LACTIC ACID FERMENTATION OF BREWERS’ SPENT GRAIN HYDROLYSATE BY LACTOBACILLUS FERMENTUM AND LACTOBACILLUS RHAMNOSUS

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Ministry of Education, Science and Technological Development of the Republic of Serbia (Project number TR-31017)

Title: Increase in bioethanol production efficiency from renewable raw materials with total usage of by-products

Leader: Prof. dr Ljiljana Mojović, Faculty of Technology and Metallurgy, University of Belgrade

2008-2011
Ultrasound-assisted production of bioethanol by simultaneous saccharification and fermentation of corn meal

Svetlana Nikolić a,*, Ljiljana Mojković a, Marica Rakin a, Dušanka Pejin b, Jelena Pejin b

DOI 10.1007/s10098-011-0366-0

Utilization of microwave and ultrasound pretreatments in the production of bioethanol from corn

Svetlana Nikolić · Ljiljana Mojković · Marica Rakin · Dušanka Pejin · Jelena Pejin
Fermentation of wheat and triticale hydrolysates: A comparative study

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Research Article

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Increase in bioethanol production yield from triticale by simultaneous saccharification and fermentation with application of ultrasound

Dušanka J. Pejin\textsuperscript{a,*} Ljiljana V. Mojović\textsuperscript{b} Jelena D. Pejin\textsuperscript{a} Olgica S. Grujić\textsuperscript{a} Siniša L. Markov\textsuperscript{a} Svetlana B. Nikolić\textsuperscript{b} and Milica N. Marković\textsuperscript{a}
How to improve the economy of bioethanol production in Serbia

Ljiljana Mojović a,*, Dušanka Pejin b, Marica Rakin a, Jelena Pejin b, Svetlana Nikolić a, Aleksandra Đukić-Vuković a
PROJECT

Ministry of Education, Science and Technological Development of the Republic of Serbia (Project number TR-31017)

Title: Production of lactic acid and probiotics on waste products of food and agro-industry

Leader: Prof. dr Ljiljana Mojović, Faculty of Technology and Metallurgy, University of Belgrade

2011-
Total usage of triticale

Triticale stillage obtained after bioethanol production was used for lactic acid production.

Liquid triticale stillage was a good raw material for lactic acid fermentation.

Microwave as a pre-treatment of triticale for bioethanol fermentation and utilization of the stillage for lactic acid fermentation

Milica Marković a,*, Siniša Markov a, Olgica Grujić a, Ljiljana Mojović b, Sunčica Kocić-Tanackov a, Maja Vukašinović b, Jelena Pejin a

a Faculty of Technology, University of Novi Sad, 1 Car Lazar Boulevard, Novi Sad 21000, Serbia
b Faculty of Technology and Metallurgy, University of Belgrade, Karnezi 1a, Belgrade 11120, Serbia
Effect of different fermentation parameters on l-lactic acid production from liquid distillery stillage

Aleksandra P. Djukić-Vuković\textsuperscript{a,}\textsuperscript{*}, Ljiljana V. Mojović\textsuperscript{a}, Maja S. Vukašinović-Sekulić\textsuperscript{a}, Marica B. Rakin\textsuperscript{a}, Svetlana B. Nikolić\textsuperscript{a}, Jelena D. Pejin\textsuperscript{b}, Maja L. Bulatović\textsuperscript{a}

Bioprocess Biosyst Eng (2013) 36:1157–1164
DOI 10.1007/s00449-012-0842-x

Original Paper

Integrated production of lactic acid and biomass on distillery stillage

Aleksandra P. Djukić-Vuković · Ljiljana V. Mojović · Maja S. Vukašinović-Sekulić · Svetlana B. Nikolić · Jelena D. Pejin
Lactic acid production on liquid distillery stillage by *Lactobacillus rhamnosus* immobilized onto zeolite

Aleksandra P. Djukić-Vuković a,*, Ljiljana V. Mojović a, Bojan M. Jokić a, Svetlana B. Nikolić a, Jelena D. Pejin b

**ARTICLE IN PRESS**

FRIN-05420; No of Pages 6

Food Research International xxx (2014) xxx–xxx

Effective valorisation of distillery stillage by integrated production of lactic acid and high quality feed

Aleksandra P. Djukić-Vuković a,*, Ljiljana V. Mojović a, Valentina V. Semenčenko b, Milica M. Radosavljević b, Jelena D. Pejin c, Sunčica D. Kocić-Tanackov c
Beer is the most commonly consumed alcoholic beverage in the world.
Beer is a malt beverage produced by an alcoholic fermentation of the aqueous extract of malted barley with hops.

