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THE POSSIBILITY OF LACTIC ACID FERMENTATION IN THE TRITICALE STILLAGE

Triticale stillage is a by-product of bioethanol production. A research study was conducted in order to see if triticale stillage is adequate for lactic acid bacteria growth and lactic acid fermentation. Three Lactobacillus strains: Lactobacillus fermentum NRRL-B-75624, Lactobacillus fermentum PL-1, and Lactobacillus plantarum PL-4 were taken into consideration. Lactic acid fermentation was monitored by measuring pH value and titratable acidity. Lactobacillus fermentum PL-1 had the greatest decrease of pH values and increase of titratable acidity so it was chosen for future work. During the research, it was investigated how nutrient composition of triticale stillage and CaCO₃ can influence lactic acid fermentation and CaCO₃ role in cell protection. The nutrient composition of triticale stillage was satisfactory for lactic acid fermentation. The addition of CaCO₃ helped in lactic acid fermentation. Although the titratable acidity in the samples with CaCO₃ was lower than in the samples without CaCO₃, the number of viable cells was higher for the samples with CaCO₃, which showed that CaCO₃ protected lactic acid cells from inhibition by lactic acid.

Key words: triticale stillage; lactic acid; Lactobacillus sp.; CaCO₃.

As a result of the industrial development there has been an increase of energy consumption. Conventional energy resources, such as fossil fuels, cannot meet the increasing energy demand. The quantities of conventional energy resources are limited, and their use is one of the main reasons for global warming [1]. Increased public concern about global warming has led to the development of renewable and clean energy all over the world [2]. Bioethanol produced from renewable biomass such as starch, sugar or lignocellulosic materials is believed to be one of the solutions. Raw starch materials can be produced each year in required quantity and stored during the whole year giving high bioethanol yields [3]. The great importance of starch-rich resources is a possibility of using by-products from bioethanol production processes. The by-product remaining after fermentation and distillation is known as whole stillage [4]. Stillage is the main by-product originating in distilleries, and its volume is approximately 10 times that of the ethanol

produced [5]. Whole stillage might be screened or centrifuged to produce thin stillage and wet distillers' grains. Distillers' by-products have excellent energy and protein sources for ruminants [6]. In order to gain a by-product of good quality, a great number of plants were reconsidered as a resource for the production of bioethanol. Triticale was proved to be a good resource for the production of bioethanol [6].

Triticale is the first successful human-made cereal grain, made by crossing wheat and rye [7]. Triticale combines the best characteristics of both parents: wheat's qualities for making various products with rye's robustness for adaptability to difficult soils, drought tolerance, cold hardiness, disease resistance and low-input requirements. Triticale is known as a crop with higher test weight and as a variety that does not need as much fertilizer (nitrogen) as some other varieties providing the same yields [8]. Triticale does not contain considerable amounts of pentosans, therefore there are no problems regarding high viscosity [9]. Triticale is known for exhibiting a high autoamylolytic enzyme activity, and this characteristic provides processing triticale without using any or less additional saccharifying enzymes than for others grains. As for nutritional characteristics, it should be mentioned that modern triticale has high values in protein

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and essential amino acids, lysine in particular, which is usually the most limiting essential amino acid in typical pig diets [7]. Triticale is also shown a suitable, high-energy source for all classes of animals, better enzymatic digestion and digestive efficiency beyond the rumen in ruminant animals, as well as a reduced amount of protein supplementation needed in the diet for monogastric animals [10].

The great problem could be storing of stillage. One of the solutions is using lactic acid bacteria (LAB) as preservation [11]. The acidification of feed by microbial metabolism may reduce the emptying rate of the stomach and stimulate the secretion of proteolytic enzymes [12,13]. The proliferation of spoilage organisms and food-borne pathogens can be prevented by low pH and high concentrations of lactic and acetic acids. Lactic acid bacteria can be homo- and heterofermentative, but only the homo-fermentative LAB are available for the commercial production of lactic acid [14]. The most LAB used for commercial productions belong with the genus *Lactobacillus*.

The state-of-art is using LAB strains with probiotics benefits. Genus, which are currently being used in probiotic preparation are *Lactobacillus*, *Bifidobacterium* and *Streptococcus (Enterococcus)* [15].

During lactic acid fermentation, the inhibition appears with lactic acid. Undissociated lactic acid passes through the bacterial cytoplasmic membrane and dissociates inside the cell. The inhibition mechanism is probably related to the solubility of the undissociated lactic acid within the cytoplasmic membrane and the insolubility of dissociated lactate, which causes acidification of cytoplasm and failure of proton motive forces [16]. As the result of this process, there is a decrease in the amount of energy which is needed for the cell growth. This is the main reason for selectively (*in situ*) removing lactic acid from the fermentative medium. A good way of removing lactic acid is adding calcium carbonate.

