Non-thermal plasma for revalorization of a complex waste substrate in open lactic acid fermentation

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Abstract

Purpose: Stillage is the main by-product of bioethanol production and cost of its treatment significantly affects the economy of bioethanol production. Utilization of stillage for fermentative lactic acid (LA) and biomass production improves sustainability of bioethanol production. Standard process of thermal sterilization of stillage for biorefinery purposes is energy demanding and is causing deterioration of valuable compounds present in stillage. Modern biorefinery processes require energy efficient recovery of nutrients from stillage at different scales.

Methods: We replaced standard sterilization with ultrasound (UT) and plasma (PT) treatments and observed a significant reduction in number of viable microorganisms in the stillage after PT and UT. After applied treatment, we initiated lactic acid fermentation (LAF) by *Lactobacillus rhamnosus* ATCC 7469. Concentration of LA is used to quantify the efficiency of stillage revalorization.

Results: We find that the highest LA productivity of 1.21 g/Lh and yield of 0.82 g/g are obtained after PT. While, UT of 10 min provides productivity of 1.02 g/Lh and LA yield of 0.69 g/g. The results are benchmarked against closed LAF. We achieved 20% better revalorization of stillage by PT when compared with conventional sterilization. An excellent L (+) LA stereoselectivity of 95.5% is achieved after PT LAF. From the aspect of energy efficiency, PT was three times lower than UT and almost ten times lower than thermal sterilization.

Conclusions: This way we achieved a simpler and energy efficient process for LA production on stillage in „open" fermentation. These are new findings in fermentative treatment of stillage or similar by-products and wastes for the benefit of industry, academia and policy making.

Keywords: biorefinery, non-thermal plasma, open lactic acid fermentation, stillage, ultrasound

1. Introduction

Distillery stillage is the main by-product of bioethanol production on renewable feedstock. It remains after distillation of bioethanol and depending on the feedstock used for bioethanol production COD values of stillage can reach up to 100 g/L [1,2]. Approximately 20L of stillage are produced per every liter of bioethanol and environmental burden of stillage disposal is high [2]. Stillage has to be treated in order to decrease its organic load and its revalorization through production of chemicals is increasing competitiveness of bioethanol [3,4]. Besides anaerobic digestion, stillage is most often used after drying for animal nutrition, as dried distillers' grains with solubles (DDGS) [2,5]. Nutrients from stillage could be recovered through other processes also, including its utilization as fertilizer [6] or as substrate for fermentative production of bacterial cellulose, fungal biomass, 1,3–propanediol, malic and lactic acid (LA) [7–12]. Among proposed fermentative processes on stillage, almost all are „closed" fermentations which include thermal sterilization of substrate [7,9–11]. Thermal sterilization is energy demanding, costly treatment; it is difficult to apply in large scale processes and part of the nutrients is lost after thermal treatment [13]. Alternative to these are „open" fermentations when substrate is used without prior sterilization and fermented under unsterile conditions by a mixed culture of microorganisms [14]. Open fermentations are simpler, lower in energy consumption than conventional closed fermentations on sterilized substrates and could be adapted to various renewable and waste substrates due to the evolution capacity of microbiota, if process is well controlled [14]. Lower productivities, lower optical purity of product and contamination are still challenges in open fermentations [14,15] in parallel with necessary improvements in extraction techniques [16]. Control of fermentation conditions and possibility to manipulate substrate microbiota are crucial for efficient open fermentations.

Non-thermal treatments are an alternative to standard thermal sterilization for control of microorganisms in agricultural and wastewater substrates [17]. These treatments should enable selective inactivation of undesired microorganisms from complex microbial communities like those in stillage [18,19] but also preserve species capable of producing desired chemicals, like lactic acid for example. Non-thermal plasmas generate abundance of highly reactive species at low temperatures thus enabling processing of sensitive materials [20] and avoidance of thermal degradation of the compounds present in treated media. Beside reactive oxidative species, the UV photons and pulsed electric field generated during plasma treatment (PT) could contribute to the overall effect [21,22]. In a complex media with high dry matter content like stillage, the effects of non-thermal plasma treatment have not been thoroughly examined since most of the studies were performed in water, water based media with low dry matter content or on solid surfaces [22–26].

The high-power ultrasound treatment (UT) can be used for decontamination due to the mechanical disruption and cavitation effects induced in samples [27]. The microbial inactivation by UT is highly dependent on substrate composition, types of microorganisms present in media and treatment conditions [26,27].

LAFs on cheap substrates like stillage attract great interest since the LA market size was valued at 2.08 billion US $ in 2016 and is expected to grow by annual growth rate of 16.3% until 2025

[28]. The demand for LA is mainly driven by increasing utilization of LA as a platform chemical and for poly-lactides – biocompatible and biodegradable polymers suitable for pharmaceutical and food applications [15,28]. The concentration of LA produced in LAF on stillage is used in our work to quantify the efficiency of stillage revalorization.

