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SCIENTIFIC PAPER

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UTILIZATION OF STILLAGES FROM BIOETHANOL PRODUCTION FROM VARIOUS SUBSTRATES

Article Highlights

- Valorization of stillage from advanced and conventional bioethanol production was examined
- The highest lactic acid and probiotics production was obtained from thin wasted bread stillage
- Solid fraction of wasted bread stillage was suitable for monogastric animals
- Solid fraction of corn stillage was more suitable for ruminants
- The energy parameters of solids of wasted bread stillage were higher than for corn stillage solids

Abstract

Stillage is a main by-product of the bioethanol industry and, depending on the origin of substrates for bioethanol production, it can be a significant pollutant affecting the profitability of bioethanol production. Directing the stillage towards the production of bio-based chemicals or high-quality feed is a preferred strategy. In this paper, a complete utilization of stillages of different origins was assessed. Thin stillages from bioethanol production from molasses, wasted bread and corn were chemically characterized, evaluated and compared as substrates for lactic acid (LA) and probiotic biomass production by Lactobacillus rhamnosus ATCC 7469, while solid fractions of wasted bread and corn stillages were analyzed for feed. The impact of pH control using $CaCO_3$ or NaOH was also examined, both in terms of LA production and valorization of the remains generated in each process.A maximal LA productivity of 1.14 g/(L h) was obtained on thin wasted bread stillage with pH control by NaOH while the number of viable probiotic bacterial cells was above 10^9 CFU/mL. The composition of the solid fraction of the wasted bread stillage was complementary with the needs of monogastric animals, while the solid fraction of corn stillage was more adequate for the nutritional requirements of ruminants.

Keywords: stillage, bioethanol, revalorization, biorefinery, lactic acid, probiotics, feed.

By-products and residues generated in bioethanol production from agricultural biomass present a significant problem from an environmental and economic point of view. The strategies for revalorization of these residues, and thus obtaining additional value, are of immense importance in terms of circular economy and overall environmental protection. The importance of utilization of residues as feedstock in biofuel production is recognized and articulated through the most recent legislation in the EU [1]. Indirect land use change directive from April 2015 [1] limits the contribution of renewable energy from biofuels produced from crops grown on agricultural land at 7%, within the total 10% target of renewable energy in road transport and a 0.5% sub-target for advanced biofuels, e.g. biofuels produced on wastes and residues. Searle and Malins evaluated in 2016 the availability of wastes and residues adequate for the pro-

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duction of advanced fuels in EU member states and concluded that the goal of 0.5% of advanced fuels in transportation could be met [2].

The production of bioethanol from residues and advances in hydrolysis of agricultural biomass and wastes were intensively studied during the last decades [3]. In addition, molasses, wasted potato, low quality crops, by-products from sugar beet industry etc. were assessed as substrates for bioethanol production [4]. The distillery wastewater remained after bioethanol production from different feedstock is traditionally known as stillage. Depending on the bioethanol production process, up to 20 L of stillage is produced per 1 L of bioethanol with a total COD of stillage around 100 g/L [5]. Hence, the amount of stillage from traditional and advanced bioethanol production is expected to grow in the future. A complexity of stillage, high concentration of metals, furfural, acids and other components can limit its utilization as a substrate in biotechnology. Therefore, the most suitable utilization concept has to be addressed and explored for every substrate-fermentation microorganism-product chain.

Although the current legislation is favoring utilization of residues and wastes as substrates for bioethanol production, the production from corn is still predominant in the world, especially in the USA, and the valorization of corn stillage has been the most widely studied [6]. Drying of corn stillage to produce dried distillers' grains with solubles (DDGS) for feed has been practiced for a long time, but the suitability of similar feed produced from stillages of different origin (such as wasted bread) was not studied in detail, as far as it is known to authors.

The utilization of the stillage as a substrate for hydrogen [7], acetic acid, proteins [5], cyanobacteria and fertilizer production [8] or for microbial fuel cells [9] were explored and reported in literature. However, smaller distilleries struggle with the cost of stillage treatment and often discharge stillage without the treatment leading to significant pollution of groundwater and soil [8], or sell the stillage as feed in wet form, due to the high cost of energy for drying. Due to high content of water and high organic load, the stillage is prone to souring and mould growth. This leads to short storage time of the stillage and causes significant losses affecting the economy and environmental aspects of bioethanol production.

Utilization of different stillages for lactic acid (LA) production, addressed in this paper, is a novel and until now unexplored approach. LA is an important chemical with a wide application in the food industry and as an ingredient in pharmaceutical formulations, particularly L(+) LA, mainly because it is naturally occurring in humans and could be processed in metabolism. It is predicted that a demand for LA will exceed 1960.1 kt in 2020 [10] and it is mainly driven by a wide application range of polylactides – biodegradable, biocompatible and thermostable polymers used for biomedical applications, but also for food packaging and controlled drug delivery [11]. Today, economical and stereo-selective production of lactic acid with respect to green chemistry principles is considered as an imperative and different wastes are being evaluated as substrates [12,13].

