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PRODUCTION OF LACTIC ACID ON LIQUID DISTILLERY STILLAGE
PROIZVODNJA MLEČNE KISELINE NA TEČNOJ DESTILERIJSKOJ DŽIBRI

Ljiljana MOJOVIĆ[*], Maja VUKAŠINOVIĆ-SEKULIĆ[*], Aleksandra ĐUKIĆ[*], Dušanka PEJIN[**], Marica RAKIN[*], Jelena PEJIN[**], Svetlana NIKOLIĆ[*]
[*]Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11120 Beograd, Serbia,
[**]Faulty of Technology,, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
e-mal: lmojovic@tmf.bg.ac.rs

SUMMARY

Bioethanol produced by fermentation of biomass is one of the most important renewable and ecologically friendly energy resources today. After bioethanol distillation a considerable amount of liquid stillage remains (1hl of the produced bioethanol, approximately 13 hl of liquid thin stillage is formed). Since the stillage contains high concentrations of organic and inorganic substances, it may cause serious environmental problems if it is disposed to water flows untreated. The aim of this work was to investigate potential of application of corn thin stillage as an inexpensive renewable feedstock for lactic acid production. In this way, it is possible to solve ecological problems and to improve the economy of bioethanol production taking into account growing demands for lactic acid for utilization in pharmaceutical, food, chemical, textile and leader industry. Lactic acid fermentation was conducted with nine different species from genera Lactobacillus and their growth, sugar utilization and lactic acid production on corn thin stillage were compared. The most productive strains were two facultatively heterofermentative species Lb. paracasei ssp. paracasei NRRL B-4564 and Lb. casei ssp. casei NRRL B- 441, which had almost identical kinetics of lactic acid production during the first 48 hours, and after that the rate of lactic acid formation decreased more for strain Lb. casei ssp. casei NRRL B- 441. For this reason the species Lb. paracasei ssp. paracasei NRRL B- 4564 was selected for further study of effect of temperature, oxygen and shaking on lactic acid production. The lactic acid production was maximal at 41°C, without shaking, under anaerobic conditions. However, the biomass production was better at 30°C, also without shaking, under anaerobic conditions.

***Key words**: lactic acid, fermentation, Lactobacillus, corn liquid stillage, bioethanol.*

REZIME

Bioetanol koji se proizvodi fermentacijom biomase predstavlja izuzetno značajno i ekološki pogodno biogorivo čija se proizvodnja danas konstantno povećava. Nakon proizvodnje bioetanola zaostaje značajna količina džibre kao otpadnog proizvoda (na 1 hl proizvedenog etanola nastaje oko 13 hl tečne džibre). Zbog prisustva značajnih količina organskih i neorganskih jedinjenja ispuštanje tečne džibre u vodene tokove može prouzrokovati ozbiljna ekološka zagadjenja. Cilj ovog rada je da se ispita mogućnost korišćenja tečne džibre iz proizvodnje bioetanola na kukuruzu kao sirovine za proizvodnju mlečne kiseline. Na ovaj način je moguće rešiti problem otpada i značajno povećati ekonomičnost procesa proizvodnje bioetanola, imajući u vidu rastući trend u proizvodnji mlečne kiseline i njenu sve veću primenu u farmaceutskoj, hemijskoj, prehrambenoj, tekstilnoj i kožnoj industriji. U radu je ispitana mlečno kiselinska fermentacija devet vrsta bakterija iz roda Lactobacillus u toku koje je praćen njihov rast, iskorišćenje šećera iz supstrata (tečne džibre) i koncentracija mlečne kiseline. Najbolja aktivnost uočena je kod fakultativno heterofermentativnih vrsta Lb. paracasei ssp. paracasei NRRL B- 4564 i Lb. casei ssp. casei NRRL B- 441, kod kojih je zapažen gotovo identičan tok mlečne fermentacije u toku prvih 48h, nakon čega brzina obrazovanja mlečne kiseline brže opada kod vrste Lb. casei ssp. casei NRRL B- 441. Zbog toga je za dalji rad izabrana vrsta Lb. paracasei ssp. paracasei NRRL B- 4564 sa kojom je ispitan uticaj temperature, kiseonika i mešanja na proizvodnju mlečne kiseline. Najveći sadržaj mlečne kiseline je postignut u fermentaciji na 41°C, u statičnim, anaerobnim uslovima. Proizvodnja biomase je bila bolja pri temperaturi od 30°C, takođe bez mešanja, pod anaerobnim uslovima.

