



Value-Added Utilization of Protein Rich Agricultural Residues—Development and Evaluation of Chemical Hydrolysis

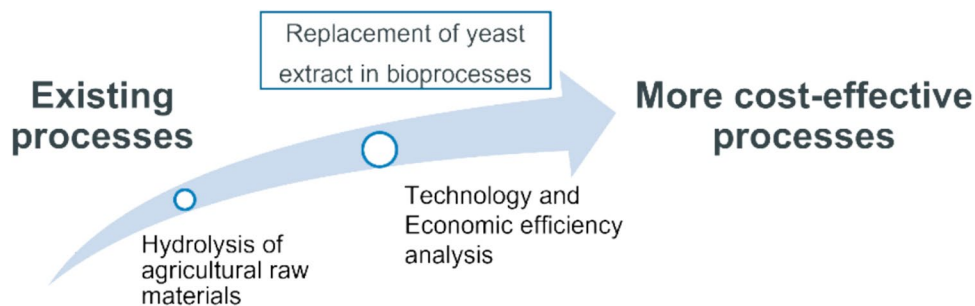
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Abstract

The chemical industry's reliance on fossil raw materials has significantly contributed to increased CO₂ levels. To reduce the climate footprint, renewable resources must replace fossil ones. Biotechnological production of industrial chemicals using renewable resources is a promising approach. However, many biotechnological processes require yeast extract, a costly nutrient source. Inexpensive, protein-rich substitutes like agricultural residues can be used instead. To improve fermentation results, a hydrolysis process was developed and tested. This paper describes the optimization of chemical hydrolysis for protein-rich agricultural raw materials like Distillers' Dried Grains with Solubles (DDGS) and rapeseed meal (RM). The hydrolysates were used as a nitrogen source in bioprocesses to verify their usability. The results show that the amount of free amino nitrogen increases with increasing molarity of the acid. In order to achieve acceptable amino nitrogen concentrations with reduced sulfuric acid molarity, the temperature was raised up to 160 °C. This temperature increase resulted in 81.3% of the amino nitrogen concentration with 1 M sulfuric acid compared to using 3 M sulfuric acid. As a result of this optimization, the costs of the hydrolysed rapeseed meal are reduced to only 8–13% of the original costs of yeast extract with the same nitrogen content.

Graphical Abstract



Keywords Hydrolysate · Distillers' dried grains with solubles (DDGS) · Rapeseed meal · Agricultural residues

Statement of Novelty

One building block for achieving a climate-neutral economy is the increased use of renewable raw materials instead of fossil ones. Biotechnological processes play a crucial role in

this—but they are relatively expensive. A major cost factor is the nutrient source yeast extract, which is widely used in biotechnological processes. However, numerous technical processes generate low-cost, protein-rich residues as by-products, which could also be used as a complex source of nitrogen. These residues cannot be used to replace yeast extract without pretreatment, due to the limited accessibility of the amino acids. Nevertheless, upstream chemical hydrolysis can significantly increase the accessibility of these amino acids. To capitalize on this opportunity, we have

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developed, optimized, and economically analyzed an acid-catalyzed hydrolysis process for agricultural residues. The goal was to obtain large amounts of free amino acids in the hydrolysates, which can be applied in various fermentation processes without compromising the final product concentration or productivity. Our results show that the hydrolyzed alternative nitrogen sources are well-suited as low-cost substitutes for yeast extract, with costs of only about 8–13% of those of yeast extract with the same nitrogen content. By utilizing these cost-effective nitrogen sources, biotechnological processes can be made more economical, and an important step can be taken towards a climate-neutral economy.