In this paper, several types of thin (liquid) stillages from bioethanol production from a standard feedstock (corn) and from alternative feedstock (wasted bread and molasses) were evaluated and compared as substrates for lactic acid fermentation (LAF), *e.g.*, production of LA and probiotics by *Lactobacillus rhamnosus* and feed. The schematic overview of the proposed concept studied in this paper is presented in Figure 1. The most important parameters of batch LAF on different stillages with different pH control agents were analyzed. In addition, the solid fractions of the stillages remaining after bioethanol production were evaluated as feed additive.

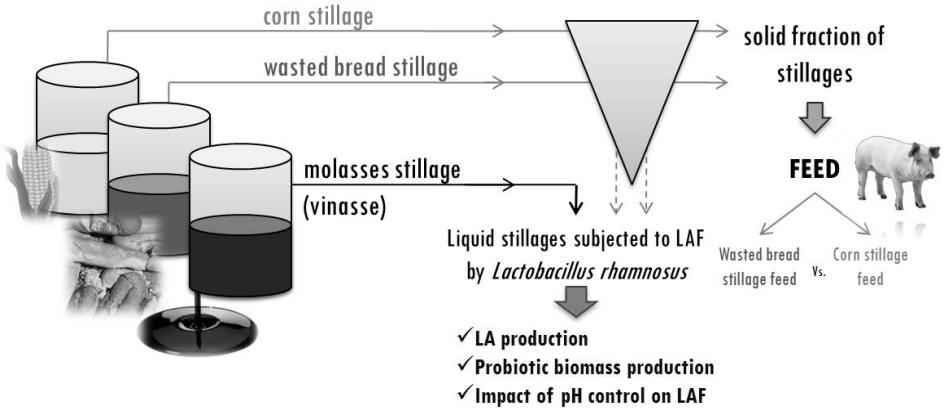

Figure 1. Proposed routes for utilization of stillages of different origin for LA, probiotic biomass and feed production.

MATERIAL AND METHODS

Substrates for lactic acid fermentations

In the first set of experiments the stillages from bioethanol production from corn and wasted bread (obtained from Reahem d.o.o., Srbobran, Serbia) and from ethanol production from molasses (Swan lake d.o.o, Kovin, Serbia) were used as substrates for LA production. Molasses-based thin stillage is a liquid fraction remaining after bioethanol fermentation of molasses substrate, distillation of ethanol and subsequent centrifugal separation (4500 rpm for 20 min) of residual yeast. Thin corn and wasted bread stillages were obtained after centrifugation of the whole stillages at 4500 rpm for 20 min (Sigma® model 2-16, Shropshire, UK) and separation of liquid fractions from solids. All thin stillage samples were chemically characterized regarding the content of dry matter, proteins, reducing sugars, lipids and ash.

The fermentation media consisted of 200 ml of liquid stillages with pH adjusted to 6.5 by 30% NaOH solution. After sterilization at 120 °C for 15 min, the substrates were cooled and sugar concentration was adjusted to approximately 20-25 g/L by addition of a sterile 40% glucose solution. Stillage samples prepared this way were used as substrates for LAFs.

In the second set of experiments, the effect of pH control on LAF of the liquid stillage using NaOH and $CaCO_3$ was evaluated. Particularly, pH control was performed by addition of 1, 2, 5 and 10% of $CaCO_3$ or addition of 30% of NaOH in 4 h intervals in order to maintain pH 6.5 in media. After pH adjustment the stillage was sterilized (120 °C, 15 min) and a sterile 40% glucose solution (up to final concentration of app. 20-25 g/L) was added. Media prepared this way was used as a substrate for batch LAF.

Microorganism

Lactobacillus rhamnosus ATCC 7469, a homofermentative L(+) LA strain (99.7% L(+) - LA) with probiotic characteristics [13] used in these experiments was obtained from American Type Culture Collection (Rockville, USA). The culture was propagated under microaerophilic conditions at 37 °C for 18 h in MRS broth before inoculation to the fermentation medium.