***Ključne reči**: mlečna kiselina, fermentacija, Lactobacillus, tečna džibra, bioetanol.*

INTRODUCTION

Bioethanol is currently one of the most important biofuels which is both renewable and environmentally friendly (Balat and Balat, 2009, Radosavljević et al, 2009). It is produced by fermentation of sugar, starch or cellulosic biomass and its utilization can significantly reduce petroleum use and exhaust greenhouse gas emission (Mojović et al, 2009). Because of that, it is expected to be one of the dominating renewable biofuels in the transportation sector within the twenty years to come (Goldemberg, 2008). The production of this fuel is increasing over the years, and has reached the level of 73.9 billion liters during the year 2009 (Semenčenko et al, 2011). Long-term predictions estimate the bioethanol production will reach more than 125 billion liters until 2020 expecting the support by governmental policies and exemptions (Demirbas, 2007).

Major byproducts of bioethanol production are stillage and carbon dioxide. Some economical analyses have shown that an adequate utilization of the by-products from bioethanol processing can significantly improve the bioethanol production economy (Mojović et al, 2007). An average stillage amount produced in the bioethanol process is approximately 13 hL per hL of bioethanol (Mojović et al, 2007, Kim et al, 1997).

There are many possibilities for valorization of stillage from bioethanol processing. Some of them are the stillage recirculation and reuse (Pejin et al, 2009, Mojović et al, 2010), production of soil fertilizers (Banković-Ilić et al, 2007), anaerobic fermentations for the production of lactic acid or butanol (Marković et al, 2011, Vukašinović-Sekulić et al, 2010, Krzywonos et al, 2009) and the production of various types of animal feed (Mojović et al 2010, Rakin et al.2009). In USA, around 85 % of the liquid stillage has been dried together with spent grains to produce dry distiller's grains with solubles (DDGS) which are being used as animal feed. In Europe, the most of the stillage for animal feed is used in wet form because the drying itself is a costly process which requires a lot of energy (Banković-Ilić et al, 2007). In the majority of industrial facilities in Serbia, the bioethanol by-products have not been utilized posing therefore a hardly solvable environmental problem. The complex composi-

tion of stillage causes high BOD_5 values which range from 15–340 g/L (Pejin et al. 2009).

One of the interesting, but quite unexplored possibilities of utilization of the liquid stillage is for the production of lactic acid. This idea is particularly supported by the world growing demand for lactic acid due to its versatile and increasing utilization in pharmaceutical, food and chemical industry (John et al, 2007). Serbia itself imports lactic acid and this approach would be of great interest from the economical point of view. The aim of this work was to investigate utilization of liquid stillage from bioethanol production for the production of lactic acid. For this purpose the selection of most appropriate lactic acid bacteria (LAB) on corn liquid stillage was assessed as well as the optimal conditions for lactic acid fermentation with the selected bacteria.

MATERIAL AND METHODS

Liquid stillage

Liquid stillage remained after bioethanol production was obtained from Reahem Ethanol Plant (Reahem Srbobran, Serbia). The composition of the corn thin stillage is shown in Table 1. Before the lactic acid fermentation, pH of the ethanol free liquid stillage was set to 6.5 (an optimal pH for grow of *Lactobacillus* sp.) and sterilized at 121°C for 20 minutes.

Microorganisms

Nine different species of *Lactobacillus* sp. used in this study are presented in Table 2. Stock cultures of lactic acid bacteria (LAB) were stored at -20^{o}C in 3 ml vials containing MRS broth (Fluka) and 50% (v/v) glycerol as a cryoprotective agent. For preparation of laboratory cultures, a drop of stock culture were transferred in 3 ml of the growth medium and incubated for 16 h under anaerobic conditions at the temperature listed in Table 2. All working cultures were pre-cultured at least three times before they were used as an inoculum for lactic acid fermentation of liquid stillage. MRS broth was sterilized at 121°C for 15 minute.

