Strategies for valorization of wastes from bioethanol production– lactic acid and probiotics as added value products

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Abstract

Lactic acid (LA) is a chemical with wide application range from chemical and food industry up to medical graft production. Due to its growing demand, lactic acid production on cheaper substrates is extensively studied.

A potential of utilization of thin stillages from bioethnol production on residues (wasted bread and molasses) was compared with the utilization of thin corn stillage as substrate for LA and probiotic biomass production. The stillages were chemically characterized and subjected to batch lactic acid fermentation (LAF) by probiotic strain *Lactobacillus rhamnosus* ATCC 7469. Effect of addition of different neutralizing agent during LAF was investigated. Also, the potential of remaining solid fraction of the stillage from bioethanol production on waste bread as a feed was analyzed in order to explore complete utilization of both thin and solid stillage in a biorefinery process.

The highest LA productivity of 1.14 g L^{-1} h^{-1} and LA yield of 0.72 g g^{-1} was obtained on thin wasted bread stillage in the process with pH control. The NaOH was selected as better neutralizing agent than $CaCO_3$ due to faster LA and biomass production, although the similar final LA concentration was achieved with using 1% $CaCO_3$. A maximal obtained number of viable *L. rhamnosus* cells of 8×10^9 CFU mL^{-1} was achieved on thin wasted bread stillage with NaOH pH control. A solid fraction of wasted bread stillage has shown high protein and nitrogen-free extract content and low content of crude fibers, which was an adequate composition of feed for monogastric animals.

Keywords: stillage, lactic acid, *Lactobacillus rhamnosus,* probiotics, biomass

1. Introduction

The residues, by-products and wastes of a complex chemical composition are produced in many processes in food and agricultural industry as well as in wood processing and forestry. With the growing population on Earth expected to reach 9 billion in 2050 [1], food chain, fuels and other energy inputs have to be reorganized to provide sustainability through rational utilization of all available resources. The importance of residues as feedstocks in biofuel production is recognized and articulated through the most recent legislation in EU [2]. Indirect land use change directive from April 2015 [2] regulates up to 7% on contribution of biofuels produced from crops grown on agricultural land, within total 10% target of renewable energy in road transport and a 0.5% subtarget for advanced biofuels, e.g. biofuels produced on wastes and residues. The production of bioethanol on residues and advances in hydrolysis of agricultural biomass and wastes were intensively studied during the last decades [3,4]. Searle and Malins [5] evaluated availability of wastes and residues adequate for production of advanced fuels in EU member states and reported that the goal of 0.5% of advanced fuels in transportation could be met. Hence, the amount of stillage as a main by-product of advanced bioethanol production is expected to grow in future.

Stillage is a main by-product of bioethanol production and depending on the process, up to 20 L of stillage is produced per 1 L of bioethanol with a total COD of stillage around 100 g/L [6]. Overall scheme of stillage production and utilization is presented in Fig. 1.