Calcium carbonate plays a crucial role in lactic acid fermentation as it buffers the medium [17]. During the production of lactic acid, calcium carbonate is converted into calcium lactate and maintains the pH [15,17]. Although there are few different neutralizing agents, the results show calcium carbonate as the preferred choice [18].

Triticale stillage has several usages. It can be used as feedstuff (as a part of more traditional feedstuff) or as a fertilizer [9]. Also, stillage has a significant role in the recirculation process because it can be used instead of technical water in the bioethanol production [19].

This research had several goals. The first one was to see which strain of lactic acid bacterium is the best choice for the production of lactic acid with triticale stillage as the growth medium. The second goal was to find out how nutritive components of triticale stillage affect lactic acid fermentation. The third goal was to see if the addition of CaCO₃ in the triticale stillage improves lactic acid fermentation. The final goal was to see if the cells were protected by the addition of CaCO₃ and in which way the lactic acid fermentation affected the cell viability.

EXPERIMENTAL

Microorganisms

Three *Lactobacillus* strains: *Lactobacillus fermentum* NRRL-B-75624, *Lactobacillus fermentum* PL-1, *Lactobacillus plantarum* PL-4 (obtained from the chair of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, Belgrade) examined to evaluate their capacity for lactic acid fermentation. All these strains were stored on MRS agar slants (HiMedia, Mumbai, India) at 4 °C.

Medium for lactic acid fermentation

Stillage was made as the main by-product during the distillation of fermented mash. Bioethanol was released as the main product of distillation. The preparation of the bioethanol production can start in several different ways.

Triticale variety Odisej, received from the Institute of Field and Vegetable Crops, Novi Sad, Serbia, was used for the bioethanol production. Mash preparation for bioethanol fermentation was done by triticale autoamylolytic enzymes, technical enzymes (Termamyl and SAN Super 360L, purchased from Novozymes®), or ultrasound.

Table 1 shows in which way the mashes were pre-treated, and, if the samples were a mixture of different stillage samples, in which ratio they were mixed.

Table 1. The pre-treatment of triticale mashes before bioethanol fermentation (TAE - triticale autoamylolytic enzymes, TE - technical enzymes, US - ultrasound)

Number of stillage sample	Pre-treatment of mash before bioethanol fermentation	Ratio of mixed triticale stillage after distillation
1	TAE, TE, TAE + US	1:1:1
2	TAE	-
3	TAE, TE	1:1

After the bioethanol fermentation, mashes were distilled, bioethanol was realised, and as the main by-product, stillage was given.

For pH correction (≈ 6.0), 1 M NaOH was added to all triticale stillage samples before enzymatic hydrolysis.

Enzymatic hydrolysis of triticale stillage samples (1-3) was conducted in the automated mashing water bath (Glasbläserei, Institut für Gärungs Gewerbe, Berlin) with following enzymes (purchased from Novozymes®): Termamyl (85 °C with 30 min rest), SAN Super 360L (55 °C with 30 min rest) and Celluclast (45 °C with 30 min rest). At the end of hydrolysis, the temperature was decreased to room temperature.

Enzymatic-treated triticale stillages were centrifuged (C-28A, BOECO, Germany) at 4000 rpm for 20 min. Supernatants were taken, sterilized by tyndallization (3 days at 100 °C for 30 min), and used as a medium for lactic acid fermentation.

Glucose concentration correction in the hydrolyzed stillage samples was done by the addition of 2 % sterile glucose solution (filtrated through a sterile filter with pore size 0.22 μm , Chromafil® CA-20/25 S, Macherey-Nagel, Düren, Germany), in samples 2 and 3.

Sterilized triticale stillage samples 2 and 3 were divided in 2 parts. CaCO_3 was added to one part of each sample (0.58 g/100 mL stillage, according to the information that each mol of glucose gives 2 mol of lactic acid and each mol of CaCO_3 utilize 2 mol of lactic acid for the creation of lactate [20]).

Inoculation and fermentation

Strains were double subcultured. The first subculturing was the incubation on MRS agar slants at 30 °C for 24 h, and after that, the second subculturing was the incubation in MRS broth at the same conditions. Triticale stillage was inoculated by a homogeneous strain suspension, with 3.33 % (v/v) inoculum, and before that, glucose and CaCO_3 were added to triticale stillage samples. Inoculated triticale stillage samples (volume of 30 mL) were divided into 50 mL narrow neck Erlenmeyer flasks and/or standard test tubes (16 mm \times 160 mm), with the volume of 15 mL triticale stillage.

