

TN02000044/3 – V6

ČLÁNEK V ODBORNÉM ČASOPISE NEBO KONFERENČNÍ PŘÍSPĚVEK

(VÝSTUP TYPU O)

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Optimization of brewery's spent grain processing for the cultivation of *Clostridium butyricum*

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The brewing and agricultural industries today generate a significant amount of waste and by-products, including brewers' spent grains (BSG). However, BSG is a raw material that can be used as a foodstuff and has great biotechnological potential. Among other things, BSG is composed of lignocellulose and proteins, and these compounds are a promising source of nutrients such as nitrogen or carbon for the cultivation of microorganisms. The structure of lignocellulose is quite compact and difficult for microorganisms to access. It is therefore necessary to disrupt its structure so that the carbohydrates present (glucose, arabinose, xylose) can be released and subsequently utilised by the microorganisms. Lignocellulose can be degraded by several methods. One of the most environmentally friendly methods for further use in the food industry is enzymatic hydrolysis, although a pre-treatment step is often carried out before the hydrolysis itself to make the carbohydrates more accessible to the enzyme. On the other hand, there is a risk that pre-treatment will produce inhibitors derived from lignocellulose, which can subsequently have a negative effect on the growth of microorganisms.

We have optimised the hydrolysis of BSG from the point of cost-effective and efficient release of monosaccharides while minimising the presence of inhibitors such as coumaric acid, ferulic acid, furfural or hydroxymethylfurfural. After enzymatic hydrolysis, the liquid phase of the hydrolysate was used to grow *Clostridium butyricum* bacteria to verify its potential as a cultivation media.

This work was supported from the grant of TAČR No TN02000044.

Valorization of agricultural and food waste in microbial biotechnology

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We are facing a substrate crisis in industrial white biotechnology. Why? Because it is unethical to use agricultural crops as substrates for the production of biofuels or bioplastic precursors when there are areas of the world where there are critical food shortages. There is a way out of this crisis and that is to use the various types of waste, which are currently undervalued, as substrates for cultivating microorganisms and obtaining value-added products. However, the use of waste is associated with various types of challenges ranging from the cleavage of difficult to use polymers into fermentable monomers (lignocellulosic complex is a typical example), to minimizing the formation of inhibitory substances during waste pretreatment and the difficult separation and purification of products from complex media. However, with knowledge of the metabolic capabilities of different types of microbial producers, valorisation of specific wastes is possible. The presentation shows a general approach to solving the problem as well as examples of specific solutions. The ideal option, which will also be discussed, would be to use raw materials in a generally biorefinery-like manner. The concept of a second-generation biorefinery will also be presented.

This work was performed thanks to the financial support of the project TACR TN02000044.

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Introduction

Brewer's spent grain (BSG) has found its way into biotechnology mainly because it is carbon and nitrogen rich compound. This waste by-product of the brewing industry is also considered as a food-grade commodity. In order to convert this raw material into a biotechnologically suitable substrate, the right methods for (pre)treatment of BSG has to be selected and optimized. The main challenge is the lignocellulosic structure of BSG, which is very compact and difficult for microorganisms to access.

Lignocellulose can be disrupted by various methods, with enzymatic hydrolysis being one of the most environmentally friendly for the food industry.

However, pre-treatment is often required to increase carbohydrate accessibility, although this can produce inhibitors that hinder microbial growth.

Objective

The aim of this project is to develop a BSG nutrient-rich medium for the cultivation of *Clostridium butyricum* by optimizing hydrolysis to reduce the economic cost of the process of accessing the nutrients contained in the brewer's spent grains.

Results

Pre-treatment

Acid hydrolysis was performed by monitoring the release of carbohydrates from the lignocellulose structure using different concentrations of sulphuric acid.



Enzymatic hydrolysis

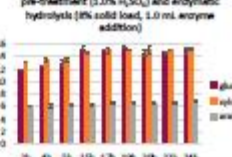
Cellic Ctec2 enzyme was used for hydrolysis of lignocellulose structure of brewer's spent grains. The filter paper unit of the enzyme was determined to be 17.6 FPU/mL.

Pre-treatment preceding the hydrolysis was also associated with the release of inhibitors including hydroxymethylfurfural, ferulic acid, coumaric acid and furfural into the medium, which were analyzed after the enzymatic hydrolysis.