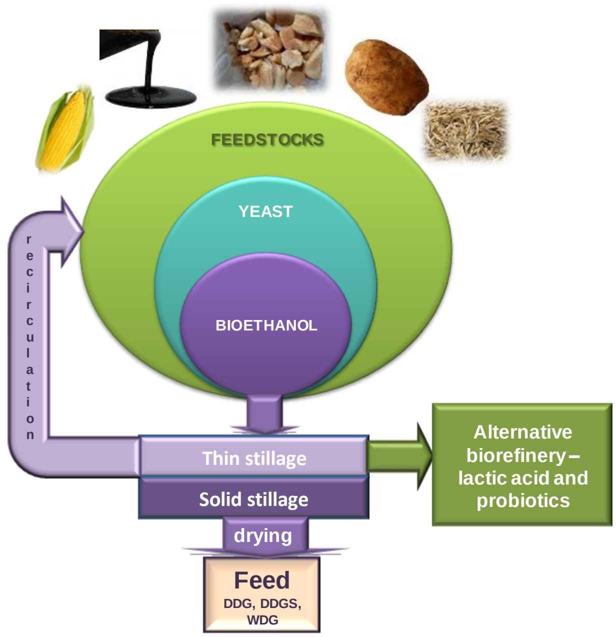

Fig.1 Schematic presentation of stillage production and utilization strategies

Because of high price of corn, bioethanol production in Serbia is mainly oriented on carbohydrate rich secondary feedstocks and residues (molasses, wasted bread, wastes from confectionary industry, wasted potato etc.) [7]. Smaller distilleries mostly sell stillage in wet form, due to their low capacity and high cost of energy for drying. With high content of water and high organic load, stillage is prone to souring and mould growth, which results in a short storage time in its raw form. Although the current legislation is pushing utilization of residues and wastes as substrates for bioethanol production, the production on corn is still predominant in the world and the valorization of corn stillage is widely studied [6,8,9]. Drying of corn stillage to produce dried distillers' grains with solubles (DDGS) for feed is practiced for long. The corn stillage is much easier to dry than rice, wheat or potato stillage. Also, utilization of stillage as a substrate for hydrogen [10], acetic acid, proteins [6], cyanobacteria and fertilizer production [11] or for microbial fuel cells [12] have been some of the strategies already described in literature. More developed and applied processes for utilization of stillage and other agroindustrial residues are mostly oriented on biogas production (anaerobic digestion) [13]. Alternative biorefinery concepts receive immense attention of scientific community to develop environmentally friendly processes for conversion of wastes to valuable chemicals and higher value products. Difficulties faced in fermentative biorefinery processes are numerous and mostly related to variability of chemical composition of wastes and residues (seasonal variability and variability due to the availability of feedstocks for ethanol fermentation), inadequate ratio of macro or micronutrients and presence of inhibitors of bacterial growth.

Lactic acid (LA) is an important chemical with a wide application in food industry and as an ingredient in pharmaceutical formulations, mainly because it is naturally occurring in humans and could be processed in metabolism [14]. It is predicted that demand for LA will exceed 1,960.1 kilo tons in 2020 [15]. The demand for LA is mostly driven by biomedical applications of LA polymers, polylactides (PLA) and poly(lactic-co-glycolic acid) (PLGA) [14]. These polymers are biodegradable, biocompatible and thermostable, used for food packaging, scaffolds production or drug delivery systems [16]. Today, economical and stereoselective production of LA with respect to green chemistry principles is considered as an imperative. Therefore, in lactic acid fermentation (LAF), the stillage, which is a serious pollutant, but of a suitable chemical composition for growth of LA bacteria [17] was studied.

In this paper, possibilities for LA production on stillages remained after production of bioethanol on corn, wasted bread and molasses were studied. *Lactobacillus rhamnosus* ATCC 7469, a homofermentative L (+) LA producing strain with probiotic characteristics [18] was used for LAF. The most important parameters of batch LAF on different stillages with or without pH control were analyzed. Also, the suitability of the solid fraction of wasted bread stillage for animal nutrition was assessed.

2. Material and methods

2.1. Substrates for lactic acid fermentations

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

Fermentation media consisted of 200ml of stillages with pH adjusted to 6.5 by 30% NaOH solution. After sterilization at 120˚C for 15 min, substrates were cooled and sugar concentration was adjusted to approximately 25 g L^{-1} by addition of a sterile 40% glucose solution. These way prepared samples were used as substrates for LAFs.

In the second set of experiments, the effect of pH control on LAF of stillage using NaOH [19] and $CaCO_3$ [20] was evaluated. Particularly, pH control was performed by addition of 1%, 2%, 5% and 10% (w/v) $CaCO_3$ and addition of 30% (w/v) NaOH to pH=6.5 in media in 4h intervals. After pH adjustment the stillage was sterilized (120˚C, 15 min) and a sterile 40% (w/v) glucose solution (up to final concentration of app. 25 g L^{-1}) was added. This way prepared media was used as a substrate for batch LAF.

2.2. Microorganism

Lactobacillus rhamnosus ATCC 7469, a homofermentative L (+) LA strain (99.7% L(+) - LA) with probiotic characteristics [18] 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 fermentation medium.

