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ANTIMICROBIAL POTENTIAL OF TRITICALE STILLAGE AFTER LACTIC ACID FERMENTATION WITH *Lactobacillus fermentum* PL-1

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This study is concerned with the testing of antimicrobial activity of triticale stillage obtained after lactic fermentation by Lactobacillus fermentum PL-1. The antimicrobial tests were performed using the disc-diffusion and agar well diffusion methods. It was found that fermented triticale stillage after lactic acid fermentation exhibited an inhibitory effect towards tested Gram positive and Gram negative bacteria: Escherichia coli, Salmonella enteritidis, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, and Enterococcus faecalis. The triticale stillage without addition of $CaCO_3$ before fermentation showed a stronger antimicrobial effect in comparison with the triticale stillage with added $CaCO_3$. Triticale stillage after lactic acid fermentation did not show any antifungal effect on the growth of tested moulds (Alternaria alternata, Aspergillus versicolor, Penicillium brevicompactum, and Fusarium subglutinans).

KEY WORDS: triticale stillage, lactic acid fermentation, antimicrobial activity

INTRODUCTION

Stillage is one of the major by-products of bioethanol production, alongside carbon dioxide. An average stillage amount produced in the bioethanol process is approximately 13 hL per hL of bioethanol (1). The high amount of stillage produced after distillation of bioethanol can cause a serious environmental problem, taking into account the products' chemical complexity, and high BOD_5 values of stillage which ranges from 15 to 340 g/L (2). Its storage represents a big ecological problem in the industrial facilities. The high costs of treatment prior to disposal into watercourses seriously affect the viability and profitability of the process. The stillage, on the other hand, can be used as an inexpensive feedstock and it can be a valuable source of nutrients for the growth of lactic acid bacteria (LAB) (3). This idea is supported by the world's growing demand for lactic acid due to its versatile and increasing utilization in pharmaceutical, food and chemical industry (4). There are several recent studies dealing with the utilization of thin corn and triticale stillage for this purpose (5-8).

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It is known that some LAB species, especially members of the *Lactobacillus* and *Bifidobacterium* genus, possess probiotic qualities which positively influence the health of humans and domestic animals. These bacteria, apart from lactic acid, which is biosynthesised by the substrate fermentation in the process of lactic acid production, also produce biocins and other antimicrobial substances that are active against microorganisms that can cause food spoilage and food-borne illnesses (9-14). It is well known that the addition of probiotics to feedstock (15) can increase the usage of antibiotics, which have a proven negative effect due to the fact that they are responsible for bacterial resistance.

The goal of this study was to test the antimicrobial activity of triticale stillage after lactic acid fermentation by *Lactobacillus fermentum* PL-1.

EXPERIMENTAL

Lactic acid fermentation

The stillage obtained after bioethanol fermentation was used as a medium for the lactic acid fermentation. The fermentation process was carried out at two different temperatures (30 and 37°C) with or without the addition of $CaCO_3$, used as a neutralising factor for lactic acid. The distillation of the bioethanol fermentation medium produced stillage as the main by-product. To adjust the pH (~ 6.0), 1M NaOH was added to triticale stillage before the enzymatic hydrolysis. The enzymatic hydrolysis was conducted in the automated mashing water bath (Glasbläserei, Institut für Gärungs Gewerbe, Germany) using the following enzymes: Termamyl 120L (30 min at 85°C), SAN Super 240L (30 min at 55°C), and Celluclast 1.5 L (30 min at 45°C). After the hydrolysis, the samples were cooled to 20°C and centrifuged (C-28A, BOECO, Germany) at 4000 rpm for 20 min. The supernatant was taken, sterilized by tyndallization (3 days at 100°C for 30 min), and used as a medium for the lactic acid fermentation. The triticale stillage was inoculated with a homogeneous strain suspension (~10^8 cfu/mL) of *Lactobacillus fermentum* PL-1 (isolated from chesse and maintained on MRS broth (HiMedia, Mumbai, India) with 20% glycerol at -20°C as a part of the collection of the Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, Belgrade)), with 3.33 mL of the inoculum/100 mL of triticale stillage. The *Lactobacillus* strain was double subcultured. The first subculturing was the incubation on MRS agar (HiMedia, Mumbai, India) slants at 30°C for 24 hours, and after that, the second subculturing was the incubation in MRS broth under the same conditions. The inoculated triticale stillage was divided into two parts. $CaCO_3$ was added to the one part (0.58 g/100 mL of stillage), according to Đukić-Vuković et al. (8). The inoculated stillage (20 mL) was transferred to 50 mL Erlenmeyer flasks which were placed in the AQUATHERM® water bath shaker (USA), and incubated at 150 rpm and 30°C for 72 hours.