Introduction

The intensive use of fossil raw materials for the production of energy and chemical products in the last years has contributed significantly to the increase in the CO₂ concentration in the earth's atmosphere and the resulting rise in temperature. In order to mitigate the effects of climate change, fossil resources must be replaced by renewable ones, a key aspect of the future bioeconomy. Many countries, including the member states of the European Union, are striving to be climate-neutral by the middle of the twenty-first century [1, 2]. National and international agencies, like United Nations Framework Convention on Climate Change (UNFCCC) and World Meteorological Organization (WMO) monitor and regulate CO₂ emissions to mitigate climate change by setting and enforcing emission reduction targets, supporting international agreements like the Paris Agreement, and improving global greenhouse gas monitoring infrastructure [3, 4]. [UNFCCC, 1992; WMO, 2025]. While energy can also be produced from other renewable resources, e.g., from wind or solar radiation, most products in the chemical industry are dependent on a renewable carbon source. In addition to CO₂, this is offered by renewable raw materials, i.e., biomass. Therefore, the usage of renewable raw materials is essential and highlighted in numerous strategies to restructure our economic systems towards a climate-neutral bio-economy [5–7].

To provide an ecological and economical alternative to petrochemical building blocks for polymer or packaging industry, not only yield, productivity and final titer of a biotechnological process for monomer production have to be optimized, but also the costs of production, especially medium costs, must be reduced. Besides the substrate, nutrient sources like yeast extract, with a price of 6–10 €/kg, cause the highest production costs [8]. Yeast extract and other complex nutrient sources are used in fermentations, because they provide organic nitrogen, like amino acids, and also vitamins, salts, trace elements, or nucleic acids [9]. Alternative sources of nutrients in the form of low-cost

and protein-rich agricultural residues [10–12], such as rapeseed meal (RM) or Distillers' Dried Grains with Solubles (DDGS), can thus be used to replace yeast extract.

RM is a by-product of the industrial production of rapeseed oil made from rapeseed [13]. Worldwide the average annual production of rapeseed oil in the years of 1996 until 2000 was 12.64 10⁶ t [14]. In recent years, interest in the processing of rapeseed has increased, as rapeseed oil is used for both food and technical purposes. During the production process large amounts, up to 48% of the mass of the processed seeds, of RM occurs. The protein content is 38–48%. The amino acid composition of RM is very balanced, so it is used as a high-protein component in the production of animal feed. But due to the presence of antinutritional compounds and high fiber content the usage as a feed additive is limited and large quantities of rapeseed meal remain unused [14, 15].

DDGS is one of the by-products of bioethanol and distillery plants [16] with a production of 22.6 million tons in the U.S in 2019 [17]. Due to its high nutritional content and low production costs DDGS is a feed ingredient substitute and its use as a feedstock could lead to the development of multi-stream processes for the production of raw materials, platform molecules or specialty chemicals within a biomass-based biorefining strategy [16, 18]. In the dry milling process, it is approximate that 100 kg of grain will yield 40.2 L of ethanol, 32.3 kg of DDGS, and 32.3 kg of CO₂ [16]. The nutrient content of DDGS varies depending on the used grain sources such as corn, wheat, barley, and sorghum. The protein content is between 15.4 (barley) and 36.2% (wheat) depending on the grain source [18]. Further research is needed to evaluate and analyze the positive and negative economic and technological impacts on agriculture and the ethanol industry. The economic value of DDGS is expected to increase for its potential use in the production of bioethanol and value-added products (e.g., organic acids). Future development of DDGS-based value-added products in the food, biocomposite, and fermentation industries would expand the market for DDGS [18].

However, the high molecular structure of the proteins in the agricultural residues has to be disintegrated by enzymatic or chemical hydrolysis prior to use [11, 19, 20]. Enzymatic hydrolysis using proteases in a separate or simultaneous hydrolysis and fermentation processes is an attractive alternative to the chemical and the predominant method. There are advantages of enzymatic hydrolysis, such as the low salt content in the hydrolysate, since no neutralization step is required [21, 22]. But the downside is e.g., the selectivity of the enzymes and possible inhibition of the enzymes. [23–25]. So far, chemical hydrolysis is rarely used for disintegration of protein-rich agricultural residues [19, 26]. The advantages like high flexibility caused by the non-selective cleavage of peptide bonds and the possibility to adapt the

reaction conditions to the requirements, provides a broad application opportunity.