The main objective of this study is to investigate plasma and ultrasound in LAF on stillage as an alternative to standard sterilization processes. The mechanism of non-thermal plasma inactivation of *Lactobacillus acidophilus* and *Escherichia coli* in water and stillage as media is thoroughly studied. Furthermore, non-thermal plasma inactivation of indigenous stillage microbiota was compared with inactivation by ultrasound treatment. The stillage after different treatments was subjected to open or closed LAF to assess its revalorization potential through LA production with high-LA producing strain of *Lactobacillus rhamnosus* [29]. The parameters of LAFs in terms of LA concentration, optical purity, yield and productivity were studied and compared for various applied treatments. In addition, all treatments were compared from the aspect of energy efficiency.

2. Materials and Methods

2.1. Preparation of distillery stillage

The distillery stillage remained after bioethanol production on wasted bread was obtained from ethanol producing facility (Reahem d.o.o., Serbia) and used for preparation of media for LAF. Chemical composition of the stillage was determined as following: dry matter content (12.79 ± 0.31%), sugar concentration (11.19 ± 0.83 g/L), protein (63.91 ± 2.81 g/L), free amino-nitrogen (295.6 ± 1.5 mg/L), ash (31.2 ± 0.1 g/L), lipids (17.36 ± 1.84 g/L). The pH value in all samples was adjusted to 6.5. The stillage was subjected to various decontamination treatments: PT, UT and thermal sterilization and it was used as the substrate for LAFs. The standard thermal sterilization was performed in an autoclave (Sutjeska, Serbia, device power 5.25 kW) at 121 ^{o}C for 20 min.

2.2. Microorganism

Lactobacillus rhamnosus ATCC 7469 and *Lactobacillus acidophilus* ATCC 4356, homofermentative LA producing strains and *Escherichia coli* ATCC 25922 were obtained from American Type Culture Collection. The *L. rhamnosus* and *L. acidophilus* cultures were propagated at 37 °C in Man Rogosa Sharpe broth (MRS) under microaerophilic static conditions. *E. coli* culture was propagated at 37 °C in nutrient broth, under aerobic static conditions. Overnight cultures were used as an inoculum in experiments.

2.3. Non-thermal plasma treatment

2.3.1. The effect of non-thermal plasma on G (+) and G (-) bacteria in water and distillery stillage

These experiments were undertaken at the beginning of the study in order to determine how non-thermal plasma acts towards *E. coli* (a representative of G (-) bacteria) and *L. acidophilus* (a representative of G (+) bacteria) in water and stillage media.

In the first set of experiments, the samples of water and stillage (6 ml) were sterilized by autoclaving (Sutjeska, Serbia) at 121 ^{o}C for 20 min and inoculated by overnight cultures of *E. coli* and *L. acidophilus* in order to set initial number of viable cells in samples at around 10^5 CFU/ml. Immediately

after inoculation, the samples were transferred in glass Petri dishes and subjected to PT for 30 min (duration of treatment was selected after preliminary studies) by using plasma needle jet. All treatments were conducted using a plasma needle operating at 25 kHz in ambient air. Argon was used as a feed gas (2 slm flow rate) in order to reduce the breakdown voltage through Penning ionization. The operating power was 2 W. The distance between the needle tip and the samples was 1 cm. Detailed description of the plasma device is provided by [30]. No significant increase of temperature in samples has been observed during treatments. During the treatments, the samples were mixed. The schematic presentation is shown in **Fig.1a**.

The second set of experiments was performed in order to determine effect of plasma generated UV photons on microbial inactivation in water and stillage. The samples were prepared and treated in the same way as previously, but quartz glass was placed between plasma jet and sample to prevent other effects of PT except UV light. Graphical presentation is provided in **Fig.1b**. The samples of sterilized water and stillage inoculated by *E. coli* and *L. acidophilus* were subjected to the same procedure but without PT, as a control.