Lactic acid fermentation

All lactic acid fermentations were performed in 100 ml flasks containing 50 ml volume of liquid stillage mainly as batch static cultures or with shaking in a laboratory water bath with shaking. The fermentation was initiated by adding 3% (v/v) of overnight cultures grown in MRS broth for 16 h. Subsequently, the flasks were incubated for 72 h in anaerobic conditions (except in experiments where the effect of oxygen was studied) at temperatures 30, 37, 41 or 45°C. A temperature applied in the experiments is indicated in the results. During the fermentations, pH was not controlled and the samples were aseptically withdrawn every 12 h for determination of number of living cells, substrate consumption and lactic acid formation.

Analytical methods

Concentration of total acids (expressed as g/L lactic acid) during lactic acid fermentation was determined by titration of 10 ml of thin stillage with 0.1 M NaOH to pH 8.2 in the presence of phenolphthalein as the indicator. The reducing sugars sugar concentration was determined by 3,5-dinitrosalicylic acid using glucose as a standard (Miller G, 1959). A standard curve was drawn by measuring the absorbance of known concentrations of glucose solutions at 570 nm. The starch content was determined by Ewers polarimetric method (International standards, 1997). The water content was determined by the standard drying method in an oven at 105°C to a constant mass (AOAC, 2000). Lipid concentration was determined according to the Soxhlet method (AOAC, 2000). The protein content was estimated as the total nitrogen by the Kjeldahl method multiplied by 6.25 (AOAC, 2000), the ash content was determined by slow combustion of the sample at 650°C for 2 h, and the fibre content was determined by the Scharrer–Kurschner method (AOAC, 2000). Hexose and pentose sugars and organic acids present in corn thin stillage were analyzed by Agilent 1100 Series HPLC system consisted of micro vacuum degasser, binary pump, thermostated column compartment, variable wavelength detector and RI detector. Column: Aminex HPX-87H (Biorad Laboratories) 7.8 mm ID x 300 mm. Elution profile: 5 mM H_2SO_4, Isocratic: The dosing volume was 20 µl, Flow rate: 0.6 ml/min, Temperature: 50 ^{0}C.

Growth of different species of *Lactobacillus* sp. during lactic acid fermentation was determined by pour plate counting method on MRS agar.

Statistical analysis

The experiments were done in triplicates. All values are expressed as means. Mean values of treatments were compared by Student's *t* test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Selection of lactic acid bacteria on liquid stillage

Physical, chemical and nutritive characteristics of the thin stillage are highly variable and depend on the raw materials and various aspects of the ethanol production process. It has around 7-10% of dry matter that mostly originates from the crops used as raw materials for bioethanol production. Although the most of the carbohydrates and sugars from the crops are utilized by yeast for the production of ethanol, CO_2 and other volatile compounds, one small part between 2-3% remains in the stillage as unusable (Pejin et al. 2009). The chemical composition of the stillage used in this study is presented in Table 1. It contained 23 g/L of reduced sugars, which were not consumed in ethanol fermentation by yeasts. The HPLC analyses of sugar from the thin stillage showed that it consisted of approximately equal amount of glucose, maltose, and raffinose. According to the chemical composition; the corn liquid stilllage could be a suitable substrate for growth of fastidious bacteria such as species from *Lactobacillus* sp. In addition, liquid stillage typically contains minerals such as zinc, magnesium, calcium, iron, selenium and the yeast residues which are rich in complex of B vitamins and in other growth supporting compounds (Pejin et al. 2009).