Lactic acid fermentation was conducted at 30 °C for 72 h in all experiments. Every 24 h, the samples of lactic acid fermentation were taken in doublets and the analyses were repeated twice.

Analytical methods

A chemical composition analysis of the average triticale stillage was done according to official methods of analysis, AOAC, Arlington, VA, USA [21].

HTPLC was done in order to see the amino acid content of used triticale stillage [22].

The HPLC analyses for the determination of maltose and glucose content in the stillage samples were performed on an Agilent 1100 Series HPLC system consisted of micro vacuum degasser, binary pump, thermostated column compartment, and RI detector. Column: Aminex HPX-87H (Biorad Laboratories), 7.8 mm ID \times 300 mm. Elution profile: 5 mM H_2SO_4 ; isocratic: the dosing volume was 20 μL ; flow rate: 0.6 mL/min; temperature: 50 °C.

pH Value was measured by an electronic pH meter (HI 9321, Hanna Instruments, Germany), double calibrated using 4.01 and 7.00 pH buffers.

Titrateable acidity (g/100mL) was measured, and calculated (multiplied with the factor for lactic acid) according to methods for determination of acidity [23,24].

The stillage sample 2 was used for viable cell count by pour plate method [25]. From chosen dilutions (10^{-7} , 10^{-8} and 10^{-9}) 1 mL of suspension was taken and transferred to a Petri dish. After that, MRS agar was transferred. With its solidification, MRS agar was transferred again, and in that way, a “sandwich” technique was done. Petri dishes were incubated at 30 °C for 72 h, with counting 2 samples of every dilution every 24 h.

After lactic acid fermentation (after 72 h), glucose and maltose quality detection was done by taking triticale stillage samples for silica gel thin-layer chromatography (TLC) [26].

RESULTS AND DISCUSSION

Chemical composition of the triticale samples

For our research it was necessary to see if there was, and in which amount, nutritional composition for lactic acid bacteria growth and metabolism. For this purpose triticale stillage which was pre-treated before bioethanol fermentation by triticale autoamylolytic enzymes, technical enzymes, and ultrasound (triticale stillage sample 1) was used.

Lactobacilli have very complex growth requirements regarding the content of sugar, proteins, amino acids, vitamins of the B complex and minerals such as Mg^{2+} , Mn^{2+} and Fe^{2+} [27]. Nutritional requirements are also seen for K^+ and PO_4^{3-} [28].

Table 2 shows the chemical composition of the observed stillage sample 1.

The results in Table 2 show the existence of several ions. All of them have an important role in lactic acid bacteria growth and metabolism. A great amount of phosphorus ions, as well as Mg^{2+} , Na^+ and

K⁺ was noticed. The amount of Se was the smallest, but it was still detectable.

Table 2. Chemical composition of stillage sample 1

Component	Result
Content of dry matter, %	21.21
Amount of proteins, %	7.25
Content of raw cellulose, %	1.77
Total invert, %	1.04
Content of phosphorus, %	0.11
Ca, mg/kg	1064.35
Mg, mg/kg	348.00
K, mg/kg	1188.00
Na, mg/kg	231.40
Cu, mg/kg	59.60
Zn, mg/kg	28.80
Fe, mg/kg	33.10

Amino acids are important for lactic acid fermentation [29]. HPTLC qualitative analysis was done to see which amino acids were present in triticale stillage samples. Lys, His, Gly or Ser, Val and Ile were detected by this method.

The HPLC analysis results for determination of maltose and glucose contents in triticale stillage samples after enzymatic hydrolysis are given in Table 3.

The HPLC analysis results for determination of maltose and glucose contents in the triticale stillage showed different values among the samples. The reason for this variation was the pre-treatment procedure for the mash before bioethanol fermentation. Triticale stillage sample 1 had a higher value for glucose than in other cases. This was due to its mixed origin. In this triticale stillage sample the stillages with triticale autoamylolytic enzymes, technical enzymes, and ultrasound pre-treatment before bioethanol fermentation were combined. Triticale stillage sample 2 was not pre-treated by technical enzymes before bioethanol fermentation, so the whole amount of glucose was utilized by yeast during bioethanol fermentation and during the stillage enzymatic hydrolysis sugar degradation was only to maltose. Triticale stillage sample 3 was triticale pre-treated by autoamylolytic enzymes and technical enzymes before bioethanol fermentation, so some

amounts of glucose and low amounts of maltose were detected.