After completing the lactic acid fermentation, the triticale stillage was centrifuged at 3000 rpm for 15 minutes. The supernatant was filtered through a sterile filter (pore size of 0.2 μm) (Chromafil® CA–20/25S) and was used for the determination of antimicrobial activity, pH value, number of *L. fermentum* PL-1 viable cells, and lactic acid content.

Test microorganisms

The microorganisms used in the antimicrobial assay were bacteria and mould species. Four Gram-positive bacteria have been tested: *Staphylococcus aureus* ATCC 252923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, and *Bacillus cereus* ATCC 10876, and three Gram-negative: *Escherichia coli* ATCC 8739, *Salmonella enteritidis* ATCC 13076 and *Pseudomonas aeruginosa* ATCC 27853. The test moulds were: *Alternaria alternata*, *Aspergillus versicolor*, *Penicillium brevicompactum*, and *Fusarium subglutinans*. The moulds were isolated from triticale stillage before the testing and identified according to the determination keys described by Klich (16), Samson et al. (17), Samson and Frisvard (18), Pitt and Hocking (19) and Leslie and Summerell (20). The isolated moulds were maintained on Czapek agar (Cz) (Merck, Darmstadt) (*Aspergillus* and *Penicillium* species) or Potato Dextrose Agar (PDA) (HiMedia, Mumbai, India) (*Alternaria* and *Fusarium* species) at 4^{o}C as a part of the collection of the Laboratory for Food Microbiology at the Faculty of Technology, University of Novi Sad, Serbia.

Preparation of spore suspensions of microorganisms

The suspension of bacteria was prepared in a saline solution that had a concentration of 10^5 cfu/mL, except for *B. subtilis* where the concentration of inoculated bacteria was 10^8 cfu/mL. McFarland‘s nephelerometer was used for the determination of the target concentration.

The suspension of the mould was prepared in a saline solution (9 mL) with the addition of 0.1% Tween 80. The suspension was filtered to remove any remaining hyphae or their parts, from the spores' suspension. The concentration of the mould spores in the suspension was determined by the haemocytometer until it reached a concentration of 10^5 conidia/mL.

Determination of antimicrobial activity

The determination of the inhibitory effect of the triticale stillage after lactic acid fermentation was carried out by the disc-diffusion (21) and agar well diffusion methods. The following media were used for these testings: Plate count agar (PCA) (LabM, Great Britain) for bacteria and Dichloran Glycerol Chloramphenicol agar (DRBC) (HiMedia, Mumbai, India) for moulds. The media were prepared according to the manufacturer’s instructions.

For the disc-diffusion method the prepared agars (approximately 12 mL of agar) were poured into Petri dishes (ϕ 90 mm) forming a 4 mm thick layer. A volume of 100 µL of suspended tested microorganisms was spread evenly on the top of the agar. Sterile paper discs with a diameter of 6 mm (Himedia, Mumbai, India), were placed on top of the agar and 15 µL of triticale stillage after lactic acid fermentation were placed on the disc surface. After 15 minutes at room temperature, the prepared Petri dishes were incubated at 30^{o}C or 37^{o}C, for 24 h for the antimicrobial testing of the bacteria and 25^{o}C, for 72 h for the mould testing. The following antibiotics were used as a positive control in the experiment: cefotaxime and clavulonic acid (30/10 mcg) (Himedia, Mumbai, India). The inhibition zone was measured in mm.

The prepared suspended test microorganisms (1 mL) were mixed with 21 mL of melted and cooled (approximately 45°C) PCA for bacteria and DRBC for moulds using the agar well diffusion method. After 15 minutes of cooling, wells (ϕ 9 mm diameter) were made in agar vith a glass tube and removed with a vacuum pump. The wells were filled with 100 μL of triticale stillage after lactic acid fermentation and left to settle for 2 h at room temperature. The Petri dishes were incubated in an upright position for 24 h at 30 and 37°C, depending on the tested bacteria. The moulds were incubated at 25°C, for 3-5 days. The inhibition zones were measured after the incubation and the results are obtained in mm.

Lactic acid solution was used as a model solution (1.57 g/100 mL) and the acid concentration was established in the fermented triticale stillage after 72 h of the fermentation process.

Statistical analysis

Excel 2010 was employed for the assessment of the average count and standard deviation. The results are presented as the average of the quadruplicate measurements ±SD.

RESULTS AND DISCUSSION

When the lactic acid fermentation was performed at 30°C and 37°C without the addition of $CaCO_3$, the pH at the end of the fermentation was 3.65 and 3.60, respectively. When $CaCO_3$ was added before the fermentation, at 30 and 37°C, the pH value was 4.90 and 4.80, respectively. The lactic acid content at the end of the fermentation without the presence of $CaCO_3$ was 1.57 g/100 mL and 1.50 g/100mL at 30°C and 37°C. When $CaCO_3$ was added before the fermentation, the lactic acid content at the end of the fermentation was 0.93 g/100 mL at 30°C and 0.92 g/100 mL at 37°C (Table 1).