Table 1. The composition of the corn liquid stillage

Component	Corn liquid stillage
Dry matter, %	8.34
Starch, % on dry matter	1.54
Total reducing sugars, g l^{-1}	23.0
Glucose (by HPLC), g l^{-1}	12.26
Maltose (by HPLC), g l^{-1}	10.30
Rafinose (by HPLC), g l^{-1}	10.12
Glicerol (by HPLC), g l^{-1}	10.21
Proteins, % on dry matter	30.39
Fibers, % on dry matter	4.95
Lipids, % on dry matter	13.14
Ash, % on dry matter	8.85

Table 2 compares tested lactic acid bacteria (LAB) regarding significant parameters achieved during the fermentation such as: the yield on the substrate (Y), volumetric productivity (P), substrate conversion and growth characteristics (number of cells). As shown in Table 2, the highest yield of 0.76 g g^{-1} and the productivity of 0.24 gL^{-1} h^{-1} were obtained with the strain *Lb. paracasei ssp. paracasei* NRRL B- 4564. After 72 h, this strain produced 17.37 g L^{-1} of lactic acid and consumed 72.90% of the sugars present in the stillage. The results showed that the strain *Lb. casei ssp. casei* NRRL B- 441 was also good candidate for lactic acid production on corn stillage, although it achieved to some extent lower yield, productivity and the substrate conversion. Both of these strains belong to the group of facultatively heterofermentative LAB. It is also interesting to note that the bacterial growth and lactic acid production were not in strict proportion e.g. the strains which exhibited the best lactic acid productivity (*Lb. paracasei* ssp. *paracasei* NRRL B- 4564 and *Lb. casei* ssp. *casei* NRRL B- 441) did not show the highest viable cell number at the end of fermentation. This could be partially due to different initial numbers of cells, but also due to differences in lactic acid production abilities and growth characteristics.

Table 2. Comparison of different strains of Lactobacillus sp. according to significant parameters obtained at the end of lactic acid fermentation (72 hours)

	Species of *Lactobacillus*	Temp.of fermentation (°C)	Y (g g^{-1})	P (g L^{-1} h^{-1})	Substrate conversion (%)	Num. of cells (N mL^{-1})
1	*Lb. paracasei ssp. paracasei* NRRL B- 4564	37	0.76	0.24	72.90	$1.43\ 10^8$
2	*Lb. casei ssp. casei* NRRL B- 441	37	0.61	0.20	60.93	$2.76\ 10^7$
3	*Lb. pentosus* NRRL B- 227	30	0.60	0.19	49.21	$8.0\ 10^8$
4	*Lb. rhamnosus* ATCC 7469	37	0.55	0.17	61.91	$6.2\ 10^7$
5	*Lb. acidophilus* ATCC 4356	37	0.47	0.15	38.96	$1.68\ 10^8$
6	*Lb. plantarum* PL-4	30	0.41	0.13	24.78	$1.8\ 10^8$
7	*Lb. helveticus* ATCC 15009	37	0.33	0.10	35.05	$8.1\ 10^4$
8	*Lb. fermentum* NRRL B-75624	30	0.27	0.09	48.86	$1.57\ 10^6$
9	*Lb. fermentum PL-1*	30	0.24	0.08	38.24	$4.88\ 10^8$

The kinetic of lactic acid fermentation with the selected strains

The kinetics of lactic acid fermentation with the two selected strains: *Lb. paracasei ssp. paracasei* NRRL B- 4564 and *Lb. casei ssp. casei* RRL B- 441 is presented in Fig. 1. Fig. 2 presents the changes in the number of cells during the fermentation.

The pH of the liquid stillage decreased during lactic acid fermentation reaching a minimum of 3.3- 3.6 at the end of fermentation. The production of lactic acid was to some extent more intensive within the first 24 hours than later during the fermentation. The most productive strains *Lb. paracasei* ssp. *paracasei* NRRL B- 4564 and *Lb. casei* ssp. *casei* NRRL B- 441 followed almost identical kinetics of lactic acid production during the first 48 hours. After that, the rate of lactic acid formation decreased more for *Lb. casei* ssp. *casei* NRRL B- 441 most probably as a consequence of a decline in total number of this bacterium in media due to product inhibition (Fig. 3).