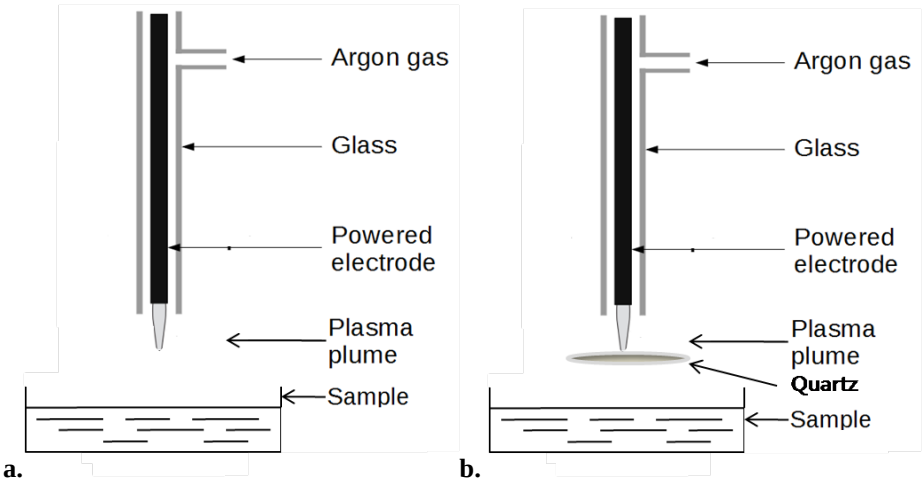

Fig.1 Schematic presentation of experimental setup for non-thermal PT (**a**) and assessment of contribution of the UV photons generated by plasma (**b**)

A number of viable cells in samples was determined using pour plate counting method on nutrient agar (for *E. coli*) and MRS agar (for *L. acidophilus*). Reduction in the number of viable microorganisms was presented as log reduction, log (N/N_0), where N - number of viable cells in samples and N_0- number of viable cells in control.

2.3.2. Non-thermal plasma treatment of distillery stillage for lactic acid fermentation

The samples of non-sterile stillage in 6 ml batches were placed in glass Petri dishes and treated by non-thermal plasma needle for 30 min (duration of treatment was selected after preliminary studies). The PT details are explained in Section 2.3.1. After the treatments and prior to LAF the samples were incubated under microaerophilic conditions at 41 ˚C (the same conditions which were used for LAF, studied in the second set of experiments and explained in section 2.5.) for 24 h in order to assess effect of

PT on the stillage microbiota. A number of total aerobic mesophilic bacteria in samples was determined using pour plate counting method as previously described on MRS agar as substrate [31].

2.4. High-power ultrasound treatment of distillery stillage

The stillage samples (60 ml) placed in 200 ml glass were treated by high-power ultrasound (Sonopuls HD 2200, Bandelin, Berlin, Germany, device power 200 W) with sonotrode TT 13 for 10 min (duration of treatment was selected after preliminary studies) at actual value of amplitude 75% and frequency of 20 kHz. After the treatment, in the first set of experiments, the samples were incubated under microaerophilic conditions at 41 ˚C for 24 h in order to examine the effect of treatment on the number of viable bacterial cells in the stillage. A number of total aerobic mesophilic bacteria in all samples was determined using pour plate counting method as previously described on MRS agar as plate substrate [31]. In the second set of experiments, the samples after the treatment were subjected to LAF (explained in section 2.5.).

2.5. Lactic acid fermentation

Stillage samples (60 ml per sample) treated by PT, UT and sterilized were subjected to fermentation for LA and probiotic biomass production. Untreated stillage was also subjected to LAF in the same way as treated samples. Initial glucose concentration in all samples was adjusted at around 35 g/L by addition of a 70% glucose solution and pH value was adjusted to 6.5. The LAFs were inoculated by 5% (v/v) *L. rhamnosus* ATCC 7469 while the untreated stillage was fermented by indigenous microbiota and considered as a control sample. The fermentations were performed as batch cultures with shaking in 200 ml flasks (100 rpm, KS 4000i control, IKA®, Germany), at 41 °C, under microaerophilic conditions maintained by using gas-pack system. These conditions were previously selected for the fermentation of stillage by *L. rhamnosus* ATCC 7469 [32]. During the LAF, the pH value in media was maintained at 6.5 by addition of 30% NaOH, in 4 h intervals.

2.6. Energy consumption calculations

The calculation of energy consumption of different treatments was performed by using manufacturers' information for lab scale equipment applied in experiments, taking into account a volume of samples subjected to the treatment. Energy of different treatments was calculated according to formula [33]:

$$E=P\times t, \quad (1)$$

where E is energy, P is power of device used for treatment and t is for duration of treatment.

The actual power of ultrasound treatment of stillage was calculated according to the procedure based on calorimetry and the following formula [26]:

$$P=m_s\times C_p\times \partial T/\partial t, \quad (2)$$

where m_s is mass of stillage media, C_p is specific heat at a constant pressure (J/gK), and $\partial T/\partial t$ is the slope at the origin of the curve (T is temperature, t is time). This equation is used to calculate the actual power of UT with a presumption that all of the power entering the system is dissipated as heat.