2.3. Lactic acid fermentation

Batch LAFs were performed in 500mL flasks with 200 mL of prepared substrates (Section 2.1.) 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% (v/v) overnight culture of *L. rhamnosus* ATCC 7469. During the fermentations samples were aseptically withdrawn and LA concentration, reducing sugar concentration and number of viable cells were determined.

2.5. Analytical methods

The dry matter percent was determined by a standard drying method in an oven at 105 °C to constant mass [21]. The protein content was estimated by Kjeldahl method as the total nitrogen and using factor 6.25 [21]. The lipid content was determined by Soxhlet method and ash content was determined by slow combustion method at 650 °C for 2h [21]. The concentration of reducing sugars, calculated as glucose, was estimated by 3,5-dinitrosalicylic acid method [22]. Calibration curve was set at 505 nm using standard glucose solutions. LA concentration was determined by enzymatic method (L-/ D-Lactic acid assay, Megazyme®, Wicklow, Ireland) after deproteinization of the sample as prescribed in the manufacturer's procedure. Chemical characterization of solid fraction of stillage after centrifugation at 4500 rpm for 20 min (Sigma® model 2-16, Shropshire, UK) was performed as described in Semenčenko et al. [23]. 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. All chemicals used in experiments were analytical grade.

2.6. Statistical analysis

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

3. Results and discussion

3.1. Chemical composition of different stillages

Stillages from bioethanol production on wasted bread stillage and molasses stillage were compared with corn stillage, as corn is the most exploited feedstock for bioethanol production generally [24, 25]. Solid part of stillage is mostly used for production of feed while liquid stillage drying is not economically feasible (Fig.1.) [8], so here the thin stillages were primarily used as LA fermentation substrates. Chemical composition of stillages subjected to LAF in this study is presented in Table 1.

Table 1. Chemical composition of studied thin stillages

	Thin corn stillage	Thin molasses stillage	Thin wasted bread stillage
Reducing carbohydrates (g L^{-1})	13.12 ± 0.70	4.20 ± 0.19	11.66 ± 0.68
Crude protein (g L^{-1})	8.42 ± 0.71	18.80 ± 0.61	21.00 ± 1.10
Ash (g L^{-1})	2.90 ± 0.32	17.60 ± 0.72	6.96 ± 0.23
Lipids (g L^{-1})	1.83 ± 0.45	0.80 ± 0.02	5.48 ± 0.81
Dry matter (%)	5.02 ± 0.29	6.61 ± 0.55	4.80 ± 0.48

The dry matter content is similar in all studied thin stillages and it is in correlation with literature data [8,26,27]. Also the amount of crude proteins, reducing carbohydrates, ash and lipids are within the values reported in literature for these substrates [8]. The protein content and reducing sugar content are the most important parameters for analysis of the substrate suitability for LAF. The lowest reducing sugar concentration was present in molasses stillage while in other stillages, it was around 12-13 g L^{-1}, because of effective previous ethanol fermentation. For the following LAF it was necessary to supplement all stillage based substrates with certain amount of glucose.

The wasted bread stillage was highest in protein content, followed by molasses stillage. Significant amount of proteins present in molasses and molasses based products originates from betain which could be beneficial for the LA production as recently reported by Xu and Xu [28]. The very high content of ash in molasses stillage is probably a result of high content of metals commonly present in molasses [6].

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

The kinetics of LA production and reducing carbohydrate utilization in thin corn stillage, thin molasses stillage and thin wasted bread stillage as substrates for LAF were presented in Figure 2. The most important parameters of fermentation processes on different substrates are given in the Table 2. The number of viable cells of *L. rhamnosus* ATCC 7469 during the LAF on three studied stillage substrates is presented in Figure 3.