Brewing is therefore a multistage process involving biological conversion of raw materials to final product.
Beer production scheme

1. Steeping
   - Water
   - Barley

2. MALTING
   - Germination
   - Kilning

3. Milling

4. Mashing
   - Spent grain
   - Hops
   - Spent hops

5. Wort cooling
   - Wort boiling with hops

6. Wort separation

7. Fermentation
   - Yeast
   - Young beer

8. Maturation
Brewers’ spent grain (BSG) is the major by-product of the brewing industry, representing around 85% of the total by-products generated.

Per 100 L of beer produced 20 kg of brewers’ spent grain are obtained.

The chemical composition of brewers’ spent grain varies according:

✓ to barley variety,
✓ harvest time,
✓ malting and mashing conditions, and
✓ the quality and type of adjuncts used in the brewing process.
Brewers’ spent grain is a lignocellulosic material rich in protein and fibre, which account for around 20 and 70% of its composition, respectively.

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<thead>
<tr>
<th></th>
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<tbody>
<tr>
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<td>15</td>
<td>16.8</td>
<td>14.7±2.2</td>
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<tr>
<td>Hemicellulose</td>
<td>23</td>
<td>28.4</td>
<td>-</td>
<td>32.5</td>
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<tr>
<td>Lignin</td>
<td>22</td>
<td>27.8</td>
<td>12.6±0.6</td>
<td>13.4±1.9</td>
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<tr>
<td>Proteins</td>
<td>18</td>
<td>15.25</td>
<td>21.5±2.1</td>
<td>-</td>
</tr>
<tr>
<td>Ash</td>
<td>-</td>
<td>4.6</td>
<td>4.8±0.5</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>52.5±4.0</td>
<td>-</td>
</tr>
<tr>
<td>Lipids</td>
<td>-</td>
<td>-</td>
<td>11.7±0.5</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>12</td>
<td>-</td>
<td>6.0±1.4</td>
<td>12.5</td>
</tr>
</tbody>
</table>
The use of brewer’s spent grain is still limited, being basically used as animal feed and in human nutrition.
Its possible applications are as a raw material in:

- biotechnology,
- energy production,
- charcoal production,
- paper manufacture,

Or

- as a brick component,
- and adsorbent.
Brewers’ spent grain

- Yeast carrier in beer fermentation
- Microorganisms
- Enzymes
- Biogas
- Phenolic acids
- Bioethanol
- Xylitol and pullulan
- Lactic acid
Lactic acid
Currently, there is an increased demand for lactic acid as a raw material for the production of biopolymer poly-lactic acid (PLA) which is a promising biodegradable, biocompatible, and environmentally friendly alternative to plastics derived from petrochemicals.

Food and food-related applications account for approximately 85% of the demand for lactic acid.

The demand for lactic acid has been estimated to grow yearly at 5–8%. 
The annual world market for lactic acid production was expected to reach 367,300 metric tons by the year 2017.

There are two optical isomers of lactic acid, \(\text{L-(+)-lactic acid}\) and \(\text{D-(+)-lactic acid}\).

Lactic acid can be manufactured either by chemical synthesis or by microbial fermentations.
Presently, almost all lactic acid produced worldwide comes from the fermentative production route.

A desired isomer of lactic acid can be produced via fermentation using selected lactic acid-producing strains.

Besides this, microbial lactic acid fermentation offers an advantage in terms of the utilization of renewable carbohydrate biomass, low production temperature and energy consumption.
Most lactic acid bacteria require a wide range of growth factors including amino acids, vitamins, fatty acids, purines, and pyrimidines for their growth and biological activity.