2.7. Methods of analysis

Chemical composition of the stillage was determined using methods described in our previous work [32]. The antioxidative activity of the stillage before and after the treatment against 2,2-Diphenyl-1-

picrylhydrazyl radical (DPPH, Sigma Aldrich, USA, CAS No. 1898-66-4) was determined as in the study of Jovanović et al. [34]. The stereoselectivity of produced LA was determined by enzymatic method (L(+)/D(-) LA assay, Megazyme®, Ireland). The LA and glucose concentrations during LAFs were determined by HPLC analysis. The samples were withdrawn from fermentation media, filtered through 0.22 μm filters (Minisart® syringe filters, Sartorius AG, Germany) and analysed by adapted HPLC method of Srivastava et al. [35]. In brief, the HPLC analysis was performed on the Dionex Ultimate 3000 Thermo Scientific (Waltham, USA) system. A reverse phase column (Hypersil gold C18, 150 mm × 4.6 mm, 5 μmL; Thermo Scientific, USA) at 65 ˚C was employed. Mobile phase was 5 mM H_2SO_4 (JT Baker, USA) with an elution rate 0.6 ml/min. Detection was performed by UV/VIS detector at 210 nm. All data acquisition and processing were done using Chromeleon Software. All chemicals used in experiments were of analytical grade and obtained from Sigma Aldrich, USA.

2.8. Statistical analysis

The experiments were done in duplicates, in three independent experiments. 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 for mean differences testing. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Interaction of non-thermal plasma and stillage – effect on chemical composition

The major challenge in processing of a complex medium such as stillage and similar wastewater is to achieve inactivation of specific microorganisms while preserving valuable compounds. Uchiyama *et al.* [36] report that argon cold atmospheric plasma as one used in our study can generate enormous amounts of $^{\cdot}OH$ radicals and H_2O_2 - the combination product of $^{\cdot}OH$ radicals in the aqueous phase even at distances of approximately 1 cm from the plasma source nozzle [36]. Superoxide $O_2^{\cdot -}$ can be present in the liquid phase in significant amounts, also [37,38]. Besides reactive oxygen species, plasma source is generating UV photons [39]. UV light has direct negative effect on bacteria and it can initiate photo dissociation of water and additional chemical reactions in treated media, therefore, interaction of non-thermal plasma with substrate like stillage is complex.

For effective fermentative processes, contents of reducing sugars and free amino-nitrogen are crucial and C/N ratio is most often determining productivity of the processes with LAB [32,40]. After the longest studied plasma treatment of 30 min, only a slight decrease in antioxidant activity was observed, from 93.5 ± 1.3% before to 90.1 ± 1.2% after the treatment. In other antioxidant rich substrates exposed to non-thermal plasma, no significant change in antioxidant activity was noted and it was confirmed using array of methods [41]. Some studies reported that antioxidants can act as scavengers for reactive species generated during PT [42]. Therefore, decrease in the antioxidant activity, as observed in our study, can be explained by a similar decrease in the concentration of enzymes and molecules associated to oxidative stress (vitamin C, polyphenol oxidase, peroxidase) which acted like scavengers in radical reactions initiated by PT [43].

The changes in the contents of reducing sugars and free amino nitrogen after the PT were not significant as confirmed by HPLC and spectrophotometric methods. Unlike other thermal methods, where

Millard reaction is occurring causing a loss of proteins and sugars by generation of melanoidins which are toxic for bacteria [44,45], non-thermal PT did not change the content of the most important compounds for stillage revalorization in LAF. Therefore, the effects of non-thermal PT on survival of different microorganisms in stillage were further examined.

3.2. Inactivation of model G (+) and G (-) bacteria in water and stillage by non-thermal plasma treatment

The effects of non-thermal PT on the survival of *E. coli*, G (-) bacteria, and *L. acidophilus*, G (+) bacteria, in different media were studied first. *E. coli*, being the most studied G (-) bacteria from the sanitary perspective [23], was used as a model of undesired G (-) microorganism in substrates. *L. acidophilus* was a representative of G (+) lactic acid bacteria (LAB) belonging to *Lactobacillus* sp. which are main constituents of indigenous stillage and food waste microbiota and responsible for LAF and silage of these substrates in uncontrolled conditions [18,19]. PTs were performed in water and stillage inoculated with the model G (-) and G (+) microorganisms.

The reduction in the number of viable *E. coli* and *L. acidophilus* in water and stillage by PT is presented in **Fig.2**. Reactive oxygen species ($^{\cdot}OH$, H_2O_2, $O_2^{\cdot -}$), reactive nitrogen species and UV photons created during PT all contribute to the antimicrobial effects of plasma [36–38]. We examined total microbial inactivation by PT and also isolated effects of UV photons generated by plasma on inactivation of bacteria (**Fig.2**).