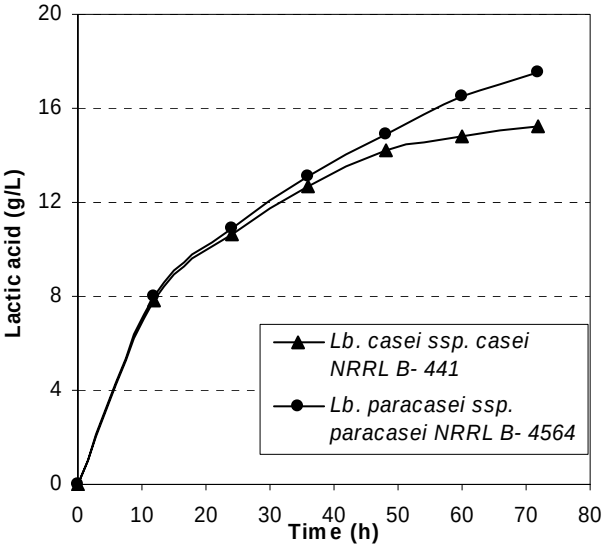

Fig. 1. Kinetics of lactic acid fermentation of corn liquid stillage with the selected LAB strains. Experimental conditions: temperature 30°C, static anaerobic batch fermentation, initial pH 6.5, inoculum concentration 3 %

The concentrations and yields of lactic acid obtained in this study are incomparable with the results of other authors since there are no reported experimental data on lactic acid production on the liquid stillage. Richter and Brethold, 1998, and Richter and Träger, 1994 studied lactic acid fermentation of media based on rye and sugar cane by Lb. paracasei. The reported concentrations of lactic acid produced were much higher than in this study and reached 84.5 and 106 g/L of lactic acid respectively. However, the amounts of sugars that could be converted into lactic acid were also proportionally higher in these feedstocks compared to the amount present in the liquid stillage.

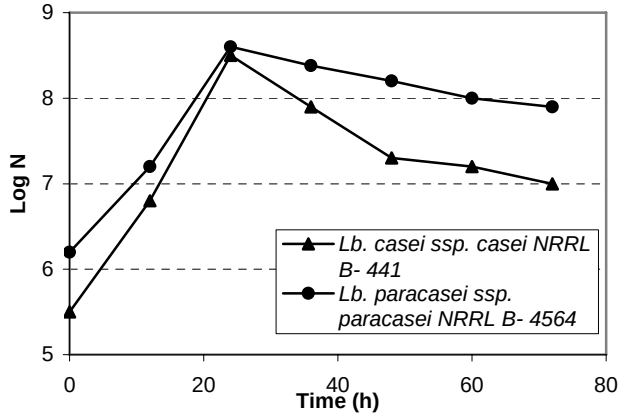

Fig. 2. Changes in cell number during lactic acid fermentation of corn liquid stillage with the selected LAB strains. Experimental conditions: temperature 30°C, static anaerobic batch fermentation, initial pH 6.5, inoculum concentration 3 %

Effect of temperature on lactic acid fermentation with *Lb. paracasei ssp. paracasei* NRRL B- 4564

The strain *Lb. paracasei ssp. paracasei* NRRL B- 4564, as the most promising candidate was selected for further optimization of the fermentation parameters. The effect of temperature was followed by determining lactic acid concentration and total number of cells after 72 h of fermentation of the liquid stillage at various temperatures and the results are presented in Fig.3.

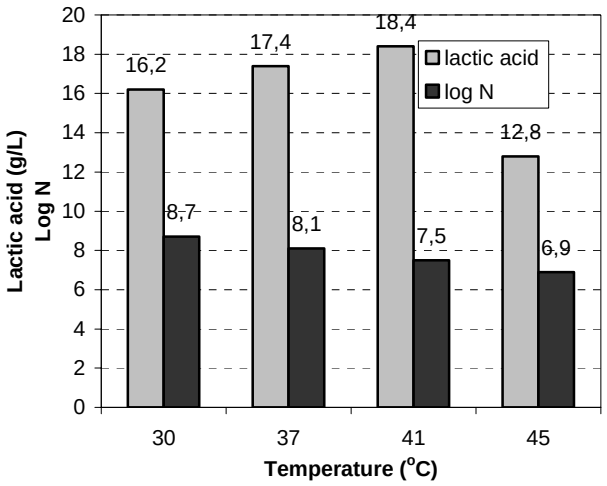

Fig. 3. Effect of temeperature on the concentration of lactic acid and number of cells after 72 h of fermentation of corn liquid stillage with Lb. paracasei ssp. paracasei NRRL B- 4564. Experimental conditions: static anaerobic batch fermentation, initial pH 6.5, inoculum concentration 3 %