The major problem of the common hydrolysates is the insufficient availability of free amino acids and small peptides, because of an insufficient hydrolysis degree, which results in a lower productivity in the fermentation process [11]. For this reason, the aim of this study is the method development as well as the optimization of the chemical hydrolysis of the protein-rich agricultural rapeseed meal (RM) and Distillers' Dried Grains with Solubles (DDGS), first growth tests by different microorganisms and an economic evaluation in order to give a brief overview of the costs compared to the usage of yeast extract.

Materials and Methods

In all cultivation experiments Fermtech® yeast extract from Merck KGaA was used as a reference. The total nitrogen concentration (TN) of Merck Fermtech® yeast extract is 9.54%. The composition of Merck Fermtech® yeast extract is presented in Klotz S (2017) [8]. The agricultural residues were purchased as follows: Distillers' Dried Grains with Solubles (DDGS) was purchased from Crop Energies AG (Mannheim, Germany) and rapeseed meal (RM) from AGRAVIS Mischfutter Leine-Weser GmbH (Braunschweig, Germany). The total nitrogen content (T_N) of DDGS was 4.88% and 5.39% for RM. The agricultural residues were completely characterized in [8, 11]. Chemicals were either purchased from Merck KGaA (Darmstadt, Germany), Carl Roth GmbH and Co. KG (Karlsruhe, Germany) or from Sigma Aldrich (St. Louis, MO, USA) in an appropriate purity for biochemistry.

For chemical hydrolysis the agricultural residues were ground with an ultra-centrifugal mill (750 μm , ZM 200, Retsch, Haan, Germany) and sieved to a particle size of lower than 710 μm .

The hydrolysis of the agricultural residues RM and DDGS was optimized in 50 mL Berghof pressure digestion vessels (Berghof Products + Instruments GmbH, Eningen, Germany). 1 g of the agricultural residues, with a particle size lower than 710 μm , were mixed with 5 mL acid or base to an initial solid content of 200 g/L. Chemical hydrolysis can be conducted under acidic or basic conditions. Depending on the hydrolysis methods, different amino acid profiles are generated. In this system, an acid and a base, the molarity and the influence of the hydrolysis time and temperature were compared. In case of acid hydrolysis with sulfuric acid, the pH value of the hydrolysate was adjusted to 6 by adding calcium hydroxide to remove the sulfate. The precipitated calcium sulfate and other solids were separated off by centrifugation (4,600 g, 20 min, 10 °C), the pellet was washed twice with water and the supernatants were collected and

diluted to a final solid content of 50 g/L. In the case of alkaline hydrolysis with NaOH the hydrolysate was neutralized to the pH value 7.3 with hydrochloric acid (30%) and diluted to a final solid content of 50 g/L. Each digestion was performed in at least duplicate.

The total nitrogen content (T_N) was determined by Kjeldahl method. 150 mg sample in a Kjeldahl reaction tube with boiling stones were mixed with an antifoam tablet (Carl Roth, Karlsruhe, Germany) and a Kjeltab S (5 g K_2SO_4 , 5 mg Se), as well as 10 mL 96% H_2SO_4 . The thermal degradation was performed in a Kjeldathrem (Gerhardt GmbH & Co. KG, Königswinter, Germany) with the following temperature gradient: 100 °C (30 min), 200 °C (60 min), 300 °C (60 min), 420 °C (60 min), RT (30 min). Distillation and titration were carried out with a Vapodest (Gerhardt GmbH & Co. KG, Königswinter, Germany) using 33% NaOH, 2% $\text{B}(\text{OH})_3$ and 0.05 M H_2SO_4 .