In order to have the same glucose content in every triticale stillage, in triticale stillage samples 2 and 3, sterile glucose solution (2.5 mL of proper glucose solution/100 mL stillage) was added, without a significant change of the volume.

Strain screening

One of the aims of this experiment was screening of given *Lactobacilli* strains in order to see which strains are the most adequate for lactic acid fermentation on triticale stillage (sample 1). Preliminary research was done with several strains of *Lactobacillus* sp., with a variety of origin that were cultured on the stillage sample 1. All the strains with measured titratable acidity value less than 0.5 g/100 mL (during 72 h) were not taken in the future research.

The results for pH values and titratable acidity are shown in Figure 1.

The results for silica gel thin-layer chromatography in this experiment are given in Figure 2.

In this experiment the main aim was to choose the best strain for lactic acid fermentation on triticale stillage. The results of pH value and titratable acidity, as well as silica gel thin-layer chromatography, confirmed *L. fermentum* PL-1 as the most appropriate strain for inoculation of triticale stillage. The greatest fall of pH value during 72 h, and the highest titratable acidity (Figure 1) was determined in this sample. In Figure 2, the weakest lines are glucose and maltose for *L. fermentum* PL-1. According to this, the conclusion is that *L. fermentum* PL-1 utilized glucose, maltose, and maltotriose during the lactic acid fermentation. The other two strains did not utilize the whole amount of maltose during the lactic acid fermentation. The reason for successful lactic acid fermentation could be the existence of maltose [30].

Based on a decrease of pH values and the rise of titratable acidity, *L. fermentum* PL-1 was chosen for future work.

Effect of triticale stillage nutritive components on lactic acid fermentation

The autoamylolytic enzyme system of triticale is maximally active at 55-60 °C and at a pH of 5.0-5.8

Table 3. HPLC determination of glucose and maltose in the stillage samples after enzymatic hydrolysis (TAE - triticale autoamylolytic enzymes, TE - technical enzymes, US - ultrasound)

Number of stillage sample	Stillage source (pre-treatment before bioethanol fermentation)	Glucose, g/100 mL	Maltose, g/100 mL
1	TAE, TE, TAE + US	2.00	0.24
2	TAE	0.00	1.86
3	TAE, TE	0.29	0.21

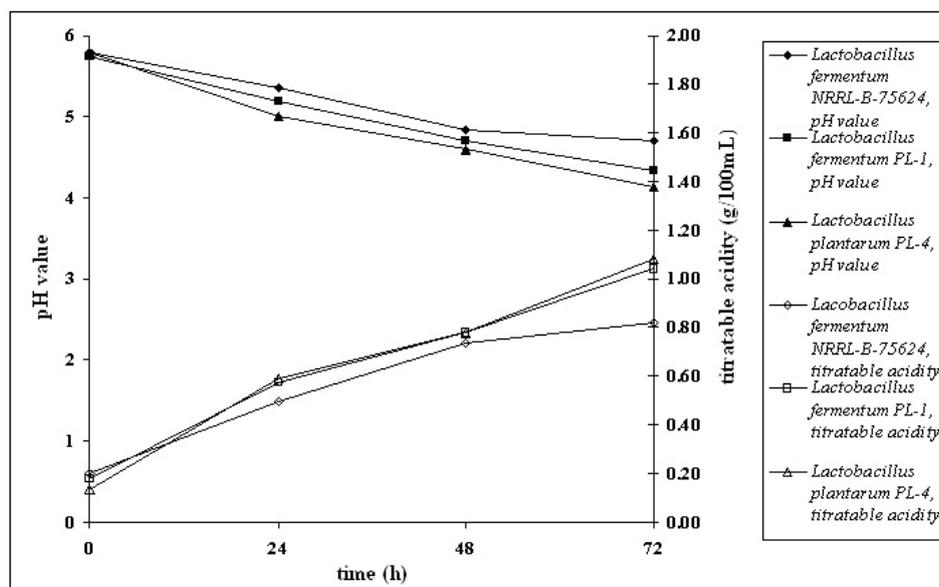


Figure 1. pH Change and titratable acidity during lactic acid fermentation period.

[9]. Starch degradation by autoamylolysis is very different from starch degradation by the action of technical enzyme preparation. Triticale mashes (autoamylolytically processed) can have a fermentable sugar content of about 80 g/L mash [9].