Lactic acid fermentation

Batch LAFs were performed in 500 mL flasks with 200 mL of prepared substrate at 41 °C, under microaerophilic conditions (maintained using gas pack system, Anaerocult® bags, Merck, Darmstadt, Germany) with shaking (100 rpm, KS 4000i control, IKA®, Werke GmbH and Co.KG, Staufen, Germany). The LAFs were initiated with addition of 5 vol.% overnight culture of *L. rhamnosus* ATCC 7469. During the fermentations the samples were aseptically withdrawn and LA concentration, reducing sugar concentration and the number of viable cells were determined. LAFs were performed without pH control, with $CaCO_3$ addition or with NaOH addition, depending on the experimental setup, as explained previously. LA yield, LA yield coefficient and LA productivity were used as parameters of LAF in this study. LA yield was calculated using Eq. (1):

$$Y_{s/p} = c_{LA}/c_s \quad (1)$$

where c_s stands for concentration of reducing sugars (substrate, g/L), c_{LA} stands for concentration of lactic acid in fermentation media, while LA yield coefficient was determined according to Eq. (2):

$$Y_{us/p} = c_{LA}/c_{us} \quad (2)$$

where c_{us} stands for concentration of utilized reducing sugars (g/L) calculated as the difference between initial reducing sugar concentration in media (c_s, g/L) and residual reducing sugar concentration in media in designated fermentation time (c_r, g/L), c_{LA} stands for concentration of lactic acid in fermentation media. LA productivity was determined using Eq. (3):

$$Q_{LA} = c_{LA}/t \quad (3)$$

where c_{LA} stands for concentration of lactic acid in fermentation media and t is the fermentation time.

Analytical methods

Thin stillages of different origin (wasted bread, corn and molasses) were chemically characterized without any previous treatment in order to evaluate them as substrates for LAF, while solid fractions of corn and wasted bread stillage, intended for feed consumption were analyzed after drying in the laboratory oven with ventilation at 60 °C for 48 h. The samples were ground in a laboratory mill (A/SKnifetec 1095; Foss, Hillerød, Denmark) with a chamber with built-in water cooling and a rotor blade. The dry matter (DM) percentage in studied thin stillages and their solid fractions was determined by a standard drying method in an oven at 105 °C to constant mass [14]. The protein content in thin stillages was estimated by Kjeldahl method as the total nitrogen and using factor 6.25 [14]. The lipid content was determined by Soxhlet method and ash content was determined by slow combustion method at 650 °C for 2 h [14]. The concentration of reducing sugars, calculated as glucose, during LAF was estimated by 3,5-dinitrosalicylic acid method [15]. Calibration curve was set at 505 nm using standard glucose solutions. LA con-

centration was determined by enzymatic method (L/D-LA assay, Megazyme®, Wicklow, Ireland) after deproteinization of the sample as prescribed in the manufacturer's procedure. During the fermentation, a number of viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique on MRS agar after incubation at 37 °C. Crude fiber content was determined by Weende method adjusted for Fibretec™ Systems, Foss, Denmark [16].

The fiber content analysis of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicellulose was performed by the Van Soest detergent method modified by Mertens [17]. *In vitro* dry matter digestibility was determined by the Aufréré method [18]. This method is based on the hydrolysis of proteins of DDGS in the pepsin acid solution (Merck 2000 FIP μg^{-1} Art 7190) at 40 °C for 24 h, and then on the hydrolysis of carbohydrates in the cellulase solution (Celullase Onozuka R10) for 24 h. All chemicals used in experiments were of analytical grade.

Calculation of the parameters significant for animal feed

The nitrogen free extract (NFE, in g/kg DM) content of the DDGS feed was calculated using Eqs. (4) and (5) [19,20]:

$$\text{Crude fiber} = \text{Cellulose}+\text{ADL} \quad (4)$$

$$\text{NFE} = 1000-(\text{Ash}+\text{Crude fiber}+\text{Oil}+\text{Protein}) \quad (5)$$

Based on the chemical composition digestible energy (*DE*, kJ/kg) and metabolizable energy (*ME*, kJ/kg) values of DDGS samples were calculated using the following Eqs. (6) and (7) [21,22]:

$$DE = 4151-(122\%\text{Ash})+(23\%\text{Protein})+(38\%\text{Oil})-(64\%\text{Crude fiber}) \quad (6)$$

$$ME = DE\times[1.003-(0.0021\%\text{Protein})] \quad (7)$$

Statistical analysis

The experiments were done in triplicates. All values are expressed as means ± standard deviation. Mean values of treatments were compared by analysis of variance. One-Way ANOVA followed by the Tukey test was applied to evaluate the effect of investigated parameters. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of different stillages

Stillages from bioethanol production from wasted bread and molasses were compared with corn stillage, as corn is the most exploited feedstock for bioethanol production globally. Solid part of the stillage is mostly used for production of feed while drying of liquid (thin) stillage is not economically feasible. Chemical compositions of the studied three types of stillages are presented in Table 1. The stillages were further subjected to LAF.

The dry matter content was similar in all studied thin stillages while the amount of crude proteins, reducing carbohydrates, ash and lipids was significantly different, but in accordance to literature data [23,24]. The protein content and reducing sugar content are the most important parameters for analysis of the substrate suitability for LAF. The reducing sugar concentrations in the range from 4.2 to 13 g/L are low because of effective previous ethanol fermentation (Table 1). During the preliminary studies we performed HPLC analysis of the carbohydrates present in all three studied stillages and detected a complex mixture of oligosaccharides and glucose as a predominant monosaccharide. Beside the glucose, traces of fructose and arabinose were detected in corn and wasted bread stillage. However, only reducing sugars were assimilated by LAB, but the stillages were rather low in the reducing sugar concentration. Therefore, the supplementation with fermentable carbon sources was necessary for efficient LAF.

