

Distillery Stillage as a New Substrate for Lactic Acid Production in Batch and Fed-batch Fermentation

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Lactic acid and biomass production on distillery stillage - a by-product of bioethanol industry - was studied in this paper. Batch and fed-batch fermentation strategies for lactic acid production on distillery stillage were performed by *Lactobacillus rhamnosus* ATCC 7469. The most appropriate initial sugar concentration in the stillage was determined and different fermentation strategies were studied under selected conditions. The most efficient sugar conversion in batch fermentation was attained with the initial sugar concentration of 55 g/L. Enhancement in lactic acid productivity (up to 1.80 g/Lh) and a very high number of viable cells (10^8 CFU/mL) were achieved in fed-batch fermentation. The high lactic acid concentration attained in fed-batch fermentation opened a new possibility for stillage processing. In addition, extensive bacterial growth achieved during fermentation of the stillage could bring additional value to the process through utilization of the spent media for animal feed formulae enriched in lactic acid bacteria.

1. Introduction

Bioethanol research in the world is driven by a constant need for cost reduction. Use of cheaper substrates such as ligno-cellulosic biomass or agro-industrial wastes could improve the profitability of the process. An alternative approach is the utilization of by-products of bioethanol facilities which could bring the additional value to the bioethanol production process. In Serbia, bioethanol production is mainly based on starch feedstock and molasses. Approximately 13 hL of stillage remains per every hL of bioethanol produced on starch substrates (Kim et al., 1997). According to Biomass Action Plan of the Republic of Serbia, bio-fuels should substitute 2.28 % (calculated on energy content) of transportation energy needs in our country by the end of 2012 (Ministry of Energy and Mining of the Republic of Serbia and NL Agency, 2010). This will result in a large amount of residual stillage with complex chemical composition and BOD₅ values in the range of 15-340 g/L depending on the source (Pejin et al., 2009).

On the other hand, the complexity of stillage makes it suitable as a substrate for fermentation. The stillage has been used for production of single cell protein (SCP); as a media component for production of acetic acid; as a fertilizer; and as a substrate for biogas and hydrogen production (Wilkie et al., 2000). Also, it has been utilised for animal feed: either as distillation wet by-products without treatment or in dried form (DDGS - Dried Distillers Grain with Solubles) (Larson et al., 1993). Because of high energy needs for drying process, alternative possibilities of stillage use are being investigated.

The lactic acid and biomass production on the stillage is a new approach. Lactic acid is used as a flavour and preservative in food and pharmaceutical industries. It is also used as a monomer for the production of poly-lactides, polymers with favourable characteristics for medical applications (biodegradability, thermo stability, elasticity etc.). On the other hand, Serbia imports lactic acid; therefore, its production on cheap and abundant substrates like stillage could be a promising strategy.

Traditional feedstock for lactic acid production is starch based substrates which are also utilized for food production (Bilanović et al., 2011). To avoid a competition with food industry, cheap by-products and waste substrates are recommended generally for fermentative production of chemicals (Ozalp and Hyman, 2009). However, they are usually deficient in nitrogen and their conversion efficiency to lactic acid is lower. Currently, the inulin-rich biomass of Jerusalem artichoke (Choi et al., 2012) which could be cultivated on a low quality soil; ligno-cellulosic feedstock like corn stover (Cui et al., 2011); and even algal biomass (Nguyen et al., 2012) are in research focus as a cheap carbon source for lactic acid production.

In order to enhance the process productivity, fed-batch fermentation is widely studied. The highest productivities to now have been achieved on synthetic media (Ding and Tan, 2006), while data on lactic acid production on waste substrates and agro-industrial by-products are very limited (Roukas and Kotzekidou, 1998). Fed-batch processes enable control of the determining parameters in the optimal range; this strategy is very effective to overcome the substrate inhibition and maintain pH (Bai et al., 2003). As far as we know, there are no reported results in literature for fermentative lactic acid production on an industrial waste bread stillage. However, some research has been performed on distillery waste water from bioethanol production on corn (Zhang et al., 2011; Mojović et al., 2011) and triticale (Marković et al., 2011). In this study, *Lactobacillus rhamnosus* ATCC 7469 was used to investigate batch and fed-batch lactic acid fermentation of the whole stillage remained after bioethanol production on waste bread. The remained fermentation media with LAB biomass could be used as an animal feed with improved nutritional characteristics.