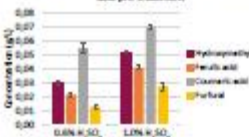
Carbohydrate release in relation to enzyme addition following 1.0% sulphuric acid pre-treatment (10% solid load)

Enzyme added	glucose	xylose	arabinose
FPU	g/ml	g/ml	g/ml
4.8	12.6 ± 0.2	15.9 ± 0.2	7.8 ± 0.2
8.8	15.3 ± 0.2	17.3 ± 0.5	7.8 ± 0.5
13.2	16.3 ± 0.1	17.4 ± 0.2	7.8 ± 0.1
17.6	17.1 ± 0.3	17.6 ± 0.3	8.0 ± 0.2

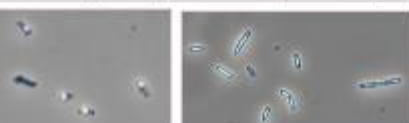
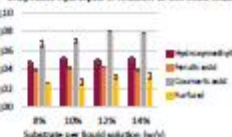
Time-dependent release of carbohydrates after pre-treatment (1.0% H₂SO₄) and enzymatic hydrolysis (8% solid load, 1.0 mL enzyme addition)



Inhibitors released depending on the solid load (10%) and enzymatic hydrolysis in relation to acid pre-treatment

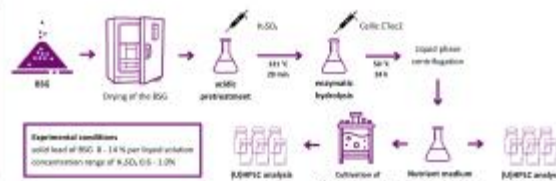


Inhibitors released depending on the pre-treatment (1.0% H₂SO₄) and subsequent enzymatic hydrolysis in relation to the solid load



Microscopic observation
Visualization of *Clostridium butyricum* bacterial spores (left) and vegetative cells (right), captured using a 100x objective lens with oil immersion.

Methods



Assessment of sporulation in BSG's hydrolysates

Bromocresol Purple was used as a pH indicator, which changes color from purple to yellow as the pH drops. In sporulation experiments it was used to quantify *C. butyricum* spores in hydrolysates. Spore germination and subsequent vegetative growth resulted in the production of acid, which acidified the medium and caused the indicator to change color.



Assessment of growth based on the production of organic acids

After acid pre-treatment and enzymatic hydrolysis, *C. butyricum* was cultivated on the hydrolysates and the saccharides (glucose, xylose, arabinose) were utilized. The amount of organic acids (lactic acid, acetic acid, butyric acid) produced was detected.

The effect of different sources of nitrogen, brewer's spent yeast (BY) and yeast extract (YE), on the growth and on the production of organic acid by *Clostridium butyricum* has been evaluated.

Organic acid production by *C. butyricum* depending on nitrogen source after pre-treatment (1.0% H₂SO₄), enzymatic hydrolysis (1 mL of enzyme added) and 10% solids loading

H ₂ SO ₄ %	nitrogen source	lactic acid g/L	acetic acid g/L	butyric acid g/L
-	-	1.3 ± 0.1	4.4 ± 0.3	4.3 ± 0.2
0.6	BY	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	YE	1.7 ± 0.8	3.7 ± 1.8	3.2 ± 2.5
1.0	-	1.5 ± 0.1	3.8 ± 0.4	4.0 ± 0.4
	BY	1.2 ± 0.1	3.8 ± 0.1	0.1 ± 0.1
	YE	2.4 ± 0.1	4.7 ± 0.1	6.6 ± 0.4

Conclusion

The concentration of the acid used in the pre-treatment affected the ratio of released carbohydrates.

The analysis shows that reducing the enzyme dosage has little effect on the release of carbohydrates meaning that 8.8 FPU is sufficient for 10% solid load.

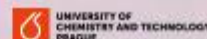
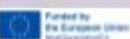
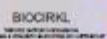
Furthermore, the amount of carbohydrates released after 15 hours reaches to very similar values as after 20 hours, indicating that the reaction time can be reduced to 15 hours.

Several inhibitors were released during the pre-treatment, but in the case where 0.6% sulphuric acid was used, the level of inhibitors determined was significantly lower than in the case of 1.0% sulphuric acid.

In assessing sporulation in BSG hydrolysates, growth was only observed up to the 10⁷ dilution.

There was a positive effect on *C. butyricum* growth and acid production when yeast extract was added to the hydrolysate growth medium. On the contrary, the addition of brewer's spent yeast had an inhibitory effect on growth and acid production was very low or absent.

Acknowledgement: This research was supported by the TACR NPO project no. TN02000044



UCT PRAGUE

RABBIT

Valorization of agricultural and food waste in microbial biotechnology

Petra Patakova and Zdenek Jandajsek

TA C B

BIOCIRKL
National Centre of Competence
"Strengthening and Growing Economy for Sustainability"

Funded by the European Union
NextGenerationEU

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FOOD OR FUEL?

Nearly a billion people will go hungry tonight, yet this year the U.S. will burn nearly 5 billion bushels of corn for ethanol. That's enough to feed 100 million people for a year.