Thus, the substrate composition and nutritional requirements of the strain considerably affect the overall performance of the fermentation.
Brewers’ spent grain

**Brewers’ spent grain hydrolysis**

- pH 5.5 and 5.0
  - Termamyl SC (1 hour at 90°C)
  - SAN Super 240 L (1 hour at 55°C)
  - Celluclast 1.5 L (10 hours at 45°C) at 180 rpm

**Centrifugation**

**Sterile liquid hydrolysate – fermentation media**

- CaCO₃ or NaOH
- Reducing sugars content
- Yeast extract content

**Fermentation**

- *L. fermentum* PL-1 at 30°C
- *L. rhamnosus* ATCC 7469 at 37°C at 150 rpm

**During the fermentation, determination of:**

- Lactic acid concentration (L-/D-lactic acid assay, Megazyme®, Wicklow, Ireland)
- Cells viability (pour plate technique, MRS agar, 30°C or 37°C)
- pH value

**Lactic acid**
BREWERS’ SPENT GRAIN HYDROLYSIS OPTIMIZATION

Brewers’ spent grain obtained in a lager beer production was dried at 40ºC for 12 hours.

Brewers’ spent grain hydrolysis was carried out under optimal conditions using the following enzymes:

1. Termamyl SC - α-amylase,
2. SAN Super 240 L – glucoamylase, and
3. Celluclast 1.5 L – cellulase (Novozymes, Denmark).
Different

**Temperatures:**
Termamyl SC – 80, 85 or **90°C** (1 hour)
San Super 240 L– 50, **55** or 60°C (1 hour)
Celluclast 1.5 L– 40, **45** or 50°C (10 hours)

**pH values:**
5.0, 5.5 and 6.0 for each enzyme

**Amounts:**
Termamyl SC – 0.1, 0.2, **0.3 mL** pre 50g
San Super 240 L– 0.1, 0.2, **0.3 mL** pre 50g
Celluclast 1.5 L– 3.0, 4.0, **5.0** or 6.0 **mL** per 50g
Reducing sugars content, g/L
Time, hours

pH 5.5 Termamyl SC and San Super 240 L, pH 5.0 Celluclast 1.5 L
pH 5.5 0.3mL TSC; 0.3mL San Super; pH 5.0 3.0mL Celluclast
pH 5.5 0.3mL TSC; 0.3mL San Super; pH 5.0 4.0mL Celluclast
pH 5.5 0.3mL TSC; 0.3mL San Super; pH 5.0 5.0mL Celluclast
pH 5.5 0.3mL TSC; 0.3mL San Super; pH 5.0 6.0mL Celluclast
Lactic acid content in *Lactobacillus fermentum* PL-1 fermentations

<table>
<thead>
<tr>
<th>Time, hours</th>
<th>Lactic acid content, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24</td>
<td>0.05</td>
</tr>
<tr>
<td>48</td>
<td>0.10</td>
</tr>
<tr>
<td>72</td>
<td>0.15</td>
</tr>
</tbody>
</table>

- **Without calcium carbonate addition**
  - L- (+)-lactic acid
  - D- (-)-lactic acid
- **With calcium-carbonate addition**
  - L- (+)-lactic acid
  - D- (-)-lactic acid

---

Lactic acid content (g/L)

<table>
<thead>
<tr>
<th>Time, hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>24</td>
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<tr>
<td>48</td>
</tr>
<tr>
<td>72</td>
</tr>
</tbody>
</table>

L- (+)-lactic acid increase 732.56-841.86%
Lactobacillus fermentum PL-1 cells viability during fermentations

**Without calcium-carbonate addition**
- 3.47% increase

**With calcium-carbonate addition**
- 33.38% increase

Viability, log CFU/mL vs. Time, hours

- 0.5% of yeast extract
- 1.0% of yeast extract
- 2.0% of yeast extract
- 3.0% of yeast extract
- 4.0% of yeast extract
- 5.0% of yeast extract
Lactic acid content in *Lactobacillus rhamnosus* ATCC 7469 fermentations