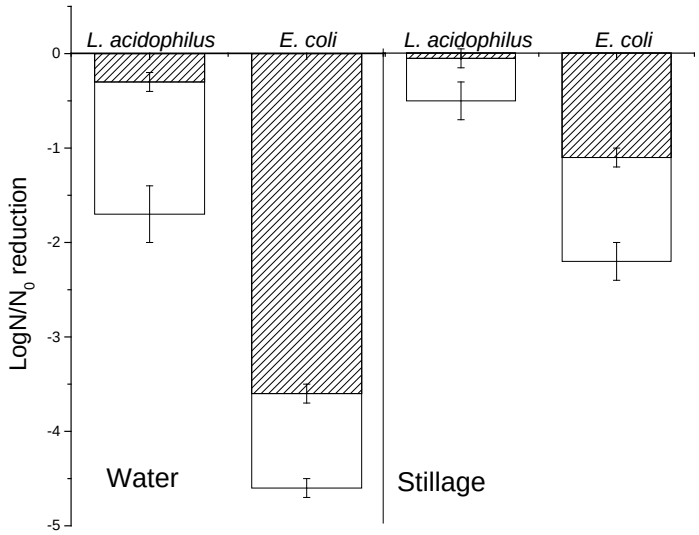

Fig.2 LogN reduction in the number of viable *E. coli* and *L. acidophilus* cells in different media. Symbols: white bars – inactivation by plasma generated radicals, patterned bars – inactivation by plasma generated UV photons

The higher reduction in viable cell number was obtained in water for both studied bacteria but in general *E. coli* was more sensitive to PT than *L. acidophilus* regardless of the media (**Fig.2**). The UV dependent inactivation of *E. coli* in water comprised up to 78% of the total logN reduction while in the

stillage it represented around 50% of the total inactivation. The dissimilarity in inactivation in different media is predominantly due to the UV dependent inactivation, especially for *E. coli*. Also, observed presence of scavengers in stillage (section 3.1.) could inactivate oxidative species produced by plasma source leading to lower microbial inactivation in stillage than in water. For *E. coli*, in absolute values, logN reduction mediated by UV photons decreased three times in stillage in comparison to water as media. The remaining contribution of reactive species to overall logN reduction has not been altered significantly with a change of media for stillage (**Fig.2**).

Because of turbidity of the stillage, the effect of UV radiation was less pronounced in stillage. These results emphasize the importance of penetration depth of UV photons and therefore a significance of the treatment chamber shape and overall treatment conditions, beside the most often reported volume of sample [39].

The inactivation of plankatonic *E. coli* cells in water obtained in this study was similar to results of Purevdorj et al. [46] and Puač et al. [23] but in more efficient time/volume ratio [23,46]. Geveke [47], Arrange et al. [48] and Mai-Prochnow et al. [49] reported higher susceptibility of G (-) bacteria to both UV light and cold PT in general. Boudam et al. [39] pointed that a fine adjustment of plasma operating conditions could increase the participation of UV light or radicals in overall bactericidal activity of PT. In this study, *E. coli* was found more susceptible to UV light component of PT compared to *L. acidophilus.* Therefore, PT in stillage enabled significant decrease in the number of *E. coli* while growth *L. acidophilus,* a representative of LAB, was minimally affected. This way, PT provided some selectivity in microbial inactivation in stillage, qualifying it as a promising strategy for treatment of stillage for revalorization in LAF.

3.3. Comparison of the effect of non-thermal plasma and ultrasound treatment on microorganisms in stillage

In the next set of experiments, PT was compared with high-power ultrasound. As an alternative non-thermal treatment, the UT method is already proven for microbial inactivation in less complex media like juices [26] and found effective in disintegration of fresh distillery spentwash [50]. The effects of non-thermal PT and UT on the growth of indigenous stillage microbiota were studied and the results are presented in **Fig.3**. The non-thermal PT caused higher reduction in viable cell number than UT. The number of viable cells did not increase during 24 h after the PT resulting in around 3 logN lower number of viable cells in PT than in control, untreated sample (**Fig.3**). Possible reason could be a slow growth and recovery of bacteria which survived the treatment. The lifetime of reactive species generated by plasma is extremely short but they initiate numerous reactions in media causing prolonged effects [51,52]. The application of plasma-activated water, water subjected to PT and then used for sanitation purposes, is based on this effect [53].

