 (*Lactobacillus fructivorans*) on MRS agar (Oxoid).

The characterization assay of BLIS

The temperature effect on activity. The crude extract of BLIS obtained above, was subjected to temperatures of 60, 80, 100 and 120 °C. The activity was then measured by transferring an aliquot of 5 µl crude BLIS onto the sterile filter paper discs placed on the seeded indicator lawn isolates of W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) and W7 (*Lactobacillus fructivorans*) on MRS agar (Oxoid) plates. The filtered crude extract was also kept at 4 °C to observe storage stability of the crude extract with time.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed by a slab-type electrophoresis apparatus (Bio-Rad Protean). Freeze-dried BLIS (20 mg) was mixed with a sample buffer containing 20% glycerol (Merck), 1% bromophenol blue, 1.5% Tris base, and 10% β-mercaptoethanol. Samples of BLIS (20 µl) were then subjected to a 7.5% gradient gel using standard proteins [lysozyme (14,300 Da), lactoglobulin (18,400 Da), trypsinogen (24,000 Da), pepsin (34,700 Da), egg albumin (45,000 Da), bovine albumin (66,000 Da) from Sigma, St. Louis, Mo.] as molecular weight markers and stained with Coomassie blue R-250. The PAGE (without SDS) was performed by a disc-electrophoresis apparatus (Buchler) to confirm the activity of the band(s) obtained in PAGE [12, 13].

The malolactic fermentation assay procedure

The detection of malolactic conversion. The conversion of malic acid to lactic acid by the operative activity of LAB was detected by HPLC. For this purpose, an LKB-Bromma model 2150 HPLC, equipped with a differential refractometer (Knauer) and Shimadzu C-R4 Chromatopack monitor was used. The column used in this experiment was a Phenomenex Rezex Cal Monosaccharide column, with a size of 300×7.8 µm. The attenuation parameter was set to 2, flow rate was 0.45 ml/min and the oven temperature was kept at 55 °C through the experiment. The diacetyl content produced by one of the isolates of LAB, responsible for malolactic conversion, W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) was detected by a gas chromatograph apparatus (Chrompack) equipped with a Porapak-Q column with a mesh size of 50–80 mm. The injection temperature ranged between 200 °C and 220 °C. Consequently the growth of the LAB in the medium was monitored by a spectrophotometer at OD_{600} (Pharmacia) and the pH of the medium was checked for each sample by a Jenway pH meter.

Effect of crude BLIS on the growth and malolactic activity of selected isolates. In order to monitor malolactic conversion a model medium consisting of 0.2 g/100 ml malic acid (Merck), 10 µM NAD^+ (Merck), 100 µM $MnSO_4$ (Panreac), 80 ml sterile MRS broth and 10 ml grape juice was prepared and a 10% inoculum of each isolate(W6-*Lactobacillus delbrueckii* subsp. *delbrueckii* and W7-*Lactobacillus fructivorans*), was added into the model medium. Samples (5 ml) from this growth media were taken and filter sterilized using 0.45 µm bacteriological filters (MFS) prior to the chromatographical assays. The effect of BLIS and nisin (Sigma) were studied by the addition of 100 IU of nisin (SIGMA) and 0.1 ml of 50 mg freeze-dried crude BLIS extract/ml. Growth was monitored by OD measurements at 600 nm.

Results and discussion

Isolation of LAB from wine

In order to find a potent inhibitor producer isolate to control the malolactic fermentation, a total number of 120 different isolates were screened. All isolates were subjected to cross-inhibition tests by the sterile toothpicks method. The results revealed that the zone-former isolates exhibited inhibition efficiencies in the range 5–41% on indicator lawn isolates. Sixteen isolates with inhibition activities were subjected to Gram-staining, endospore staining, catalase, temperature dependence tests, and subsequently, evaluation by API CH 50 kits. Tests revealed three different presumptive LAB, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactobacillus fructivorans*. One of the isolates, W3 (*Leuconostoc mesenteroides* subsp. *cremoris)* was selected for further investigation as it had shown the highest inhibitory activity among the sixteen presumptive LAB isolates.

The production of BLIS from the selected isolate W3 (*Leuconostoc mesenteroides* subsp. *cremoris*)

The optimal synthesis of the BLIS was found to occur in 8 h when W3(*Leuconostoc mesenteroides* subsp. *cremoris*) was grown at 30 °C and pH 2. The presence of in-

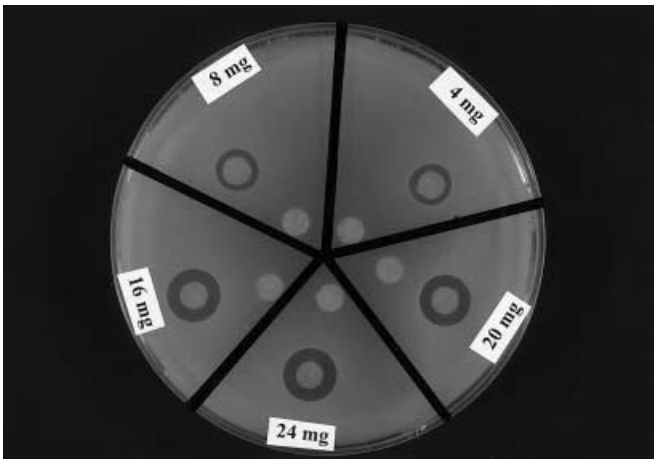

Fig. 1 The effect of freeze-dried, bacteriocin-like inhibitory substance (BLIS), produced by W3 (*Leuconostoc mesenteroides* subsp. *cremoris*) at different concentrations (milligrams/milliliter) on the seeded lawn of W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*), grown at 30 °C for 24 h on MRS agar (Oxoid)

hibitory activity was shown by filter paper disc assay after microfiltration, on the growth of the isolate W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) (Fig. 1).