**L-(+)-lactic acid content increase 1677-1936%**

- **0.5% of yeast extract**
  - L-(+)-lactic acid
  - D-(−)-lactic acid

- **1.0% of yeast extract**
  - L-(+)-lactic acid
  - D-(−)-lactic acid

- **2.0% of yeast extract**
  - L-(+)-lactic acid
  - D-(−)-lactic acid

- **3.0% of yeast extract**
  - L-(+)-lactic acid
  - D-(−)-lactic acid

- **4.0% of yeast extract**
  - L-(+)-lactic acid
  - D-(−)-lactic acid

- **5.0% of yeast extract**
  - L-(+)-lactic acid

**Lactic acid content, g/L**

**Time, hours**

**Lactic acid content in *Lactobacillus rhamnosus* ATCC 7469 fermentations**
Lactobacillus rhamnosus ATCC 7469 cells viability during fermentations

Viability, log CFU/mL vs. Time, hours

- Without calcium carbonate addition
- With calcium carbonate addition

- 105.14% increase
- 120.56% increase

- 0.5% of yeast extract
- 1.0% of yeast extract
- 2.0% of yeast extract
- 3.0% of yeast extract
- 4.0% of yeast extract
- 5.0% of yeast extract
Without CaCO\textsubscript{3} \quad With CaCO\textsubscript{3} \\
0.5\% of yeast extract \quad 30.68 \quad 34.88 \\
1.0\% of yeast extract \quad 36.42 \quad 38.50 \\
2.0\% of yeast extract \quad 38.50 \quad 37.78 \\
3.0\% of yeast extract \quad 39.22 \quad 39.50 \\
4.0\% of yeast extract \quad 39.50 \quad 44.00 \\
5.0\% of yeast extract \quad \quad 97.52
During the fermentation, determination of:
- Lactic acid concentration (L/D-lactic acid assay, Megazyme®, Wicklow, Ireland)
- Cells viability (pour plate technique, MRS agar, 30°C or 37°C)
- pH value
L(-)-lactic acid content, g/L

Time, hours

2.7% of reducing sugars

38.22-53.63% increase

0 12 24 36

Without yeast extract addition
0.5% of yeast extract
1.0% of yeast extract
2.0% of yeast extract
3.0% of yeast extract
4.0% of yeast extract
5.0% of yeast extract
The graph illustrates the L-(+)-lactic acid content in g/L over time (hours) for varying amounts of yeast extract addition. The data points show that the lactic acid content increases with time and yeast extract concentration.

- Without yeast extract addition: 5.4% of reducing sugars
- 0.5% of yeast extract
- 1.0% of yeast extract
- 2.0% of yeast extract
- 3.0% of yeast extract
- 4.0% of yeast extract
- 5.0% of yeast extract

The data shows a 68.39-90.33% increase in lactic acid content.
8.1% of reducing sugars

L-(-)-lactic acid content, g/L

Time, hours

0 24 48 72

Without yeast extract addition
0.5% of yeast extract
1.0% of yeast extract
2.0% of yeast extract
3.0% of yeast extract
4.0% of yeast extract
5.0% of yeast extract

77.49-103.61% increase

60.33
2.7% of reducing sugars

3.89-6.89% increase

Viability, log CFU/mL

Time, hours

Without yeast extract addition
0.5% of yeast extract
1.0% of yeast extract
2.0% of yeast extract
3.0% of yeast extract
4.0% of yeast extract
5.0% of yeast extract
Viability, log CFU/mL vs. Time, hours

5.4% of reducing sugars

1.30-4.65% increase

Without yeast extract addition
0.5% of yeast extract
1.0% of yeast extract
2.0% of yeast extract
3.0% of yeast extract
4.0% of yeast extract
5.0% of yeast extract
8.1% of reducing sugars

Viability, CFU/mL

Time, hours

Without yeast extract addition
0.5% of yeast extract
1.0% of yeast extract
2.0% of yeast extract
3.0% of yeast extract
4.0% of yeast extract
5.0% of yeast extract

1.93-4.07% increase
Without yeast extract addition, 0.5% of yeast extract, 1.0% of yeast extract, 2.0% of yeast extract, 3.0% of yeast extract, 4.0% of yeast extract, 5.0% of yeast extract.

Lactic acid fermentation.