8 bushels of corn = 21.6 gallons of ethanol OR 100,000 BTUs of energy

CONVERTED TO ETHANOL OR COMBUSTED TO PRODUCE ELECTRICITY

Grown for Biofuel

Crisis of white biotechnology feedstock not only for biofuels case

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Content of the presentation:

- 1)Crisis of substrates for industrial (white) biotechnology
- 2)Waste as a feedstock and source of carbon and nitrogen for biotechnologies
- 3)Which strategy to use for waste processing, examples
- 4)Is biorefinery approach a solution?

2

Processing of non-food biomass as a feedstock

Agricultural and forestry waste - wheat and rice straw, corn stover, bagasse, sawdust, twigs...

Municipal waste, paper

Animal breeding waste

Industrial waste

Local processing of waste

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Traditional industrial (white) biotechnological processes

a) Yeast *Saccharomyces cerevisiae* based:
Ethanol production, food and feed biomass

b) Use of other microorganisms:
Acids production (lactic, citric, acetic) but also itaconic, propionic, gluconic..
Alcohols production - butanol, isopropanol, propandiol..
Polyhydroxyalkanoates
Polysaccharides

3

Complex structure of waste, Low concentration

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Food biotechnology feedstock

Sucrose containing

Starch containing

food/feed

4

Pre-treatment approach to waste containing non-digestible polymers (lignocellulose example)

- 1.Mechanical - cutting, milling
2. Physico-chemical - acid/alkaline hydrolysis, ammonium expansion (AFEX), steam explosion...
- 3.Enzyme decomposition

CBP (consolidated bio-processing) - use of microorganism or microbial consortium able to decompose complex material and produce required metabolites

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Process selection for waste digestion



Different hydrolysates

Inhibitors (degradation products) formation

c1ccc(cc1)C(=O)O Formic acid
CC(=O)O Acetic acid
c1ccc(cc1)C(=O)O Benzoic acid
c1ccc(cc1)C(=O)O Vanillin

Selection criteria:

- Feedstock
- Minimization of inhibitors formation
- Economic demands (chemicals, heat, equipment...)
- Environmental concerns (waste...)

Paulova et al., *Biotechnol. Adv.* 2015, doi:10.1016/j.biotechadv.2014.12.002

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Animal Waste

blood, horns, hooves, bones, feather, skin, residues after mechanical separation of meat... - source of proteins (amino acids)
Nitrogen source for microbial culture



residues after mechanical separation of meat

after sterilization

After alkaline hydrolysis and centrifuging

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Combined approaches toward substrate pre-treatment and fermentation

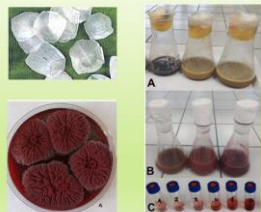
SHF (separate hydrolysis and fermentation) - optimal conditions for both hydrolysis and fermentation X time consuming

SSF (simultaneous saccharification and fermentation) - simultaneous application of enzymes and microorganisms X compromised conditions for both processes

CBP (consolidated bio-processing) - use of microorganism or microbial consortium able to decompose cellulose and produce required metabolites

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Use of common carp waste for pigment production by *Monascus purpureus*

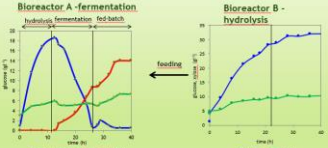


Kalek et al., 2023
<https://doi.org/10.18832/kp2023.69.686>

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Fed-batch delayed SSF (dSSF) for ethanol production from wheat straw

FED-BATCH PROCESS - increase of sugar concentration by feeding new (concentrated) substrate

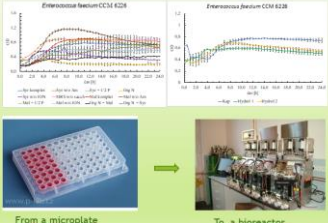


yield from cellulose 0.37 g/g

Paulova et al., *Fuel* 122, 294 (2014)

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***Enterococcus faecium* (how to avoid product separation)**

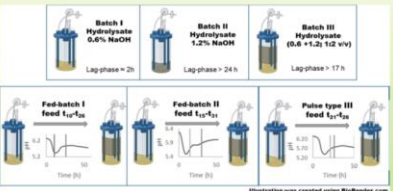


From a microplate

To a bioreactor

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Fed-batch strategy to fully eliminate the negative effect of lignocellulose derived inhibitors in ABE fermentation



Batch I Hydrolyzate 0.6% NaOH
 Batch II Hydrolyzate 1.2% NaOH
 Batch III Hydrolyzate (0.6 + 1.2) 1.2 v/v

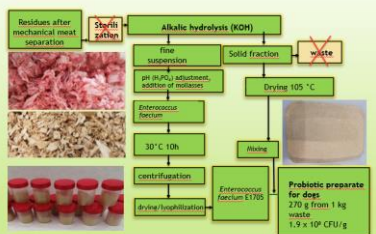
Feed-batch I feed $t_{0-t_{24}}$
 Feed-batch II feed $t_{24-t_{36}}$
 Pulse type III feed $t_{36-t_{48}}$

Illustration was created using BioRender.com

Branska B et al. *Biotechnol Biofuels* Bioprod. 2024 Jun 25;17(1):87. doi: 10.1186/s13068-024-02520-6.