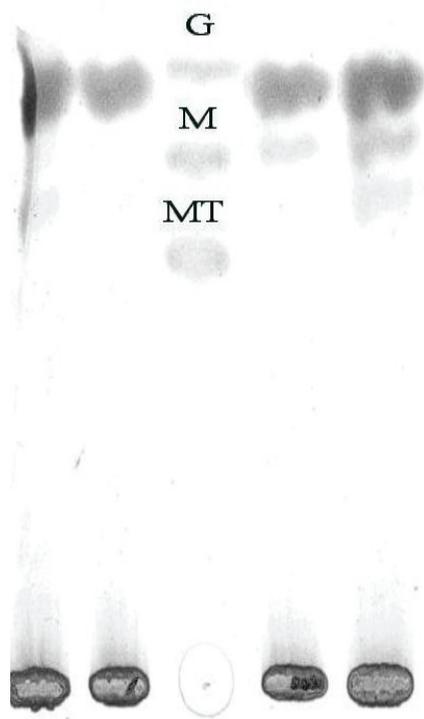


Figure 2. Results for silica gel thin-layer chromatography. From left to right: *Lactobacillus fermentum* NRRL-B-75624, *L. fermentum* PL-1, standard solution ((G) glucose + (M) maltose + (MT) maltotriose), 1 mg/mL, *L. plantarum* PL-4, fermentation medium (stillage sample 1).

The main observation point was to see if triticale stillage (sample 2) is a good medium for lactic acid fermentation, as well as if it has enough biogenic nutritive components for lactic acid fermentation. This was also done for triticale stillage sample 3. The reason for this kind of research was to see if there was any difference (as a medium with satisfactory nutritive components for lactic acid fermentation) between triticale stillage (from distillation of fermented mash, as the result of bioethanol releasing; mash was pre-treated before bioethanol fermentation by triticale autoamylolytic enzymes) sample 2 and triticale stillage made by mixing 2 triticale stillages (from distillation of fermented mash, as the result of bioethanol releasing; mashes were pre-treated differently before bioethanol fermentation, one by triticale autoamylolytic enzymes and another one by technical enzymes), sample 3.

Fermentations were done in doublets with 2 different types of vessel, test tubes and Erlenmeyer flasks.

Figure 3 shows pH values and titratable acidity during lactic acid fermentation without the addition of CaCO_3 (triticale stillage sample 2).

Figure 4 shows pH values and titratable acidity during lactic acid fermentation with the addition of CaCO_3 (triticale stillage sample 2).

A decrease of pH value was the same in the samples without CaCO_3 , while pH value was lower in test tubes in samples with CaCO_3 . Higher titratable acidity was achieved in the test tubes, with or without CaCO_3 (Figures 3 and 4). This was due to the geometry of the vessel. Standard test tubes have cylindrical geometry which gives smaller surface exposed

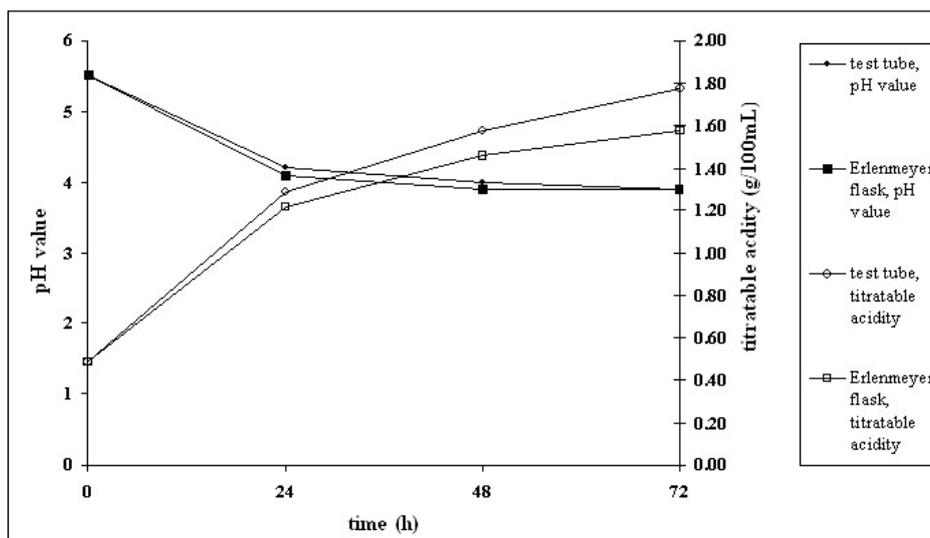


Figure 3. pH Values and titratable acidity during lactic acid fermentation without the addition of CaCO_3 for *Lactobacillus fermentum* PL-1 (sample 2).