6.12% increase.
L-(+)-lactic acid yield, %

<table>
<thead>
<tr>
<th>Yeast Extract Addition</th>
<th>Lactic acid fermentation</th>
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<tr>
<td>Without yeast extract</td>
<td>81.13%</td>
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<tr>
<td>0.5% of yeast extract</td>
<td>85.90%</td>
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<td>1.0% of yeast extract</td>
<td>86.88%</td>
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<td>2.0% of yeast extract</td>
<td>89.52%</td>
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<tr>
<td>3.0% of yeast extract</td>
<td>89.78%</td>
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<td>4.0% of yeast extract</td>
<td>90.70%</td>
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<tr>
<td>5.0% of yeast extract</td>
<td>91.29%</td>
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10.16% increase
Lactic acid fermentation

- Without yeast extract addition: 80.71%
- 0.5% of yeast extract: 83.23%
- 1.0% of yeast extract: 83.57%
- 2.0% of yeast extract: 84.64%
- 3.0% of yeast extract: 85.27%
- 4.0% of yeast extract: 86.38%
- 5.0% of yeast extract: 87.33%

6.62% increase
<table>
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<th>2.0% of yeast extract</th>
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<td>0.74</td>
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<td>Time, hours</td>
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<td>0.5% of yeast extract</td>
<td>1.0% of yeast extract</td>
<td>2.0% of yeast extract</td>
<td>3.0% of yeast extract</td>
<td>4.0% of yeast extract</td>
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<td>1.14</td>
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<td><strong>1.69</strong></td>
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<td>1.0% of yeast extract</td>
<td>2.0% of yeast extract</td>
<td>3.0% of yeast extract</td>
<td>4.0% of yeast extract</td>
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<td>1.06</td>
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<td>48</td>
<td>0.60</td>
<td>0.91</td>
<td>0.99</td>
<td>1.02</td>
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<td>1.12</td>
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<td>0.77</td>
<td>0.78</td>
<td>0.83</td>
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</table>
Brewers’ spent grain

Brewers’ spent grain hydrolysis
pH 5.5 and 5.0
Termamyl SC (1 hour at 90°C), SAN Super 240 L (1 hour at 55°C), Celluclast 1.5 L (10 hours at 45°C) at 180 rpm

Centrifugation

Sterile liquid hydrolysate – fermentation media
NaOH
Brewers’ yeast (0.5-5.0%)
Reducing sugars content

Fermentation
$L.\text{ rhamnosus ATCC 7469}$ at 37°C
150 rpm

During the fermentation, determination of:
- Lactic acid concentration (L-/D-lactic acid assay, Megazyme®, Wicklow, Ireland)
- Cells viability (pour plate technique, MRS agar, 30°C or 37°C)
- pH value
The possibilities of using spent brewers’ yeast (Spent Brewer’s Yeast and Beta-Glucans Isolated From Them as Diet Components Modifying Blood Lipid Metabolism Disturbed by an Atherogenic Diet, B. Waszkiewicz-Robak, 2013)
The graph illustrates the lactic acid content, measured in grams per liter (g/L), over time (in hours) for different concentrations of brewers' yeast. The y-axis represents the lactic acid content, while the x-axis shows time in hours.

The graph shows a significant increase in lactic acid content with the addition of brewers' yeast. The line for the base case (without brewers' yeast addition) remains the lowest throughout the observation period.

Key notes from the graph:
- The lactic acid content increases with the addition of brewers' yeast.
- The percentage increase ranges from 6.61% to 73.46%.
- The line for 1.0% of brewers' yeast is the highest, indicating the most substantial increase compared to the base case.
- The line for 0.5% of brewers' yeast is the lowest, showing minimal increase.

Overall, the graph demonstrates a positive correlation between brewers' yeast concentration and lactic acid production.
The diagram shows the relationship between time (in hours) and L-(-)-lactic acid content (g/L) for different concentrations of brewers' yeast added to a solution with 5% reducing sugars. The x-axis represents time in hours (0 to 36), and the y-axis represents L-(-)-lactic acid content (0 to 30 g/L). The y-axis includes markers at 0, 5, 10, 15, 20, 25, and 30 g/L.

Different concentrations of brewers' yeast are indicated by different line colors and markers: 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0%. The addition of brewers' yeast results in an increase in lactic acid production, with a notable increase of 13.24-30.64% over time.