On the other hand, the stillages are good sources of nitrogen, with the wasted bread stillage being the highest in protein content, followed by molasses stillage (Table 1). Significant amount of proteins present in molasses and molasses-based products originates from betaine which could be beneficial for the LA production as reported by Xu and Xu, [25]. A very high content of ash in molasses stillage is a result of high content of metals commonly present in molasses, especially high content of Fe [5].

The carbon/nitrogen ratio is an important parameter for LAF and in order to compare different stillages as substrates for LAF, we adjusted initial reducing sugar concentration at around 25 g/L. The stillages are nitrogen- and mineral-rich substrates, how-

Table 1. The chemical composition of studied thin stillages

Stillage	Reducing carbohydrates, g/L	Crude protein, g/L	Ash, g/L	Lipids, g/L	Dry matter, %
Thin corn	13.12±0.70	8.42±0.71	2.90±0.32	1.83±0.45	5.02±0.29
Molasses	4.20±0.19	18.80±0.61	17.60±0.72	0.80±0.02	6.61±0.55
Thin wasted bread	11.66±0.68	21.00±1.10	6.96±0.23	5.48±0.81	4.80±0.48

ever, lacking the fermentable sugars that should be externally supplemented. For further optimization of the processes and scale up of the LAF on waste bread stillage it would be necessary to further examine and optimize the initial sugar concentrations, as well as a possibility to use alternative renewable carbon sources rich in fermentable sugars (e.g. hydrolysates of lignocellulose substrates).

Assessment of different stillages as substrates for lactic acid fermentations by *L. rhamnosus* ATCC 7469

The kinetics of LA production and reducing sugar utilization from thin stillages from corn, molasses and wasted bread are presented in Figure 2. Further, the growth of *L. rhamnosus* ATCC 7469 cells during the fermentation of three studied substrates was followed and presented in Figure 3.

The most important parameters of fermentation processes from different substrates are given in Table 2.

Kinetics of LA production and sugar consumption were similar for three studied substrates, however, the LA production from wasted bread stillage was faster, and after 30 h of fermentation the LA concentration was around 13% higher than from other stillages (Figure 2). Differences in LA production during the fermentations of molasses and corn stillages were not significant. The LA yield was the highest in LAF from thin wasted bread stillage corresponding to the 69% of the maximal theoretical yield. Also, the highest LA productivity was obtained from the same substrate, so it could be considered as the most adequate for LA production by *L. rhamnosus* ATCC 7469. These results imply that the thin stillages from advanced bioethanol production could be valorized in a biorefinery process for LA production as comparable, or even better, substrates than the thin corn stillage, which is the most abundant type of stillage worldwide. High concentration of metals and melanoidins in molasses-based stillage caused neither decline in LA productivity nor a lower final LA concentration in comparison to these parameters obtained in LAF of corn stillage. The substances present in molasses-based stillage were often reported as inhibitory for bacteria [26] but the results of our study have shown that *L. rhamnosus* ATCC 7469 strain could convert glucose to LA in molasses stillage substrate with the same effectiveness as in corn thin stillage.

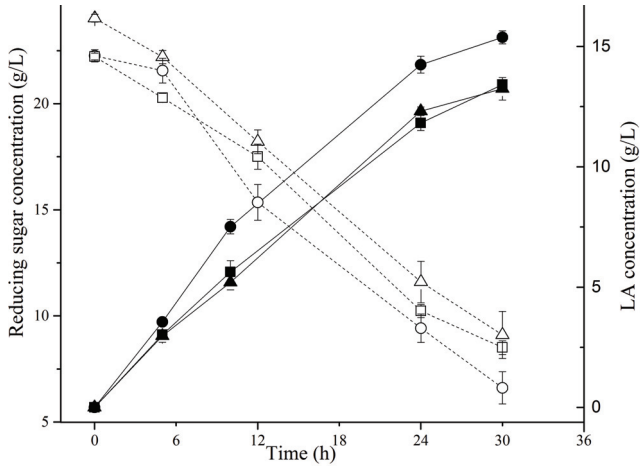

Figure 2. The kinetics of LA production and reducing carbohydrate utilization on three different stillage-based substrates. Experimental conditions: batch fermentation, microaerophilic, shaking (100 rpm), 41 °C, 5 vol.% inoculum concentration; Symbols: circle – thin wasted bread stillage, triangle – thin corn stillage, square – molasses stillage; open symbols – sugar concentration, solid symbols – LA concentration.