Table 1. The pH value, total viable *Lactobacillus fermentum* PL-1 cells count and lactic acid content in the triticale stillage after 72 h of lactic acid fermentation

Sample	pH	*L. fermentum* **PL-1** (cfu/mL)	**Lactic acid content** (g/100mL)
Lactic acid fermentation without the addition of $CaCO_3$ at 30°C	3.65	123×10^6	1.57
Lactic acid fermentation without the addition of $CaCO_3$ at 37°C	3.60	1×10^6	1.50
Lactic acid fermentation with the addition of $CaCO_3$ at 30°C	4.90	87×10^6	0.93
Lactic acid fermentation with the addition of $CaCO_3$ at 37°C	4.80	70×10^6	0.92

The results obtained for the antimicrobial activity suggest that triticale stillage after lactic acid fermentation has an inhibitory effect on the bacterial growth (Tables 2 and 3).

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Table 2. Antimicrobial activity of the triticale stillage after lactic acid fermentation on the tested microorganisms, using disc-diffusion method

Microorganism	Lactic acid fermentation at 30°C		Lactic acid fermentation at 37°C		Control sample
	With the addition of $CaCO_3$	Without the addition of $CaCO_3$	With the addition of $CaCO_3$	Without the addition of $CaCO_3$	
	Inhibition zones (mm)				
E. coli	ND	ND	ND	ND	35.0
S. enteritidis	ND	ND	ND	ND	40.0
P. aeruginosa	ND	ND	ND	ND	18.5
B. subtilis	ND	8.66±0.57	ND	8.16±0.76	23.66±1.15
B. cereus	ND	ND	ND	ND	ND
S. aureus	ND	ND	ND	ND	ND
E. faecalis	ND	ND	ND	ND	ND
A. alternata	ND	ND	ND	ND	ND
A. versicolor	ND	ND	ND	ND	ND
P. brevicompactum	ND	ND	ND	ND	ND
F. subglutinans	ND	ND	ND	ND	ND

ND – inhibiotion zone not determined

Table 3. Antimicrobial activity of the triticale stillage after lactic acid fermentation on the microorganisms tested using the agar well diffusion method

Microorganism	Fermentation at 30°C		Fermentation at 37°C		Model solution
	with the addition of $CaCO_3$	without the addition of $CaCO_3$	with the addition of $CaCO_3$	without the addition of $CaCO_3$	
	Inhibition zones (mm)				
E. coli	ND	14.25±0.95	ND	14.75±0.5	11.75±0.5
S. enteritidis	ND	14.50±1.29	ND	14.75±0.5	11.5±0.57
P. aeruginosa	ND	14.75±0.95	ND	14.75±0.5	11.00±1.0
B. subtilis	ND	15.00±0.81	ND	15.50±0.58	13.5±0.58
B. cereus	ND	11.0±0.00	ND	11.0±0.00	ND
S. aureus	ND	ND	ND	12.75±1.25	ND
E. faecalis	11.50±0.58	14.75±0.95	11.0±0.00	14.75±0.5	13.0±0.81
A. alternata	ND	ND	ND	ND	ND
A. versicolor	ND	ND	ND	ND	ND
P. brevicompactum	ND	ND	ND	ND	ND
F. subglutinans	ND	ND	ND	ND	ND

ND – inhibiotion zone not determined

The inhibitory effect depends on the application of $CaCO_3$ during fermentation, as well as on the applied methods for testing antimicrobial activity.

When the disc-diffusion method was applied for the determination of antimicrobial activity, in the fermentation of the medium to which $CaCO_3$ was not added, the triticale stillage after lactic acid fermentation had an inhibitory effect on the growth of *B. subtilis* (Table 2). However, when the agar well diffusion method was applied, the triticale stillage after lactic acid fermentation involving $CaCO_3$ had also an inhibitory effect on the

growth of *E. coli*, *S. aureus*, *Bacillus* spp., *E. faecalis*, and *P. aeruginosa* (Table 3). This result was expected, considering that the agar well diffusion method requires a greater amount of sample (100 μL) compared to the disc-diffusion method (15 μL).

The strongest antimicrobial effect of triticale stillage after lactic acid fermentation obtained in the absence of $CaCO_3$ was determined for *B. subtilis*. The triticale stillage after lactic acid fermentation carried out at 37°C and without the addition of $CaCO_3$ showed an inhibitory effect on *S. aureus* (Table 3).

The antibacterial activity of the tested triticale stillage after lactic acid fermentation is attributed to the action of the antimicrobial products of *L. fermentum* PL-1, produced in the lactic acid fermentation process such as, lactic acid, acetic acid, fatty acids, protein compounds, peptides, etc. (22).