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Probiotic prepare for dogs from animal waste

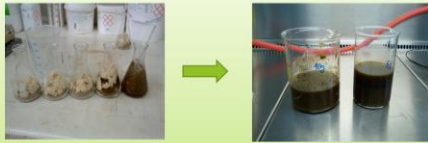


Residues after mechanical meat separation
 Sterilization
 Alkaline hydrolysis (KOH)
 Fine suspension
 pH (H₂PO₄) adjustment, addition of medium
 Enterococcus faecium
 30°C 10h
 centrifugation
 drying/lyophilization
 Enterococcus faecium 5.795
 Probiotic prepare for dogs
 270 g from 1 kg waste
 1.9 x 10⁹ CFU/g

- Waste valorisation
- Zero additional waste generation
- Low energy consumption

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Mixed hydrolysate straw + feather - joint hydrolysis to prepare complete culture medium



Bramska et al., 2017 Czech patent No. 306795

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Biorefinery

Refinery

- Heterogeneity of feedstock
- Different approaches toward material decomposition
- Low volumetric density of most materials
- Difficult to use all components of the material

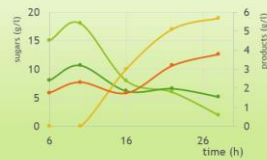
- Unique feedstock
- Unique approach
- High density of the feedstock
- Total amount of material is used



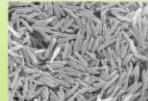
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Production of ABE from joint hydrolysate

ABE - total amount of acetone, butanol and ethanol
Acids - total amount of acetic and butyric acids



Yield of solvents 20%
Yield of all liquid products 33%
Productivity 0.2 g/l/h for solvents
0.36 g/l/h for all products

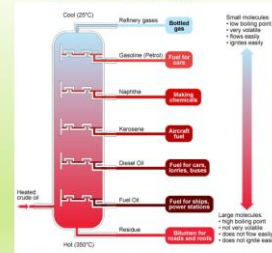


Bramska et al., 2020 <https://doi.org/10.1016/j.renene.2019.07.094>

Clostridium beijerinckii

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Petroleum refinery



<http://www.energybc.ca/profiles/oil.html>

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Production of lactic acid using straw + feather

Source of carbon - hydrolysate of wheat straw
Source of nitrogen - chicken feather hydrolysate

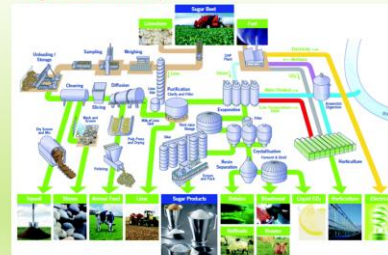


Yield 69%
Specific productivity 1.3 g/l/h
Price of medium reduced in 60%

Chorwalova et al., 2018 [doi: 10.17221/461/2017-CJFS](https://doi.org/10.17221/461/2017-CJFS)

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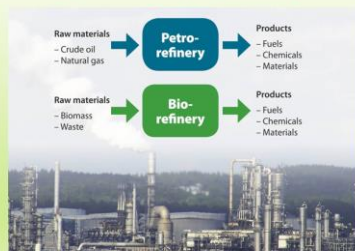
Sugar beet biorefinery



Fareed Dissanayake, 2017, 302, 415-431

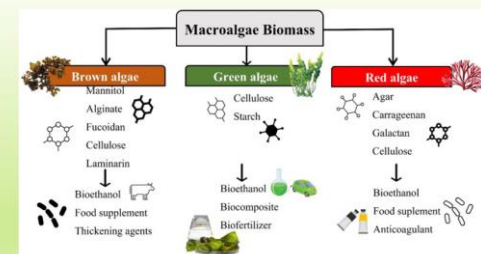
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Is biorefinery a solution?



<https://www.euratomic.com/en/euratomic-expert-articles/towards-a-circular-economy-with-multiple-product-biorefineries.html>

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<https://www.sciencedirect.com/science/article/pii/S2589014X21002413>

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Next generation biorefinery Steelanol - production of ethanol
from steel off gases mixture



<http://www.steelanol.eu/en>

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Thanks to all my colleagues, students
and friends including microbial ones ☺



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