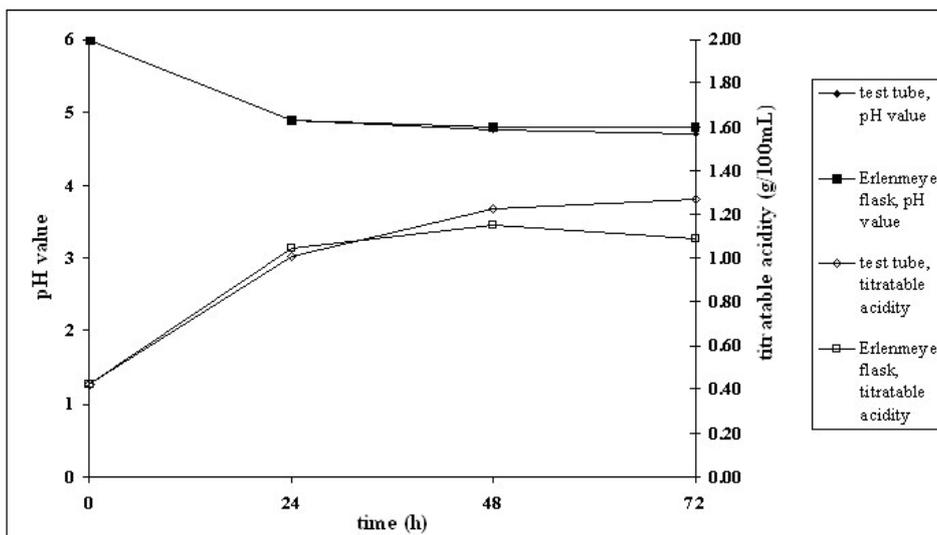


Figure 4. pH Values and titratable acidity during lactic acid fermentation with the addition of CaCO_3 for *Lactobacillus fermentum* PL-1 (sample 2).

to oxygen. These conditions are favorable for lactic acid bacteria due to their anaerobic properties.

Thin-layer chromatography confirmed that glucose was more utilized for lactic acid fermentation in the samples with CaCO_3 (data not shown). This occurrence could be explained by the fact that CaCO_3 transformed lactic acid into Ca-lactate during the fermentation. Ca-lactate reduces the inhibition of lactic acid fermentation, which appears by produced lactic acid. In this way, only a part of lactic acid stayed dissolved in the triticale stillage and only that part was measured during titration.

These results show that triticale stillage sample 2 (from distillation of fermented mash, as the result of

bioethanol releasing; mash was pre-treated before bioethanol fermentation by triticale autoamylolytic enzymes), is a very good medium for lactic acid fermentation.

Figure 5 shows the pH values and titratable acidity during lactic acid fermentation without the addition of CaCO_3 (triticale stillage sample 3).

Figure 6 shows the pH values and titratable acidity during lactic acid fermentation with the addition of CaCO_3 (triticale stillage sample 3).

A decrease of pH value was the same in the samples without CaCO_3 , while the pH value was lower in test tubes in the samples with CaCO_3 . Bigger titratable acidity was achieved in the test tubes, with or

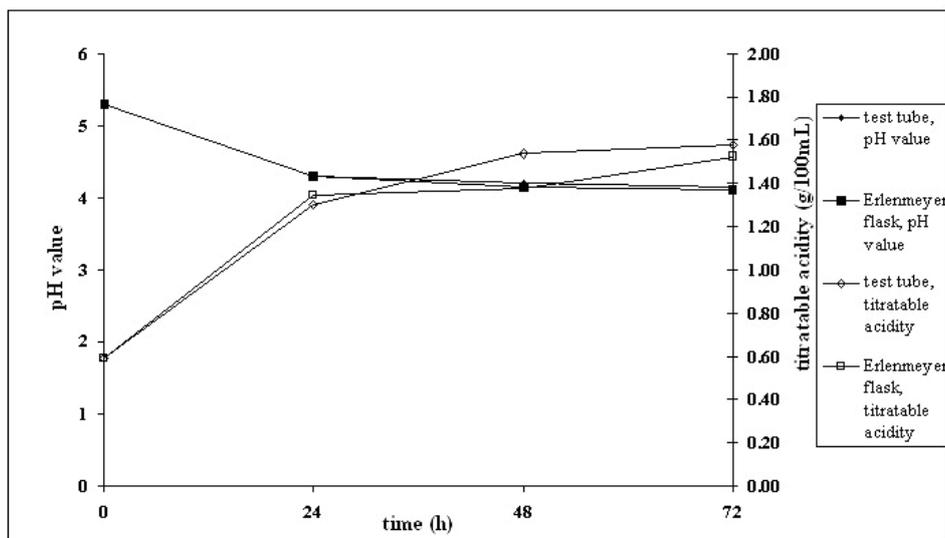


Figure 5. pH Values and titratable acidity during lactic acid fermentation without the addition of CaCO_3 for *Lactobacillus fermentum* PL-1 (sample 3).