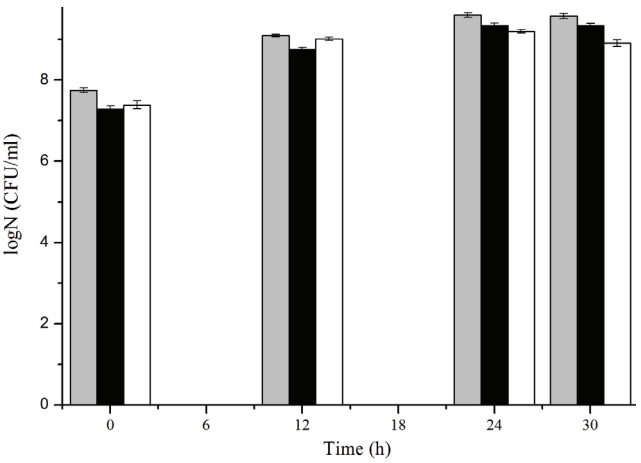

Figure 3. The number of viable L. rhamnosus ATCC 7469 during LAF on three different stillage-based substrates. Experimental conditions: batch fermentation, microaerophilic conditions, shaking (100 rpm), 41 °C, 5 vol.% inoculum concentration; Symbols: light grey bars – thin wasted bread stillage, white bars – thin corn stillage, black bars – molasses stillage.

Table 2. The parameters of lactic acid fermentation on liquid corn, molasses and wasted bread stillages after 30 h of fermentation without pH control; the calculation of the parameters LA yield, LA yield coefficient and LA productivity is presented in Material and methods, subsection Lactic acid fermentation

Stillage	LA concentration, g/L	LA yield, g/g	LA yield coefficient, g/g	LA productivity, g/(L h)
Thin corn	13.24±0.20	0.55 ±0.02	0.89±0.03	0.44±0.02
Molasses	13.41±0.47	0.60±0.03	0.98±0.05	0.45±0.02
Thin wasted bread	15.38±0.27	0.69±0.02	0.98±0.03	0.51±0.02

Further, the growth of *L. rhamnosus* ATCC 7469 cells during the fermentation of three studied substrates was followed and is presented in Figure 3.

L. rhamnosus ATCC 7469 growth was the most intense on thin wasted bread stillage attaining more than 3×10^9 CFU/mL in the media without pH control (Figure 3). The lowest number of *L. rhamnosus* ATCC 7469 cells was obtained on corn stillage at the end of fermentation, although the bacterial growth on corn stillage was the fastest during first 12 h of fermentation. The strain *L. rhamnosus* ATCC 7469 exhibits a probiotic potential [13] and thus the utilization of the biomass together with other remains after LAF as animal feed or feed additive could give an additional value to the process. The number of viable probiotic cells in all studied substrates was within recommended values of 10^6–10^9 CFU/g of feed [27].

Data for LA production in batch fermentation of thin stillage are limited and here obtained values of LA productivity and yield (Table 2) were similar to the values reported for kitchen waste (LA yield of 0.39 g/g and LA productivity of 0.60 g/(L h)) [28] and higher than the values obtained on food waste by indigenous *Lactobacillus* sp. (LA yield 0.46 g/g and LA productivity of 0.28 g/(L h)) [29]. The LA productivity of around 0.5 g/(L h) is not high, implying that further improvement of the fermentation parameters should be employed to increase the productivity. The results presented in Table 2 were obtained in fermentations without pH control and are expected to be improved by implementing the means of pH control. The highest amount of proteins in wasted bread stillage (Table 1), the highest LA concentration and productivity obtained during LAF (Figure 2) and the most suitable C/N ratio favored thin wasted bread stillage for further investigation of the effect of pH control.

Effect of the choice of neutralizing agent in lactic acid fermentation of thin wasted bread stillage

Different neutralizing agents such as ammonia, $CaCO_3$, Na_2CO_3, NaOH, etc. were used for pH control in LAFs [29]. The best neutralizing agent should be selected and empirically evaluated for every substrate used in LAF. Also, the selection of the neutralizing agent is very important from the aspect of LA extraction from fermentation broth, since the presence of residues such as solid $CaCO_3$ or Na ions remaining after the fermentation could direct the routes for valorization or treatment before disposal. In order to improve performances of LAF on thin wasted bread stillage, NaOH and $CaCO_3$ as neutralizing agents were studied and compared. The effect of different concentrations of $CaCO_3$ on LA production and growth of *L. rhamnosus* biomass is presented in Figure 4.