At 5% of reducing sugars, the lactic acid content is observed to increase significantly with the addition of brewers' yeast, reaching a peak of 27.03 g/L. The diagram indicates that the highest concentration of brewers' yeast (5.0%) yields the greatest increase in lactic acid content over the 36-hour period.
1.22-5.00% increase

Viability, log CFU/mL

Time, hours

Without brewers' yeast addition
0.5% of brewers' yeast
1.0% of brewers' yeast
2.0% of brewers' yeast
3.0% of brewers' yeast
4.0% of brewers' yeast
5.0% of brewers' yeast
Viability, log CFU/mL

Time, hours

5% of reducing sugars

Without brewers' yeast addition
0.5% of brewers' yeast
1.0% of brewers' yeast
2.0% of brewers' yeast
3.0% of brewers' yeast
4.0% of brewers' yeast
5.0% of brewers' yeast

0.76-3.03% increase
Without brewers' yeast addition, the L-(+)-lactic acid yield is 80.39%. With the addition of brewers' yeast, the yield increases to 82.13% for 0.5%, 82.65% for 1.0%, 83.06% for 2.0%, 83.55% for 3.0%, 83.96% for 4.0%, and 85.90% for 5.0% of brewers' yeast. There is a 5.51% increase overall.
Without brewers' yeast addition, 81.13% yield. When 0.5% of brewers' yeast is added, the yield increases to 84.69%. A 1.0% addition yields 84.85%, and this continues up to 89.01% with 5.0% addition. The highest yield, 89.01%, is seen with 5.0% addition. The chart shows a 7.88% increase overall.
<table>
<thead>
<tr>
<th>Time, hours</th>
<th>Without brewers' yeast addition</th>
<th>0.5% of brewers' yeast</th>
<th>1.0% of brewers' yeast</th>
<th>2.0% of brewers' yeast</th>
<th>3.0% of brewers' yeast</th>
<th>4.0% of brewers' yeast</th>
<th>5.0% of brewers' yeast</th>
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<tr>
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<td>0.63</td>
<td>0.65</td>
<td>0.66</td>
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<td>0.73</td>
<td>0.74</td>
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</table>
Brewers’ spent grain hydrolysate – fermentation media

NaOH

Glucose (after 12, 24, 36, and 48 hours) to the initial content

Glucose and yeast extract (after 12, 24, 36, and 48 hours) to the initial content

High gravity wort (10 mL every 4 hours)

During the fermentation, determination of:
- Lactic acid concentration (L-/D-lactic acid assay, Megazyme®, Wicklow, Ireland)
- Cells viability (pour plate technique, MRS agar, 30°C or 37°C)
- pH value

Fermentation

L. rhamnosus ATCC 7469 at 37°C
150 rpm

Centrifugation

Lactic acid

Brewers’ spent grain hydrolysis

pH 5.5 and 5.0

Termamyl SC (1 hour at 90°C), SAN Super 240 L (1 hour at 55°C), Celluclast 1.5 L (10 hours at 45°C) at 180 rpm
With glucose and yeast extract addition during fermentation
With glucose addition
With glucose and yeast extract addition
With wort addition

L-(-)-lactic acid content, g/L

Without glucose and yeast extract addition during fermentation
With glucose addition
With glucose and yeast extract addition
With wort addition

66.10-86.41% increase
114.79
156.96-191.49% increase

Time, hours
Viability, log CFU/mL

Time, hours

Without glucose and yeast extract addition during fermentation
With glucose addition
With glucose and yeast extract addition
With wort addition
Without glucose and yeast extract addition during fermentation

With glucose addition

With glucose and yeast extract addition

With wort addition

L-\((+)-lactic acid yield, \%\)

Lactic acid fermentation
<table>
<thead>
<tr>
<th>Time, hours</th>
<th>Without glucose and yeast extract addition during fermentation</th>
<th>With glucose addition</th>
<th>With glucose and yeast extract addition</th>
<th>With wort addition</th>
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<tr>
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Colleagues:

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Carlsberg Serbia

Novozymes, Denmark
THANK YOU KINDLY!