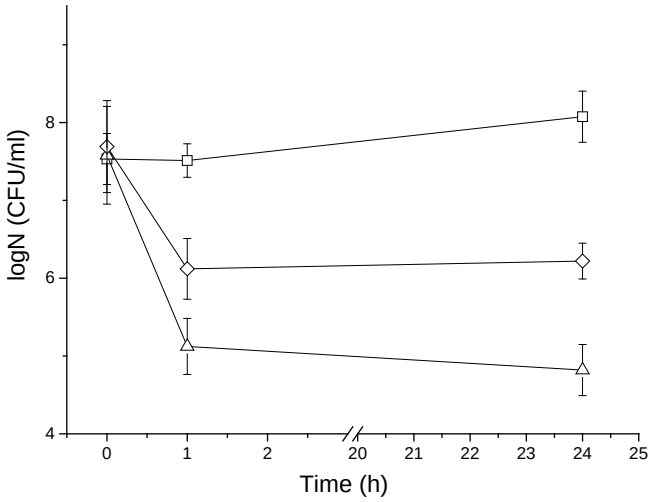

Fig.3 Number of viable cells of stillage microbiota in time after different treatments. Symbols: square – untreated stillage, romb – ultrasound treated stillage, triangle – plasma treated stillage

The decrease in the number of microorganisms achieved after PT (**Fig.3**) could be very useful for the control of contamination of the stillage. This provides longer storage time of stillage and more versatility in its utilizations. On the other hand, the presence of reactive species in stillage after PT can maybe negatively affect growth of LAB responsible for LA production and limit valorization potential of stillage for fermentation. Therefore, we inoculated stillages subjected to different treatments with a high L (+) LA producing strain *Lactobacillus rhamnosus* ATCC 7469 [7] and compared number of viable bacteria 24h after different treatments against untreated stillage also inoculated with *L. rhamnosus*. These results are presented in **Fig.4**.

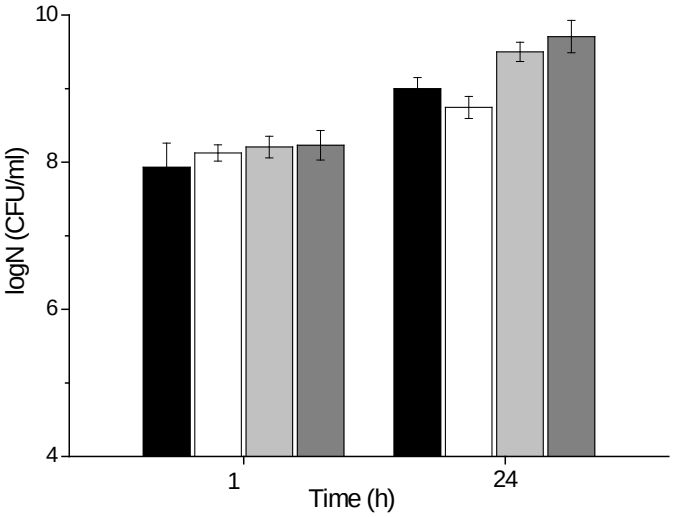

Fig.4 Number of viable cells in stillage after different treatments and inoculation with high LA producing strain *Lactobacillus rhamnosus* ATCC 7469. Symbols: black bars-untreated stillage, white bars- sterilized stillage, light grey bars- plasma treated stillage, dark grey stillage- ultrasound treated stillage.

When we compare the number of viable microorganisms after 24h with *L. rhamnosus* (**Fig.4**) and without *L. rhamnosus* (**Fig.3**), the higher number of bacteria was obtained with addition of *L. rhamnosus* than without it. Also, the growth was enhanced in PT and UT samples over untreated or sterilized (**Fig.4**). However, LAF performance of bacterial populations in PT and UT samples has to be benchmarked against untreated and conventional thermally treated samples in order to evaluate their potential for effective LA production.

3.4. Lactic acid fermentation of treated stillage by *Lactobacillus rhamnosus* ATCC 7469

The concentration of LA produced in LAF is used to quantify the efficiency of stillage revalorization. Additionally, optical purity of the produced LA is very important criteria for selection of the most promising process. *L. rhamnosus* ATCC 7469 is a high L (+) LA producing strain in mono culture, while in open fermentations on a complex media with mixed populations it can be difficult to achieve desired optical purity because different LAB produce different LA isomers. Two key factors have to be taken into account to achieve best treatment results - high sugar to LA conversion rate and stereoselective LA production.

After performing the treatments of the stillage (UT, PT, sterilization), all treated stillage samples and untreated control were inoculated with *L. rhamnosus* ATCC 7469 and subjected to LAF. The LA concentrations during open LAF, performed on PT, UT stillage and untreated stillage as substrates, and closed LAF, performed on sterilized stillage, are presented in **Fig.5**. In Table 1. the most important parameters of all studied LAFs are presented.