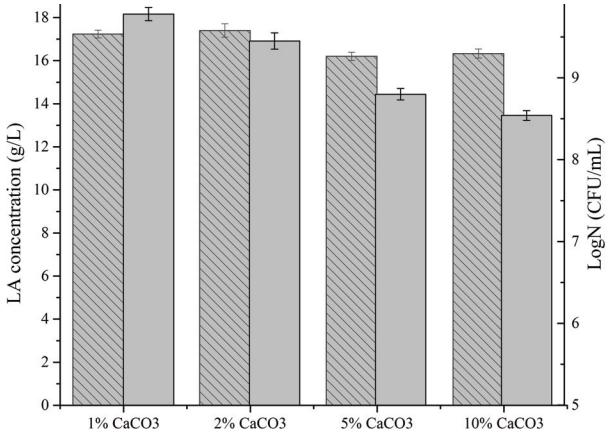

Figure 4. LA concentration and number of viable L. rhamnosus ATCC 7469 cells in LAF of thin wasted bread stillage with $CaCO_3$ addition after 36 h of fermentation. Experimental conditions: batch fermentation, microaerophilic conditions, shaking (100 rpm), 41 °C, 5 vol.% inoculum concentration; Symbols: light grey patterned bars – LA concentration, light grey bars – number of viable L. rhamnosus cells.

The addition of 2% of $CaCO_3$ resulted in the highest LA concentration of 17.40 g/L, with corresponding LA productivity of 0.57 g/(L h). With increase of $CaCO_3$ concentration in media, LA concentration did not change significantly, but the number of viable *L. rhamnosus* cells decreased. The difference in LA concentration obtained by addition of 1 and 2% of $CaCO_3$ was not significant and the number of *L. rhamnosus* cells was significantly higher in samples with 1% of $CaCO_3$, so in the next set of experiments a concentration of 1% of $CaCO_3$ was used for pH control. Also, the effect of residual $CaCO_3$ concentration after fermentations should be addressed and analyzed from the point of application of the fermentation residues in animal nutrition.

The $CaCO_3$ is used in animal nutrition, especially during the intensive growth or lactation of animals [30]. A maximal dietary allowance for $CaCO_3$ in feed is not established and the recommended dietary allowance for cattle is around 0.31% on dry matter basis of daily feed intake [31]. The application of $CaCO_3$ in human and animal nutrition is generally accepted as safe [32], but the intake of 5.9% on dry matter basis of daily feed intake is considered very high [31]. Based on these findings, the residues of stillage fermented by probiotic biomass in the process of LA production with addition of 1% of $CaCO_3$ or less could be considered as a supplement in the feed.

Additionally, in the process of LA extraction from fermentation media with $CaCO_3$ as a neutralizing

agent, a significant amount of gypsum ($CaSO_4$) remains after addition of H_2SO_4 in order to extract LA. This is very important since the gypsum is a resistant environmental contaminant and only small quantities of produced $CaSO_4$ could be used for soil conditioning or feed. The other approaches have been studied such as utilization of $CaSO_4$ as micro-filler in composites with PLA, polymers based on LA for bone grafts and implants [33]. Valorization of the gypsum remaining in the downstream processing of LA into high-value PLA-$CaSO_4$ composites for medical applications represents a great example of green technology through waste valorization. The necessity for further processing of the remained gypsum is an additional reason for application of as low concentration of $CaCO_3$ in LAF as possible.

To compare utilization of NaOH and $CaCO_3$ as neutralizing agents, in the next set of experiments the amount of 1% of $CaCO_3$ was used and 30% NaOH was added in 4 h intervals for maintaining pH value of 6.5. The obtained kinetics of LA production is presented in Figure 5.

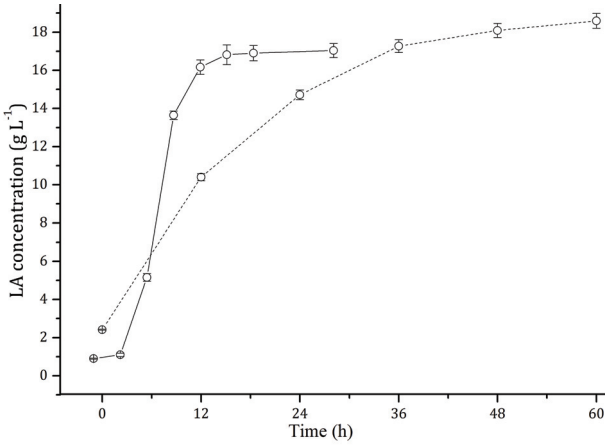

Figure 5. The kinetics of LA production from thin wasted bread stillage with 1% of $CaCO_3$ and addition of 30% NaOH in 4 h intervals as a neutralizing agent. Experimental conditions: microaerophilic conditions, shaking (100 rpm), 41 °C; Symbols: dot line – 1% of $CaCO_3$, solid line – NaOH as neutralizing agent.