Other researchers have also pointed out the antibacterial activity of *L. fermentum* and other LAB (13, 23-27). Savadogo et al. (23) indicated that *L. fermentum* had the strongest inhibiting effect on the growth of *E. faecalis*. Also, Belal et al. (27) mentioned a strong antimicrobial effect of *L. fermentum* Te1007 on *B. subtilis*, *E. coli, S. aureus*, and a moderate effect on *E. aerogenes, Staphylococcus epidermidis, S. thyphimurium, Shigella sonei,* and *Klebsiealla pneumonia*. However, Pascual et al. (28) reported that *L. fermentum* L23 did not have an inhibitory effect on *E. coli*.

As it is shown in Table 3, a stronger inhibitory effect on tested microorganisms was achieved when the lactic acid fermentation was carried out without the addition of $CaCO_3$ at both investigated temperatures. This could be explained by the fact that produced lactic acid binds in Ca-lactate when the fermentation is conducted in the presence of $CaCO_3$. This leads to a reduction of lactic acid content in the fermentation, and hence to a lower antimicrobial effect.

Triticale stillage after lactic acid fermentation did not show any antifungal effect on the tested fungi (Tables 2 and 3), probably due to the low lactic acid content, as well as to the fact that the studied fungi are more resistant in comparison to the bacteria. In contrast to the obtained results, other researchers reported a strong antifungal activity of lactic acid bacteria (LAB). De Muynck et al. (29) found that LAB, including *L. fermentum,* showed antifungal activity towards *Aspergillus flavus*, *Penicillium* spp., and *Rhizopus oryzae*. These authors suggested that the antifungal effect depends on the lactic acid content. Magnuson et al. (30), Rouse et al. (31) and Gerez et al. (32) also indicated an antifungal activity of cyclopeptides biosynthesised by LAB, including *L. fermentum* on *Aspergillus fumigatus*, *A. nidulans, Penicillium commmune, P. digitatum, Fusarium sporotrichoides, F. graminearum*, and *F. oxysporum*. Belal et al. (33) reported on the synergistic effect of *L. fermentum* Te007 and *L. pentosus* G004 in the inhibition of the growth of *Aspergillus niger* and *A. oryzae*. These authors also reported that the antifungal components of these bacteria were thermostable at 90 and 120°C and at a pH from 3 to 7, which suggests that they can be applied in the production of heat-treated food as an alternative to chemical preservatives.

CONCLUSION

It has been determined that fermented triticale stillage after lactic acid fermentation has an inhibitory effect towards tested Gram positive and Gram negative bacteria: *Esche-*

richia coli, Salmonella enteritidis, Pseudomonas aeruginosa, Bacillus cereus, B. subtilis, Staphylococcus aureus, and *Enterococcus faecalis*. Triticale stillage to which no $CaCO_3$ was added before the lactic acid fermentation showed a stronger antimicrobial effect in comparison with the triticale stillage with the addition of $CaCO_3$. Triticale stillage after lactic acid fermentation did not show any antifungal effect on the growth of tested moulds (*Alternaria alternata*, *Aspergillus versicolor*, *Penicillium brevicompactum*, and *Fusarium subglutinans*). Generally, triticale stillage after lactic acid fermentation, besides its primary role in the production of lactic acid, may have a role in preventing the growth of some pathogenic microorganisms.

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АНТИМИКРОБНИ ПОТЕНЦИЈАЛ ЏИБРЕ ТРИТИКАЛЕА ПОСЛЕ МЛЕЧНО-КИСЕЛЕ ФЕРМЕНТАЦИЈЕ ПОМОЋУ *Lactobacillus fermentum* PL-1

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У овом раду је испитана антимикробна активност џибре тритикалеа после млечно-киселе ферментације са *Lactobacillus fermentum* PL-1. Анитимикробна испитивања су изведена диск-дифузионом методом и методом бунарчића. Утврђено је да џибра тритикалеа после млечно-киселе ферментације има инхибиторни ефекат на

испитиване Грам-позитивне и Грам-негативне бактерије: *Escherichia coli, Salmonella enteritidis, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus* и *Enterococcus faecalis*. Џибра тритикалеа у коју није додат $CaCO_3$ пре млечно-киселе ферментације је имала јачи антимикробни ефекат од џибре у коју је додат $CaCO_3$ пре млечно-киселе ферментације. Џибра тритикалеа после млечно-киселе ферментације није имала антифунгални ефекат на раст испитиваних плесни (*Alternaria alternata*, *Aspergillus versicolor*, *Penicillium brevicompactum и Fusarium subglutinans*).

Кључне речи: џибра тритикалеа, млечно-кисела ферментација, антимикробна активност

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