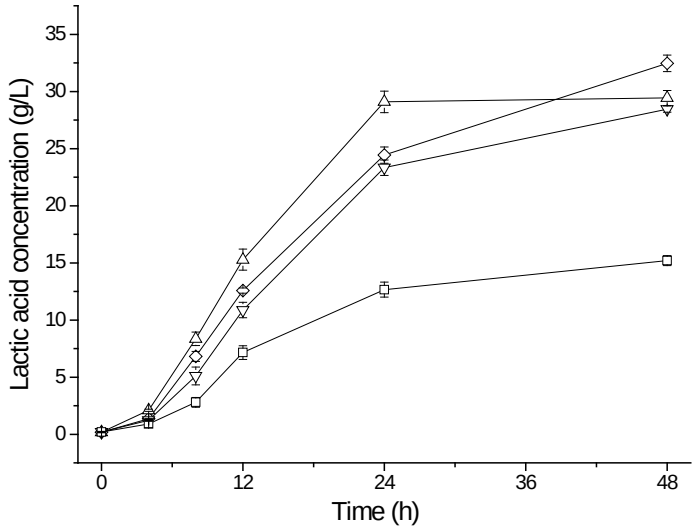

Fig.5 Kinetics of LA production during closed and open LAF on stillage subjected to different treatments. Symbols: square – untreated stillage, non-inoculated, open LAF; up triangle – non-thermal PT, inoculated by *L. rhamnosus* ATCC 7469, open LAF; diamond – non-thermal UT, inoculated by *L. rhamnosus* ATCC 7469, open LAF; down triangle – sterilized stillage, inoculated by *L. rhamnosus* ATCC 7469, closed LAF.

The most productive LAF was in PT samples directly followed by LAF in UT sample (**Fig.5**). Obviously, indigenous LAB preserved after treatments enhanced LA production. The highest LA

productivity in PT sample is a result of minimal deterioration of substrate because of absence of substrate heating during the treatment (section 3.1.) and significant suppression of competition of microorganisms in the substrate (**Fig.3**, **Fig.4**). Competition between indigenous microbiota of stillage and inoculated *L. rhamnosus* in untreated samples resulted in very low productivity of 0.57 g/Lh (Tab.1.) although this is still higher than the values reported for open LAF of kitchen waste [19] implying suitability of stillage as a substrate for LAF. Higher final LA concentration (in 48h) after UT can be explained by the presence of higher number of microorganisms in samples after UT (**Fig.4**). Although UT is often considered as non-thermal technique, ultrasound (10 min) elevated the temperature of stillage to 70˚C similarly to the reported UT of other substrates [26]. This plays additional role in microbial inactivation, deterioration of stillage and finally results in very similar productivities achieved in closed fermentation on sterilized stillage (0.97 g/Lh) and open fermentation on UT stillage (1.02 g/Lh). Besides yields and productivities, stereoselectivity of obtained LA is very important issue to be addressed.

In closed LAF, 97.2% of produced LA was L (+) isomer, while in the open LAF with PT, 95.5% of produced LA was L (+) LA, suggesting the prevalence of L (+) LA producing species after PT. Stereoselectivity of produced LA was lower in other samples. Although the final concentration was higher after UT, the diversity of LA producing strains (**Fig.4**) resulted in less stereoselective LA production, which is not desired. The highest values of LAF parameters were obtained in an open LAF performed after PT, being around 20% higher than in closed LAF with sterilized media. Therefore, PTs could be recommended as a good alternative to sterilization in order to achieve higher overall LA production with still high stereoselectivity. Inoculation by *L. rhamnosus* was necessary at the beginning of fermentation; otherwise the productivities on the stillage fermented solely by stillage microbiota were very low (Tab.1.).

The interaction of PT and UT with the stillage used for LAF was not previously studied, especially in the context of substrate pretreatments for open fermentations. The open LAF but without any physical treatment of substrate were studied with *Bacillus* sp., *Lactobacillus* sp. or *Streptococcus* sp. until now [13,19,54]. The highest LA productivities of around 2 g/Lh have been obtained in open LAF on food waste by *Streptococcus* sp. [13] and on synthetic substrate by *Enterococcus mundtii* QU 25 [55]. The average LA productivity of 1.04 g/Lh on lignocellulosic hydrolyzates by *Bacillus* sp. NL01, in an open fed-batch fermentation was reported [54] while on mixed restaurant food waste maximal reported LA productivity by *Lactobacillus* sp. was between 0.27–0.53 g/ Lh [13]. These are significantly lower values than the LA productivity of 1.2 g/Lh obtained in batch LAF on stillage after PT (**Fig.5**,Tab.1).

L. rhamnosus ATCC 7469 used in our study to produce LA has a proven probiotic potential [7]. After the separation of liquid fermentation broth with dissolved LA for extraction, solid remains with probiotic biomass of *L. rhamnosus* ATCC 7469 could be used as valuable additive for feed. It is demonstrated that in open LAF, the selected PT can significantly decrease the number of undesired G (-) microorganisms (**Fig.2**). Therefore, the PT could be a valuable tool to increase the safety of biomass and solid remains for utilization in animal nutrition.