The LAF was faster with using NaOH as neutralizing agent, but the final LA concentration was higher in the fermentation with $CaCO_3$ addition. The most significant parameters of LAF with different neutralizing agents are presented in Table 3. Maximal LA productivity of 1.14 g/(L h) was achieved in the sample with NaOH, while the highest LA productivity of 0.81 g/(L h) in the sample with $CaCO_3$ addition was attained after 12 h of fermentation. Also, by using NaOH for pH control, a complete LA production was finished after just 16 h with a yield of 0.76 g/g or 76% of the maximal theoretical LA yield and residual sugar concentration of around 5 g/L (Table 3). Based on these findings, the addition of NaOH as a neutralizing agent is considered better with respect to LA and biomass production (8×10^9 CFU/mL) from thin wasted bread stillage. Also, the separation of probiotic biomass and extraction of LA is easier if NaOH is used as neutralizing agent, hence a complete utilization of all by-products in the process is more prospective.

Because of the higher yield coefficient and productivity of the fermentation with pH control, it could be expected that even higher LA concentrations could be obtained through further optimization of media composition, particularly fermentable sugar concentration and application of advanced fermentation strategies. Also, continuous pH adjustment, instead of NaOH addition in intervals, could result in higher LA productivity by avoiding pH drops which are causing stress to bacterial cells. Reviewing literature data, lower productivities than the values obtained in this study were reported in batch LAF from waste sugarcane bagasse (maximal productivity of 0.93 g/(L h)) [34] or from wastewater sludge (maximal productivity 0.23 g/(L h)) in simultaneous saccharification and fermentation (SSF) mode [35]. However, higher LA productivities up to 105.1 mg/g of rice husks and 90.1 mg/g of agave bagasse after HCl hydrolysis and supplementation by yeast extract were attained after 24 h of LAF [36]. Therefore, compared to the literature, it could be pointed out that the thin stillage from adv-

Table 3. Significant fermentation parameters on wasted bread stillage obtained with using different neutralizing agents

Neutralizing agent	Final LA concentration[a], g/L	Final logN[a], CFU/mL	LA yield[a], g/g	LA productivity[a], g/(L h)	Maximal LA productivity[b], g/(L h)
1% $CaCO_3$	16.89±0.35	9.78±0.08	0.75±0.01	0.47±0.01	0.81±0.02
2% $CaCO_3$	17.4±0.31	9.45±0.1	0.77±0.01	0.48±0.01	-
5% $CaCO_3$	16.2±0.19	8.8±0.07	0.72±0.01	0.45±0.01	-
10% $CaCO_3$	16.33±0.21	8.54±0.06	0.73±0.01	0.45±0.01	-
NaOH	17.04 ± 0.37	9.91±0.09	0.76±0.01	0.47±0.01	1.14±0.01

[a]After 36h LAF; [b]obtained after 12h of LAF. The calculation of the parameters LA yield, LA yield coefficient and LA productivity is presented in Material and methods, subsection Lactic acid fermentation

anced bioethanol production could be rather well revalorized in LA production.

Analysis of solid fractions of wasted bread and corn stillages for use in animal nutrition

Beside the utilization of thin fraction for LA and biomass production, solid fraction of the stillage was assessed for feed in order to evaluate a whole potential of wasted bread stillage. Therefore, it was chemically analyzed from the aspect of utilization in animal nutrition and the most important parameters of animal feed were calculated. Also, all relevant chemical characteristics of solid fraction of wasted bread stillage were compared with the same characteristics of the solid fraction of corn stillage and presented in Figure 6, in order to measure up the potential of wasted bread DDG as an alternative to commonly produced DDG as feed.

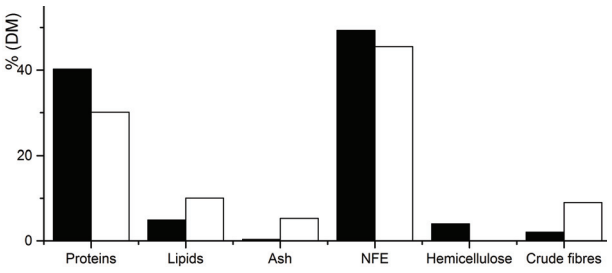

Figure 6. Comparison of chemical composition of the solid fraction of wasted bread and corn stillage based on dry matter.

The proteins amounted to around 40% of all components present in the solid fraction of wasted bread stillage, while more than 50% of dry matter presented easily assimilative non-protein components (nitrogen-free extract). The amounts of proteins and NFE were higher in the solid fraction of wasted bread stillage than in the solid fraction of corn stillage. The composition of dry matter of the solid fraction of corn stillage revealed significantly higher amount of crude fibers and ash in comparison to solid remains of wasted bread stillage, thus implying better suitability of corn stillage remains for ruminants, while wasted bread stillage remains could be recommended for monogastric animals due to lower content of fibers. Also, the solid fraction of wasted bread stillage with over 40% of protein could be a high protein feed. This is probably a result of the presence of yeast (rich in proteins) from the initial bread production and/or from subsequent bioethanol production. The dry matter content of the solid fraction of wasted bread stillage was around 34%, while in corn stillage, which is mostly used for DDGS production, dry matter content varies from 7-10%, therefore the cost of drying in order to stabilize the remains is expected to be significantly lower.