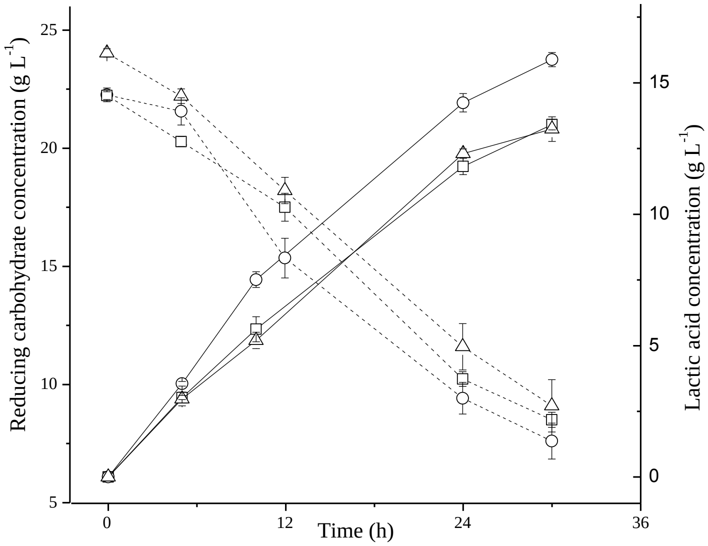

Fig. 2 The kinetics of LA production and reducing carbohydrate utilization on three different stillage based substrates. Experimental conditions: batch fermentation, micoraerophilic, shaking (100 rpm), 41˚C, 5% (v/v) inoculum concentration; Symbols: circle – thin wasted bread stillage, triangle – thin corn stillage, square – thin molasses stillage; dashed lines – reducing carbohydrate's concentration, solid lines – LA concentration

Kinetics of LA production and sugar consumption were similar for three studied substrates although the LA production on wasted bread stillage was faster and after 30h of fermentation the LA concentration was around 13 % higher (Fig. 2). Differences in LA production during the fermentations of molasses and corn stillage were not significant. The LA productivity and yield were highest in LAF on thin wasted bread stillage, so it could be considered as the most adequate for LA production by *L. rhamnosus* ATCC 7469. These results imply that thin stillage from advanced bioethanol production on wasted bread and molasses could be valorized in biorefinery process for LA production as comparable or even better substrate than thin corn stillage, which is the most abundant type of stillage worldwide.

The growth of *L. rhamnosus* ATCC 7469 cells was also the most intense on thin wasted bread stillage with more than 3×10^9 CFU mL^{-1} in the media without pH control and without supplementation with external nitrogen sources. 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 the first 12h of fermentation. The strain *L. rhamnosus* ATCC 7469 has a probiotic potential [18] and thus the utilization of the biomass together with other remains after LAF as animal feed could give an additional probiotic value. The number of viable probiotic cells attained in all studied substrates was within recommended values of $10^6 – 10^9$ CFU g^{-1} of feed [29,30].

The highest amount of proteins in wasted bread stillage (Tab.1.) and the absence of high content of metals which are commonly present in molasses [6] favors thin wasted bread stillage as the substrate for growth of LAB and LA production. 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 $g^{–1}$ and LA productivity of 0.60 g $L^{–1}$ $h^{–1}$) [31] and higher than the values obtained on food waste by indigenous *Lactobacillus* sp. (LA yield 0.46 g $g^{–1}$ and LA productivity of 0.28 g $L^{–1}$ $h^{–1}$) [32]. However, results obtained here are without pH control in media and without addition of external nitrogen sources. Amongst three studied stillages, thin wasted bread stillage was selected as the best substrate for LAF.