2. Experimental

2.1. Stillage preparation

The stillage remained after bioethanol production on wasted bread was obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia). The pH of the stillage was adjusted to 6.5 with 30 % solution of NaOH and sterilized at 120 °C for 15 min. The concentrations of reducing sugars in the stillage were set at 55, 75 and 85 g/L by addition of sterile 70% glucose solution in the first set of batch experiments, in order to select optimal initial sugar concentration for lactic acid production. In second set of experiments, fed-batch fermentation strategy was studied. Feeding solution was supplied to after decline in sugar concentration in fermentation media below 20 g/L with aim to maintain the sugar concentration around 50 g/L. Feeding solution was a sterile stillage enriched with glucose at concentration of 140 g L⁻¹. The feeding was performed until the fermentation flask was filled up to 70 % of the complete volume.

2.2. Microorganism

Lactobacillus rhamnosus ATCC 7469 used in this experiment was obtained from American Type Culture Collection (ATCC, Rockville, USA). The culture was propagated in Man Rogosa Sharpe medium (MRS) (Fluka, USA), under anaerobic conditions in a gas pack system at 37 °C for 18 h before inoculation to fermentation medium. The fermentation was initiated by addition of 5 % (v/v) of inoculum.

2.3. Lactic acid fermentation

All lactic acid fermentations were performed under previously optimised conditions (pH, temperature and shaking) (Djukić-Vuković et al., 2012). During the fermentations, flasks were shaken (150 rpm, KS 4000i control, IKA®, Germany) at temperature of 41 °C. In fermentation media, pH was maintained at 6.5 by addition of 30 % solution of NaOH in two hour intervals. The fermentations were performed in 1000 mL flasks with initially 400 mL of the fermentation media in a gas pack system (Anaerocult® bags, Merck KGaA, Darmstadt, Germany). The fermentation experiments were done in triplicates. During the fermentation: pH, sugar consumption, lactic acid concentration and the number of living cells were analyzed.

2.4. Analytical methods

The dry matter percent was determined by a standard drying method in an oven at 105 °C to constant mass (AOAC, 2007). The protein content was estimated by Kjeldahl method result multiplied by factor 6.25 (AOAC, 2007). The lipid content was determined by Soxhlet extraction method and ash content was determined by slow combustion method at 650 °C for 2 h (AOAC, 2007). The concentration of reducing sugars was estimated by 3, 5-dinitrosalicylic acid method (Miller, 1959). Alpha-amino nitrogen was determined by ninhydrin method (Kalant, 1956). Lactic acid concentration was determined by enzymatic method (L-/ D-Lactic acid assay, Megazyme®, Wicklow, Ireland) after deproteinization of the sample according to procedure prescribed in assay. Number of viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique on MRS agar. All chemicals used in experiments were analytical grade. The measurements 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. Differences were considered significant at $p < 0.05$. Vertical bars in figures represent standard deviation of three measurements.

3. Results and discussion

3.1. Chemical composition of stillage

The chemical composition of the stillage used as a fermentation media in our study is presented in Table 1. Because of the modest content of reducing sugars due to advanced previous alcoholic fermentation, it was necessary to supplement it with glucose. Relatively high amount of proteins (more than 50 % of dry matter), indicated that the stillage could be suitable as a substrate for the growth of nutritionally fastidious lactic acid bacteria (LAB).

Table 1: Chemical composition of distillery stillage

Chemical composition of stillage	Content
Total reducing sugar (% of dry matter)	9.74 ± 0.04
Lipid (% of dry matter)	8.49 ± 0.12
Protein (% of dry matter)	58.50 ± 0.12
Ash (% of dry matter)	21.47 ± 0.20
Dry weight (% of total stillage)	11.55 ± 0.30

Residuals of yeast cells which are present in distillery stillage are rich in nitrogen and vitamins and could improve the fermentation by LAB. Comparing the content of alpha amino nitrogen in the stillage prior to fermentation (236.8 mg/L) and at the end of fermentation time (274.3 mg/L), it could be presumed that this particular strain could degrade proteins and provide nitrogen in the form accessible for utilization and growth. It has been documented that *L. rhamnosus* strains possess proteolytic activity (Savijoki et al., 2006) which can improve accessibility of nitrogen from different sources present in the fermentation media.

3.2. Batch fermentation

The effect of different initial sugar concentrations on lactic acid production during the fermentation is presented in Figure 1. For evaluation of the most promising initial sugar concentration, efficiency of sugar to lactic acid conversion is presented in Figure 2.