3.4. Comparison of energy efficiency of different treatments

The estimated values of energy inputs of the performed pretreatments as well as amounts of LA

produced are presented in **Fig.6**. After different treatments all samples were subjected to LAF in the same way, therefore only energy consumption of pretreatments was included in calculation.

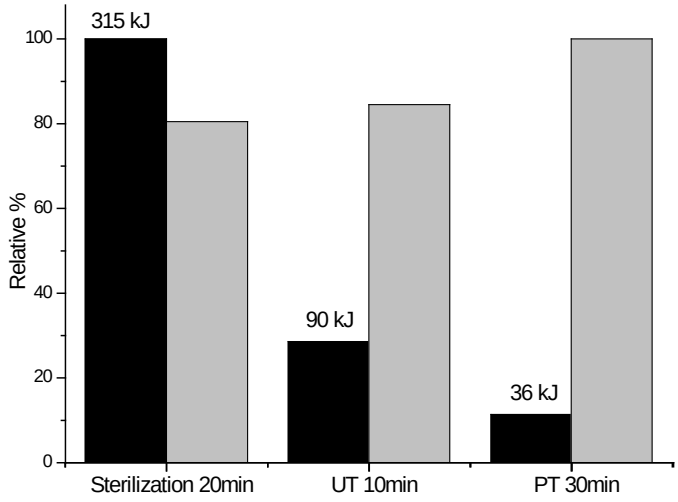

Fig.6 Estimate of required energy for different processes at laboratory level and mass of LA produced. Symbols: black bars – energy consumption of treatment in relative %, above bars is actual energy of treatment; grey bars – amount of LA produced in LAF after treatments in relative %

The lowest in energy consumption is non-thermal PT while, as expected, sterilization is the highest energy-consuming treatment. The actual values of energy consumption of PT, UT and sterilization amounted 36 kJ, 90 kJ and 315 kJ, respectively. The productivity of closed LAF solely by *L. rhamnosus* strain did not justify a high cost of energy for media sterilization. In the case of UT, only the part of energy spent during the treatment was actually delivered to the sample and this part is called actual energy of UT which is calculated based on calorimetric data for every studied sample [26]. Hence, although the UT was the second best regarding LA productivity, actual energy of UT delivered to stillage media is lower than the energy spent for UT (90 kJ) and amounts around 29.5 kJ. Therefore, only one third of spent energy was used in the process for improvement of LAF.

4. Conclusion

The microbial inactivation by non-thermal PT is highly influenced by substrate. We find that there are two reasons for lower reduction in stillage than in water. The turbidity of the stillage is causing a lower penetration of plasma generated agents decreasing their microbial inactivation efficiency. The high concentration of antioxidants in the stillage is also explaining lower logN reduction noticed in the stillage in comparison to water.

E. coli was more susceptible to PT than *L. acidophilus,* regardless of substrate. PT has shown selectivity towards G (-) microorganism and a resistance of G (+) LAB. This recommends the PT as a promising technique for the control of microorganisms present in distillery wastewater.

By comparing PT and UT we find that a 30 min long PT shows superior characteristics of up to 2.5 logN reduction, while UT also induced a decrease in the number of viable microorganisms in stillage

but to lower extent. A 20% higher LA productivity was achieved in open LAF by *L. rhamnosus* ATCC 7469 after PT than in open LAF after UT of distillery stillage. Besides being the most effective, the PT was the lowest in energy consumption and maintains stereoselectivity of LA production.

The PT provided the most effective revalorization of stillage through LAF with the highest LA productivity and the lowest energy consumption. This was achieved in the open fermentation mode which is much simpler process. Further adaptations for PT in larger scale could significantly influence effectiveness and improve the economy of process with integrated PT.

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Tables

Table 1. Important parameters of open and closed LAFs performed on distillery stillage media

Characteristics of LAF	Distillery stillage treatment	LA concentration [c] (g/L)	LA yield (g/g)	LA volumetric productivity [c] (g/Lh)
Open LAFs [d]	non-thermal PT [a]	29.09±0.94	0.82±0.03	1.21±0.04
	UT [b]	24.43±0.71	0.69±0.02	1.02±0.03
Closed LAF [d]	sterilization	23.33±0.68	0.66±0.02	0.97±0.03
Open LAF	untreated	13.66±0.65	0.38±0.02	0.57±0.03

[a] 30 min PT, [b] 10 min UT, [c] after 24 h of LAF, [d] Samples inoculated by *L. rhamnosus* ATCC 7469 after treatments