As shown in Fig. 3., the highest concentration of lactic acid of 18.4 g/L was achieved at the temperature of 41°C, which was selected as an optimal. The concentration of lactic acid increased with the temperature rise from 30 to 41°C, after that it started rapidly to decline (Fig. 3.). If we compare number of cells obtained at different temperatures, we can conclude that the highest amount of biomass can be produced at 30°C, and much less by further increasing the temperature to 45°C. Thus, for the purpose of production of LAB biomass or probiotics in the liquid stillage, or for parallel production of biomass and lactic acid, lower temperatures should be selected. These findings are in agreement with reports of Hofvendahl and Hahn-Hägerdal, 2000 and Richter and Brethold, 1998 regarding the effect of temeperature on growth of *Lactobacillus* strains.

Effect of oxygen and mixing conditions on lactic acid fermentation with *Lb. paracasei ssp. paracasei* NRRL B- 4564

In these experiments, the fermentation was performed with *Lb. paracasei ssp. paracasei* NRRL B- 4564 at temperature of 41°C which was already determined as an optimal. It is known that *Lb. paracasei* ssp. *paracasei* NRRLB 4654 is a microaerophilic bacteria, and the aim was to examine the effect of oxygen on the production of lactic acid and bacterial biomass in liquid stillage, particulary because of the fact that it is much easier to perform the fermentation under aerobic condition if it is possible. Three types of batch fermentations were compared, static anaerobic, static aerobic and aerobic fermentation with shaking. The results are presented in Fig. 4. The concentration of lactic acid obtained in the fermentations (after 72 hours) under static aerobic and anaerobic conditions was quite close, while the lowest concentration of lactic acid was obtained under aerobic conditions with shaking. The best growth was attained in static anaerobic fermentation (Fig. 4.). The number of viable cells rapidly decreased close to zero after 72 hours in aerobic fermentations with shaking, most probably due to adverse effect of oxygen which is more prominent by shaking. The results suggest that the best conditions for bacterial growth are static anaerobic.

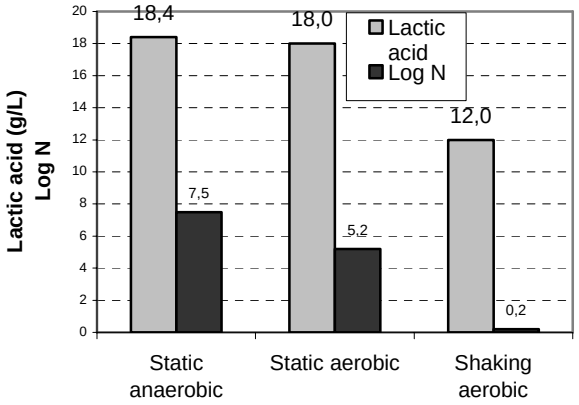

Fig. 4 Effect of oxygen and shaking conditions on the concentration of lactic acid and number of cells after 72 h of fermentation of corn liquid stillage with Lb. paracasei ssp. paracasei NRRL B- 4564. Experimental conditions: temperature 4°C , initial pH 6.5, inoculum concentration 3 %

CONCLUSIONS

According to the results obtained in this study, liquid corn stillage could be a good substrate for growth of lactic acid bacteria and lactic acid production. The best lactic acid producer *Lb. paracasei* ssp. *paracasei* NRRL B- 4564 produced 18.4 g L^{-1} of lactic acid and consumed 72.90% of the sugars present in thin stillage after 72 h of fermentation indicating that could be a promising species for larger scale production of lactic acid on the stillage. The lactic acid production was maximal at 41°C, without shaking, under anaerobic conditions. However, the biomass production was better at 30°C, also without shaking, under anaerobic conditions.

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