During the first 34 h of fermentation, the lactic acid production was faster in samples with lower initial sugar concentration. The highest productivity of 1.49 g/Lh was achieved with initial sugar concentration of 55 g/L and lactic acid yield of 92.3 % was reached with almost complete utilization of sugar after 34 h of fermentation. Lactic acid concentration continued to rise in samples with initial sugar concentration of 75 g/L and 85 g/L after 34 h of fermentation, although the productivities achieved (1.18 g/Lh and 1.29 g/Lh, respectively) were lower than those obtained in samples with 55 g/L.

The highest lactic acid concentration of 75.06 g/L was attained with highest initial sugar concentration of 85 g/L, but the yield (88 %) and productivity (1.29 g/Lh) were lower. This is probably a result of substrate inhibition. The significant inhibitory effect of sugar concentrations above 50 g/L on lactic acid production in batch fermentations was annotated by other authors, too. In fermentation by *L. lactis*, the highest productivity was achieved with initial sugar concentration of 30 g/L and it progressively declined by raising the concentrations up to 90 g/L (Bai et al., 2003).

Because of complex chemical composition of waste substrates and their variability, it is difficult to compare reported lactic acid concentrations and yields without a thorough analysis. Certainly, the chemical variability of used substrates is an important issue. Coelho et al. (2010) reported a maximal lactic acid concentration of 41.65 g/L after 48 h of fermentation on cassava wastewater with initial sugar concentration of 50 g/L. Enrichment of media with complex nitrogen sources resulted in very high productivity of 3.73 g/Lh of a batch fermentation by *L. rhamnosus* on white rice (Li et al., 2012). The yield in their fermentation was lower (81.72 %) than that achieved in our study, probably due to better balance of important nutrients for lactic acid production in the whole waste bread stillage.

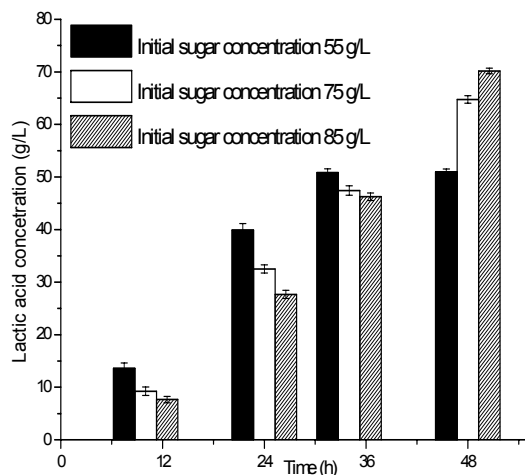


Figure 1: The lactic acid production on distillery stillage with different initial sugar concentrations.

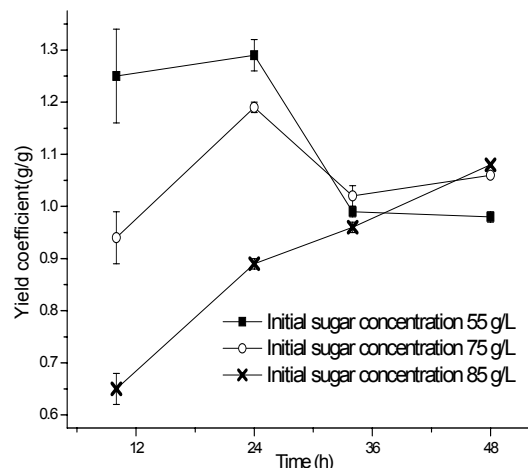


Figure 2: Time course of yield coefficient values in lactic acid fermentation with different initial sugar concentrations.

Zhang et al. (2011) reported lactic acid concentration of 68 g/L and yield of 0.78 g/g on a very high density distillery wastewater (with sugar concentration of 87 g/L), the substrate relatively similar to the whole stillage used in our study. In contrast to our study, though, this substrate was supplemented with mineral salts, yeast extract and pepton and fermented with newly isolated strain *Enterococcus hawaiiensis* CICIM-CU B0114 (Zhang et al., 2011). Utilization of corn, triticale and waste bread liquid stillages as substrates resulted in lactic acid yields of 0.50 g/g (Mojović et al., 2011), 0.76 g/g and 0.80 g/g, and these values are lower than the maximal yield obtained in this study (Djukić-Vuković et al., 2011).