The other important parameters for evaluation of the solid fraction of stillage for feed are: digestible, metabolizable energy and digestibility. The digestible energy for the solid fraction of corn stillage was 17307.1 kJ/kg and for the solid fraction of wasted bread stillage it was 17760.7 kJ/kg. Metabolizable energy was 17249.5 and 17663.9 kJ/kg for solid remains of corn and wasted bread stillage, respectively. The digestibility of the solid fraction of wasted bread stillage was 916.23 g/kg of dry matter, while for corn stillage it was 743.8 g/kg of dry matter. The obtained values for both digestible and metabolizable energies were lower for corn stillage solids, and digestibility of corn stillage was also lower than the value for wasted bread stillage. The obtained values of energy parameters for wasted bread stillage from 3556 to 4140 kcal/kg of dry matter (14878.3 to 17321.76 kJ/kg of dry matter) were similar and within the range reported for corn DDGS [37,38] and wheat DDGS [39]. Previously, Semenčenko *et al.* [40] have shown variability in the DDGS composition depending on the corn hybrid used for bioethanol production, however, in the commercial processes for valorization of corn stillage, it has been often difficult to specify the hybrid used, since often a mixture of available corn biomass have been used. The remains of wasted bread stillage have shown also higher digestibility in comparison to corn stillage remains, probably due to the lower amount of fibers.

Finally, it can be emphasized that this study demonstrated suitability of wasted bread stillage for its complete utilization even over corn stillage. The thin stillage (liquid part) was suitable for the production of LA and probiotic biomass while the solid part of the stillage was found adequate as a feed for monogastric animals.

CONCLUSIONS

Thin stillages from advanced bioethanol production from wasted bread, molasses and corn-based bioethanol production were studied as substrates for LAF. It was shown that wasted bread and molasses stillages were similar or even better substrates for LAF than the most abundant corn stillage. A maximal LA productivity obtained from thin wasted bread stillage of 1.14 g/(L h) was similar or higher to that obtained from many other waste substrates previously studied. A high number of viable probiotic bacterial cells of above 10^9 CFU/mL was achieved. Solid fraction of the stillage, separated before LAF, was

suitable for monogastric animals according to the composition, especially with respect to high nitrogen-free extract and protein content, and low crude fiber content. The solid fraction of corn stillage was more adequate for nutritional requirements of ruminants. The energy parameters have shown that the solid fraction of wasted bread stillage is higher in digestible, metabolizable energy and digestibility compared to the solid corn stillage.

The results of our study have clearly shown that agri-food wastes such as thin stillages from bioethanol production could be revalorized as substrates for LA and probiotic biomass production while residual solid fraction could be dried and used in animal nutrition.

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NAUČNI RAD

MOGUĆNOSTI UPOTREBE DŽIBRE IZ PROIZVODNJE BIOETANOLA NA RAZLIČITIM SUPSTRATIMA

Džibra je najznačajniji otpadni ili sporedni proizvod industrije bioetanola. U zavisnosti od porekla supstrata koji se koriste u proizvodnji bioetanola, može biti značajan zagađivač, pa utiče na profitabilnost proizvodnje bioetanola. Upotreba džibre u biotehnološkim postupcima proizvodnje hemikalija ili visoko vredne stočne hrane je poželjna strategija za smanjenje negativnih efekata džibre na životnu sredinu i profitabilnost proizvodn je bioetanola. U ovom radu je analizirana mogućnost kompletnog iskorišćenja džibre. Tečne džibre iz proizvodnje bioetanola na melasi, otpadnom hlebu i kukuruzu su hemijski okarakterisane i upoređene kao supstrati za proizvodnju mlečne kiseline (MK) i probiotske biomase soja Lactobacillus rhamnosus ATCC 7469, dok je čvrsti ostatak hlebne i kukuruzne džibre analiziran kao hrana za životinje. Takođe je ispitan uticaj pH kontrole pomoću $CaCO_3$ ili NaOH sa aspekta proizvodnje MK i sa aspekta iskorišćenja ostataka iz oba procesa. Maksimalna produktivnost MK od 1,14 g/(L h) je dobijena na tečnoj džibri iz otpadnog hleba sa pH kontorolom pomoću NaOH, dok je broj živih probiotskih bakterija bio preko 10^9 CFU/mL. Rezultati su pokazali da je sastav čvrste frakcije otpadne hlebne džibre komplementaran sa potrebama monogastričnih životinja, dok sastav čvrste frakcije kukuruzne džibre više odgovara nutritivnim potrebama preživara.

Ključne reči: džibra, bioetanol, revalorizacija, biorafinerija, mlečna kiselina, probiotici, stočna hrana.