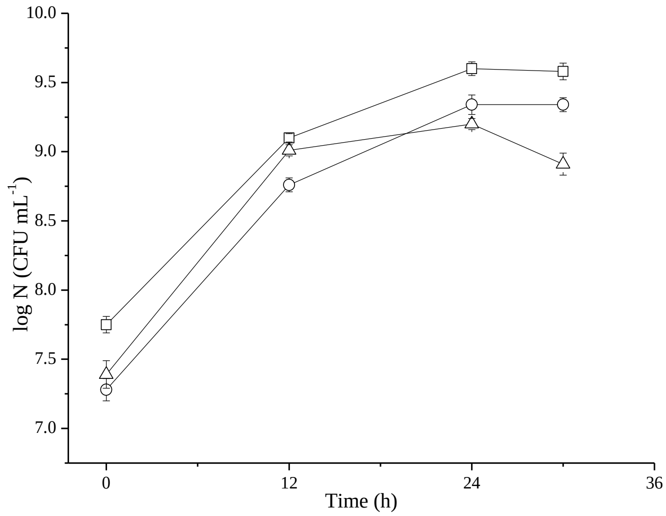

Fig. 3 The number of viable *L. rhamnosus* ATCC 7469 during LAF of three different stillages based substrates. Experimental conditions: batch fermentation, microaerophilic conditions, shaking (100 rpm), 41˚C, 5% (v/v) inoculum concentration; Symbols: circle – thin wasted bread stillage, triangle – thin corn stillage, square – molasses stillage

Tabel 2. The parameters of LAFs on thin corn stillage, thin molasses stillage and thin wasted bread stillage after 30h of fermentation

	LA concentration (g L^{-1})	LA yield (g g^{-1})	LA yield coefficient (g g^{-1})	LA productivity (g L^{-1} h^{-1})
Thin corn stillage	13.24 ± 0.20	0.55 ± 0.02	0.89 ± 0.03	0.44 ± 0.02
Thin molasses stillage	13.41 ± 0.47	0.60 ± 0.03	0.98 ± 0.05	0.45 ± 0.02
Thin wasted bread stillage	15.38 ± 0.27	0.69 ± 0.02	0.98 ± 0.03	0.51 ± 0.02

3.3. Effect of different neutralizing agents on lactic acid fermentation of thin wasted bread stillage

Importance of pH control in LAFs was extensively elaborated in literature [33,32] and different neutralizing agents were used: ammonia, $CaCO_3$, Na_2CO_3, NaOH etc. The $CaCO_3$ and NaOH were mostly used for pH control at around 6.5 which is an optimal value for the growth of *L. rhamnosus* ATCC 7469 and LA production. In order to improve performances of LAFs on thin wasted bread stillage NaOH and $CaCO_3$ as neutralizing agents were compared. The effect of different concentrations of $CaCO_3$ on LA production and growth of *L. rhamnosus* biomass is presented in Figure 4.

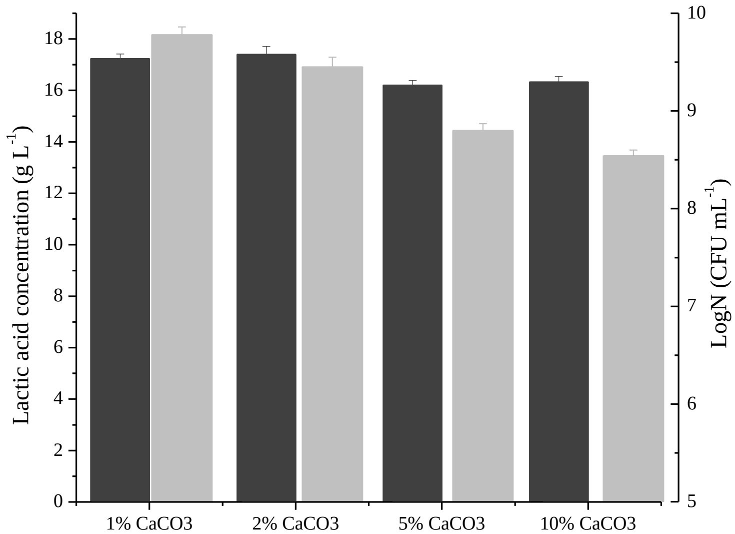

Fig. 4 LA concentration and number of viable *L. rhamnosus* ATCC 7469 cells in LAF of thin wasted bread stillage with $CaCO_3$ addition after 36h of fermentation. Experimental conditions: batch fermentation, microaerophilic conditions, shaking (100 rpm), 41˚C, 5% (v/v) inoculum concentration; Symbols: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

The addition of 2% $CaCO_3$ resulted in highest LA concentration of 17.40 g L^{-1}, with corresponding LA productivity of 0.57 g L^{-1} h^{-1}. 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% $CaCO_3$ and 2% $CaCO_3$ was not significant and the number of *L. rhamnosus* cells was significantly higher in samples with 1% $CaCO_3$, so in the next set of experiments 1% $CaCO_3$ concentration was used for pH control. Also, the effect of residual $CaCO_3$ concentration after the LAFs should be addressed and analyzed for potential application of the fermentation residues in animal nutrition.