The highest yield coefficient of 1.31 g/g was obtained in samples with initial sugar concentration of 55 g/L after 24 hour of fermentation (Fig. 2). The trend of decline of yield coefficient in samples with lower initial sugar concentration (55 g/L and 75 g/L) after first 24h of fermentation time implicates that the conversion of sugars was efficient from the beginning of fermentation, because the optimal conditions were achieved at the start of fermentation. On the contrary, in samples with high initial sugar concentration (85g/L), at the beginning of fermentation sugar could not be converted into lactic acid efficiently because of substrate inhibition effect and the yield coefficient was rising during the fermentation in accordance with the decline in sugar concentration. The sugar concentration of 55 g/L was therefore found optimal for efficient lactic acid production, an in the following set of experiments fed-batch fermentation was performed with sugar concentration of around 50 g/L.

3.3. Fed-batch fermentation

Time course of fed-batch fermentation is presented in Figure 3. Maximal lactic acid concentration of 97.1 g/L, a yield of 0.87 g/g, productivity of 1.80 g/Lh and viable cell number of 10^9 CFU/mL were achieved after 54 h of fermentation. The lactic acid concentration was significantly higher (47.6 %) compared to that obtained in batch fermentation. Fed-batch fermentation mode enabled control of substrate concentration in optimal range without inhibition effect, so it could be a strategy for intensification of lactic acid production. Meng et al. (2012) reported increased lactic acid productivity on a synthetic media supplemented with peanut meal as a nitrogen source. In their study, a single-pulse fed-batch fermentation mode was selected as the most effective and it resulted in a final lactic acid concentration of 180 g/L. An average productivity of 1.61 g/Lh within 112 h of fermentation time was achieved (Meng et al., 2012). Furthermore, Zhang et al. (2010) obtained 96.3 g/L of lactic acid in pH controlled feeding fed-batch fermentation on a synthetic media enriched with yeast extract, peptone and minerals. A high lactic acid concentration of 180 g/L was also reported by Ding et al. (2006) during 84 h of fed-batch fermentation on a synthetic media with exponential addition of glucose and yeast extract.

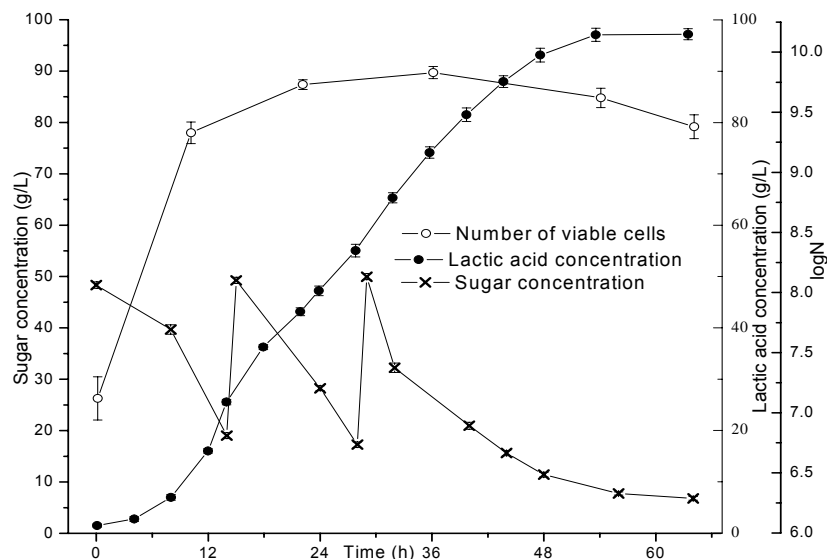


Figure 3: Time course of fed-batch fermentation on distillery stillage. Vertical bars represent \pm standard deviation of three measurements.

In contrast to synthetic substrates, a feasibility of the fermentations on the real waste substrates is a challenge and data on these processes are limited. Deproteinized whey was studied as a substrate in fed-batch fermentation and the yield of 0.77 g/g and lactic acid concentration of 46 g/L were achieved (Roukas and Kotzekidou, 1998). Productivity of 1.80 g/Lh obtained in our study is even superior compared to the productivity obtained in the study of Meng et al. (2012) on synthetic substrates.

4. Conclusions

Distillery stillage coming as a by-product from bioethanol production on wasted bread could be a good substrate for production of lactic acid and biomass. In batch fermentation, the most effective conversion of sugars to lactic acid was achieved with initial sugar concentration of approximately 55 g/L. Fed-batch fermentation with sugar concentration control improved the fermentation productivity. Final lactic acid concentration and productivity were increased for 47.6 % and 17.2 %, respectively compared to batch process, while high viable cell number above 10^9 CFU/mL of *L. rhamnosus* ATCC 7469 was reached at the end of the fermentation.

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