The characterization of BLIS

Temperature, pH and storage stability assays. The crude extract of the BLIS was subjected to temperatures of 60, 80, 100 and 120 °C. The BLIS was found to be minimally affected by heating for 20 min at 120 °C, indicating the active substance to be heat stable. pH stability was consistent with properties reported for the bacteriocins produced by other LAB such as pediocin PA-1 [14] and lactacin [15]. It has also been confirmed that the crude extract of the BLIS was stable at 4 °C for a period of 1 month; however, room temperature storage was not appropriate due to rapid loss of activity.

Polyacrylamide gel electrophoresis. PAGE was used to estimate the molecular mass of the BLIS. A Coomassie blue stained PAGE gel showed a single sharp band for the BLIS, and a sliced portion of the protein band corresponding to the single band obtained in the PAGE indicated a zone of inhibition on indicator lawn isolates of W6(*Lactobacillus delbrueckii* subsp. *delbrueckii*) and W7(*Lactobacillus fructivorans*). The molecular weight of the BLIS was found to be around 32,000 Da, which is a large enough molecular weight that BLIS can be categorized as a type III bacteriocin [5].

The control of malolactic fermentation with BLIS, nisin, and nisin-BLIS combination

The results have demonstrated that the isolate,W6 (*Lactobacillus delbreuckii* subsp. *delbreuckii*) is capable of

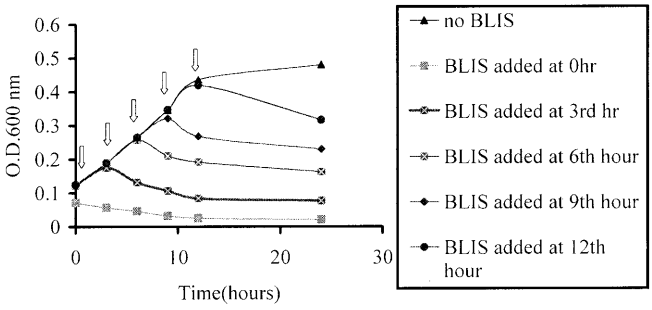

Fig. 2 The effect of *BLIS* addition at different time intervals on growth of a lactic acid bacteria (W6) (*Lactobacillus delbrueckii* subsp. *delbrueckii*) in MRS broth medium at 30 °C. *Arrows* indicate the time of inhibitor addition

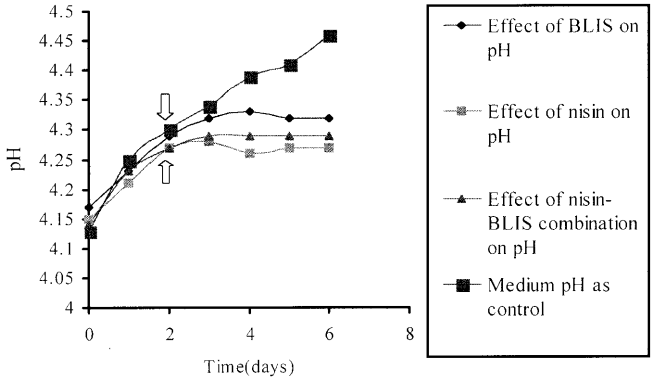

Fig. 3 The effect of nisin, BLIS and nisin-BLIS combination on the pH of the model wine medium (18 °C) bearing malolactic isolate W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*). All inhibitory substances were incorporated at the end of day 2. *Arrows* indicate the time of inhibitor addition

malolactic fermentation in the model wine medium. After the malolactic fermentation had begun, when nisin and BLIS, solely or in combination, was added into the medium, the lactic acid production from malic acid through the operative activity of the LAB was almost abolished. pH and growth analysis have demonstrated inhibition of growth and no change in pH after the addition of nisin and BLIS, solely or in combination. When BLIS was added into the growth medium at different time intervals, it was observed that W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) was affected by the addition of the BLIS, resulting in a sharp decline in growth (Fig. 2).

The activity of BLIS isolated from W3 (*Leuconostoc mesenteroides* subsp. *cremoris*) on malolactic conversion was shown by addition of the BLIS at different growth stages of the isolate W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) in the model medium. It is apparent that BLIS and nisin, or their combination, have a detrimental inhibitory activity on the growth, and as a consequence on the malolactic fermentation at desired stages of the growth (Table 1). This effect of BLIS can also be seen by the leveling of pH change after the addition of the BLIS into the growth media (Fig. 3). These results

Table 1 Lactic acid and malic acid content of the model wine medium after addition of nisin, bacteriocin-like inhibitory substance (*BLIS*) and nisin-BLIS combination, at the 2nd day of malolactic fermentation, conducted by W6 (*Lactobacillus delbrueckii* subsp. *delbrueckii*) in model wine medium

Applications	Malic acid in the medium %				Lactic acid in the medium %			
	Day 0	Day 2	Day 4	Day 6	Day 0	Day 2	Day 4	Day 6
Nisin	100	73	70	70	0	27	30	30
BLIS	100	77	74	70	0	23	26	30
BLIS-nisin combination	100	75	73	74	0	25	27	26
Control	100	74	34	8	0	26	66	92

show that the BLIS produced by W3 (*Leuconostoc mesenteroides* subsp. *cremoris*) has a potential for controlling the malolactic activity. Its effect, like nisin, is directly on the cell growth; consequently there is inhibition of the malolactic conversion and changes in the wine pH.

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