$CaCO_3$ is used in animal nutrition, especially during the intensive growth or lactation of animals [34]. The maximal dietary allowance for $CaCO_3$ in feed is not established and recommended dietary allowance for cattle is around 0.31% on dry matter basis of feed daily intake [35]. The application of $CaCO_3$ in human and animal nutrition is generally accepted as safe [36], but intake of 5.9% on dry matter basis of feed daily intake is considered very high [37] and based on these findings more than 1% $CaCO_3$ in residual fermentation broth is considered high amount. Therefore, the residues of stillage fermented by probiotic biomass in the process of LA production with addition of 1% $CaCO_3$ could be used as a supplement in feed, in order to achieve good performances of LAF and good survival of probiotic biomass.

Additionally, in the process of LA extraction from fermentation media with $CaCO_3$ as neutralizing agent, significant amount of gypsum ($CaSO_4$) is remaining after addition of H_2SO_4. This is very important since gypsum represents resistant environmental contaminant and 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 microfiller in composites with PLA, polymers from LA for bone grafts and implants [38,16]. Valorization of the gypsum remaining in the LA production 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. This is an additional reason for application of as low as possible concentration of $CaCO_3$ in LAF.

For further comparison of NaOH and $CaCO_3$ as neutralizing agents, 1% $CaCO_3$ was used and the NaOH was added in 4 h intervals up to pH value of 6.5 is reached and the obtained kinetics of LA production is presented in Figure 5.

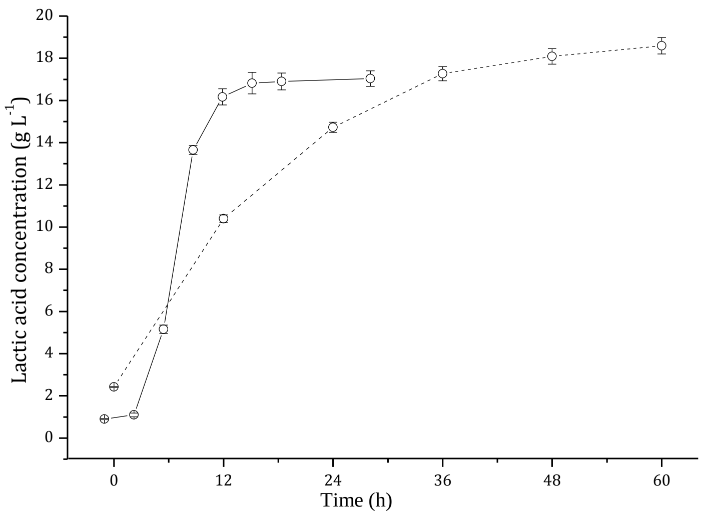

Fig. 5 The kinetics of LA production on thin wasted bread stillage with 1% $CaCO_3$ and addition of NaOH in 4h intervals as neutralizing agent. Experimental conditions: initial sugar concentration 22.26 g L^{-1}, microaerophilic conditions, shaking (100 rpm), 41˚C; Symbols: dot line – 1% $CaCO_3$, solid 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 $CaCO_3$. The maximal LA productivity of 1.14 g L^{-1} h^{-1} was achieved in with NaOH after 12h of fermentation while the highest LA productivity of 0.81 g L^{-1} h^{-1} with $CaCO_3$ addition was attained also after 12h of fermentation. Based on these findings, addition of NaOH as a neutralizing agent is better with respect to LA and biomass production on thin wasted bread stillage, with maximal LA yield of 0.77 g g^{-1} and very high maximal obtained number of viable cells (8×10^9 CFU mL^{-1}).