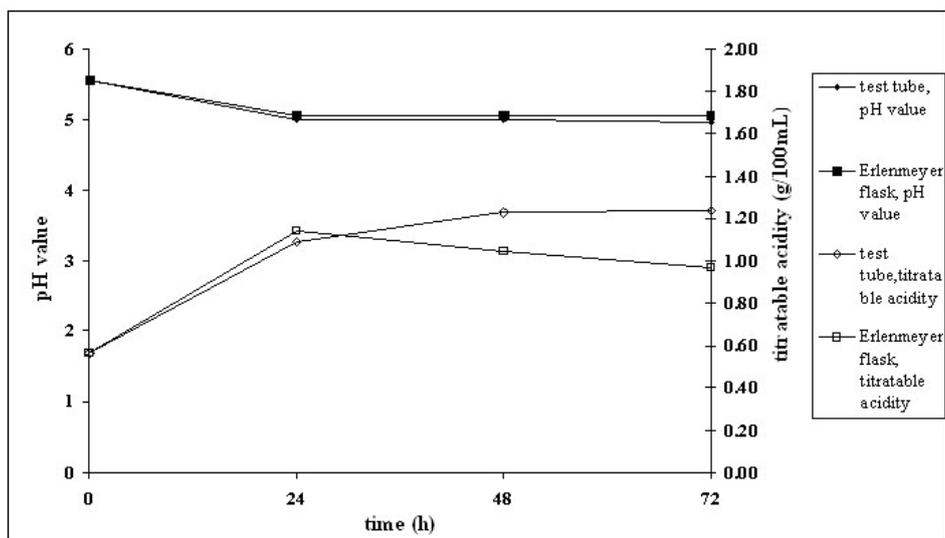


Figure 6. pH Values and titratable acidity during lactic acid fermentation with the addition of CaCO_3 for *Lactobacillus fermentum* PL-1 (sample 3).

without CaCO_3 (Figures 5 and 6). The values were higher for the samples without CaCO_3 .

Here we can notice the same phenomena seen in triticale stillage sample 2. The geometry of the vessel and addition of CaCO_3 had the same influence as described above.

Based on the achieved results, the conclusion is that triticale stillage sample 2 is a better choice as a medium for lactic acid fermentation. In Figures 3-6 it can be noticed that regardless of the type of the vessel or the presence of CaCO_3 , higher titratable acidity was achieved from the 24th hour to the end of observation in triticale stillage sample 2. This research showed even better results than screening. This shows

that triticale stillage prepared by autoamylolytic enzymes before bioethanol fermentation has enough and better biogenic nutritive components for lactic acid fermentation by *L. fermentum* PL-1.

It has been reported that peptides are more favourable for the growth of lactic acid bacteria than mixtures of pure amino acids [28]. During the centrifugation, peptides were separated. *L. fermentum* utilized most of nitrogen by free amino acids released by autolysis of the yeast [26].

Cell protection by CaCO_3 and cell viability

The main observation point was to see in which way CaCO_3 takes part in pH value variations and tit-

ratable acidity. Figure 7 shows the pH values and titratable acidity during lactic acid fermentation with and without the addition of CaCO_3 .

Viable plate count was done by a pour plate method every 24 h, in doublets (samples were taken from the flasks). These count results are given in Table 4.

CONCLUSION

The research results showed that triticale stillage can be used for lactic acid bacteria growth and the production of lactic acid. Three *Lactobacillus* strains: *L. fermentum* NRRL-B-75624, *L. fermentum* PL-1, and *L. plantarum* PL-4 were taken into consi-