The highest attained LA productivity of 1.14 g L^{-1} h^{-1} was achieved after just 12h of fermentation and almost complete lactic acid production was finished after just 16h with LA yield of 0.72 g g^{-1} and residual sugar concentration of around 5 g L^{-1}. Although the LA concentration was not very high, this result was obtained with substrate supplementation with only low amount of glucose and without addition of expensive sources of nitrogen such as commonly used yeast extract. Because of high yield coefficient and productivity of this fermentation it could be expected that higher lactic acid concentrations could be obtained with higher supplementation of media with glucose or application of advanced fermentation strategies [39]. In similar batch LAF on waste sugarcane baggase maximal productivity of 0.93 g L^{-1} h^{-1} was obtained [40] and on wastewater sludge the maximal productivity was 0.23 g L^{-1} h^{-1} in simultaneous saccharification and fermentation (SSF) mode [41].

3.4. Analysis of solid fraction of wasted bread stillage for use in animal nutrition

In order to evaluate whole potential of wasted bread stillage, beside the explored utilization of thin fraction of the wasted bread stillage as substrate for lactic acid production, solid fraction was chemically characterized for use in animal nutrition. In the Figure 6, chemical composition of solid fraction of stillage is presented.

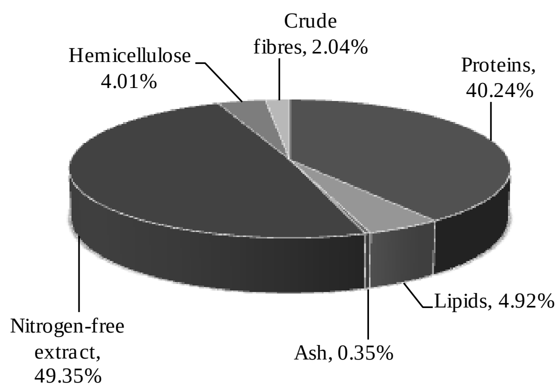

Fig. 6 Chemical composition of solid fraction of wasted bread stillage (dry matter basis)

Proteins amounted more than 48% of all components present in solid fraction of stillage and more than 40% of dry matter presents easily assimilative non protein components (nitrogen-free extract). The solid fraction could be considered a high value feed, especially for monogastric animals, due to low content of crude fibres and cellulose (Fig.6). Also, with around 80% of dry matter in solid fraction of stillage, the cost of its drying are not expected to be very high and content of proteins as well as NFE content is higher than in corn DDGS which has a long standing tradition of utilization as animal feed [23].

Adequacy of solid fraction of stillage as a feed for monogastric animals and intense growth and production of probiotic biomass and LA in LAF on thin wasted bread stillage enable valorization of complete wasted bread stillage from advanced bioethanol production.

4. Conclusions

Thin stillages from advanced bioethanol production of wasted bread and molasses production and from biothanol production on corn were studied as substrates for LAF. It has been shown that wasted bread and molasses stillages are similar or better substrates for LAF than the most abundant corn stillage. Because of recent legislation, advanced bioethanol production will grow and significant amounts of stillages will remain. The environmentally and economically challenging issue of stillage disposal could be possibly addressed through its utilization in lactic acid and probiotic biomass production. The productivity obtained in this study on thin wasted bread stillage of 1.14 g L^{-1} h^{-1} is similar or higher to that obtained on many other waste substrates previously used for LA production. In parallel, a high number of viable probiotic bacterial cells of above 10^9 CFU mL^{-1} was achieved. Solid fraction of the stillage, separated before LAF, was analyzed and it was found complementary with the needs of predominantly monogastric animals, especially in respect to high nitrogen-free extract and protein content and low crude fibre content. Therefore, thin stillage employment in integrated process for biomass and LA production with potential of solid fraction utilization as valuable animal feed could be a promising strategy for further optimization.

Acknowledgments

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