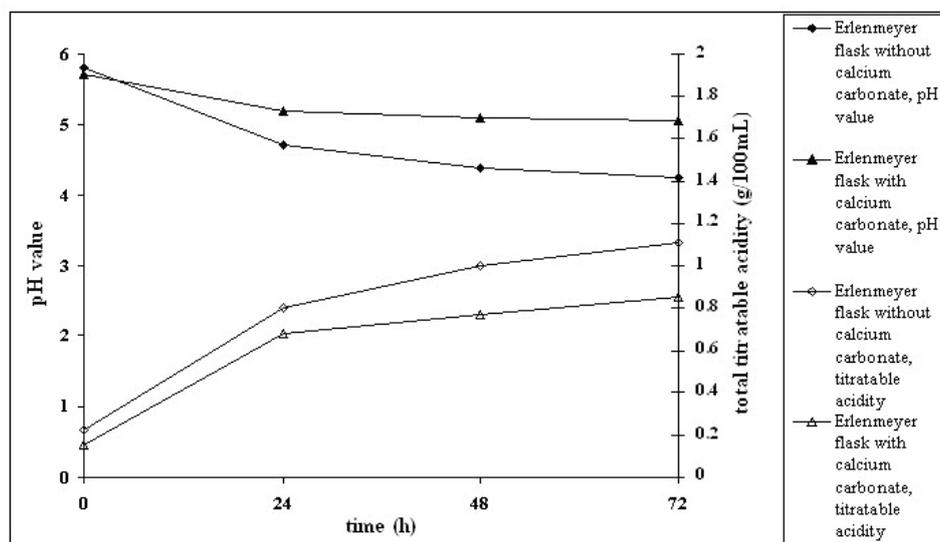


Figure 7. pH Values and titratable acidity during lactic acid fermentation with and without the addition of CaCO_3 for *Lactobacillus fermentum* PL-1.

Figure 7 shows that pH values, during the whole experiment, were lower for the sample without CaCO_3 . The results for titratable acidity were the same as for the second and third one (Figures 3–6), which means that the results were better for the samples without CaCO_3 . On the other hand, the viable plate count (Table 4) showed that the colony number at the end of the process was higher for the samples with CaCO_3 , due to the protection effect of precipitated CaCO_3 .

Table 4. Viable plate count (10^8 CFU/mL) for *Lactobacillus fermentum* PL-1

Type of stillage	Time, h			
	0	24	48	72
Without CaCO_3	0.81	1.33	1.65	0.45
With CaCO_3	0.81	1.83	1.88	1.43

Silica gel thin-layer chromatography for this experiment confirmed these results (data not shown). The presence of even 10% (w/v) CaCO_3 in the production medium was found to be good for lacticing of the pH, which adversely affected the bacterial growth [17].

deration. *L. fermentum* PL-1 which was used for further research showed to be the best choice. The addition of CaCO_3 in the triticale stillage before lactic acid fermentation showed that CaCO_3 is important for successful lactic acid fermentation. CaCO_3 prohibits the inhibition of lactic acid bacteria. The samples with CaCO_3 had lower value for titratable acidity, and this was due to Ca-lactate formation. The results from viable cell count done by a pour plate method confirmed this by the fact that the higher number of colonies was for samples with CaCO_3 .

The nutritional components present in the triticale stillage were adequate for growth and metabolism of lactic acid bacteria. Triticale stillage sample with pre-treatment only by triticale autoamylolytic enzymes before bioethanol fermentation showed better results than the samples that were made from triticale stillage samples pre-treated by technical enzymes before bioethanol fermentation.

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NAUČNI RAD

MOGUĆNOST MLEČNO-KISELINSKE FERMENTACIJE NA DŽIBRI TRITIKALEA

*Džibra tritikalea predstavlja jedan od sporednih produkata tokom proizvodnje bioetanola. Glavna ideja istraživanja je bila da se istraži pogodnost džibre tritikalea kao podloge za uzgajanje bakterija mlečne kiseline i mlečnokiselinsku fermentaciju. Tokom rada su ispitivana tri soja bakterija mlečne kiseline: *Lactobacillus fermentum* NRRL-B-75624, *Lactobacillus fermentum* PL-1 i *Lactobacillus plantarum* PL-4. Tok mlečno-kiselinske fermentacije praćen je merenjem pH vrednosti uzorka, kao i određivanjem titrabilne kiselosti. Kod *Lactobacillus fermentum* PL-1 je zabeleženo najveće sniženje pH vrednosti, kao i porast titrabilne kiselosti, te je iz tih razloga izabran za dalji tok rada. Istraživanje je bilo usmereno i na to kako hranjivi sastav podloge i dodatak CaCO_3 utiču na mlečno-kiselinsku fermentaciju, kao i na zaštitnu ulogu CaCO_3 na ćelije bakterija mlečne kiseline. Hranjivi sastav džibre tritikalea se pokazao kao kompletna podloga za odvijanje mlečno-kiselinske fermentacije. Titrabilna kiselost je bila niža kod uzoraka sa dodatkom CaCO_3 , u odnosu na uzorke kojima nije dodat CaCO_3 , zbog formiranja Ca-laktata. Istovremeno je zabeležen veći broj vijabilnih ćelija u uzorcima kod kojih je dodat CaCO_3 , što ukazuje da CaCO_3 ima zaštitnu ulogu na ćelije bakterija mlečne kiseline.*

*Ključne reči: džibra tritikalea; mlečna kiselina; *Lactobacillus* sp., CaCO_3 .*