The free amino nitrogen content (AN) was determined by the ninhydrin method. Therefore, 2 mL hydrolysate (diluted 100-fold) were mixed with 1 mL ninhydrin solution (5.0 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 6.0 g KH_2PO_4 , 500 mg ninhydrin and 300 mg fructose are dissolved in water, pH is adjusted to 6.6–6.8, and the volume is adjusted to 100 mL) in a sealable reaction tube and heated in a boiling water bath for 16 min. Afterwards, the mixture is cooled down in a 20 °C tempered water bath for 20 min and then 5 mL potassium iodate solution are added (1.0 g KIO_3 dissolved in 300 mL water and 200 mL ethanol 96%). After a further reaction time of 3 min at room temperature, the absorption at 570 nm is measured. The blank was subtracted from the measured value and the method was calibrated with glycine standards. All measurements were performed in minimum in triplicates. The mean values calculated from these triplicate measurements and the corresponding standard deviations (error bars) are presented in the figures.

The degree of hydrolysis was determined ($(\text{AN}/T_N) \cdot 100$, with AN = free amino nitrogen content and T_N = total nitrogen content). A digestion at 110 °C for 24 h in 3 M sulfuric acid served as reference.

For the determination of free amino acids an analysis kit (EZ:faast™ GC-FID Physiological) from Phenomenex (Torrance, USA) was used. For peptide bounded amino acids 50 mg of a dried sample was hydrolyzed with 1 mL 6 M HCl + 0.02% phenol in a headspace vial (cleaned with conc. HCl and water). The vial was sealed with a septum, deep-frozen in liquid nitrogen, evacuated and heated to 110 °C for 48 h. The hydrolysate was diluted to 100 mL and filtered. Free amino acids were analyzed according to the manufacturer's instructions. A gas chromatograph with a flame ionization detector GC-17A from Shimadzu (Kyoto, Japan) with a Zebron™ ZB-AAA column (10 m \times 0.25 mm \times 0.25 μm) from Phenomenex (Torrance, USA) was used for the measurement with H_2 as carrier gas with a flow rate of 2.15 mL/

min. The temperature of 110 °C was heated up to 250 °C with a gradient of 20 °C min⁻¹, further up to 320 °C with 10 °C min⁻¹ and was held for 1 min.

In order to evaluate the potential of acidic and basic hydrolysates, growth tests with *Lactobacillus casei*, *Lactobacillus reuteri*, as well as *Clostridium diolis* were carried out. As an example of these growth experiments, the results of cultivations with the strain *C. diolis* are shown.

The strain *C. diolis* DSM 15410, available at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), was stored in 50% pure glycerol at -80 °C. In order to built up the inoculum, three generations of precultures were grown without shaking at 32 °C. The precultures, 50 mL glass vials sealed with butyl rubber septums, were filled under anaerobic conditions with 30 mL medium.

All of the used media components were p.a. quality and purchased from either Merck (Germany), Sigma-Aldrich (USA), or Roth (Germany).

Per liter of distilled water, the preculture medium included: 9.09 g KH₂PO₄, 0.53 g NH₄Cl, 0.123 g MgSO₄·7H₂O, 0.017 g CaSO₄·2H₂O, 0.01 g FeSO₄·7H₂O, 1 g yeast extract, 1 mL of a 1 g/L resazurin solution, 0.2786 g L-cysteine·HCl·H₂O, and 2 mL trace element solution. Per liter of distilled water, the trace element solution (DSMZ medium 144, 5 times concentrated) contained: 64 g nitrilotriacetic acid, 1 g FeCl₂·4H₂O, 0.5 g MnCl₂·4H₂O, 0.85 g CoCl₂·6H₂O, 0.5 g CaCl₂·2H₂O, 0.5 g ZnCl₂, 0.1268 g CuCl₂·2H₂O, 0.05 g H₃BO₃, 0.05 g Na₂MoO₄·2H₂O, 0.13 g NiCl₂·6H₂O, 5 g NaCl, and 0.1 g Na₂SeO₃·5H₂O. The pH value of the trace element solution was adjusted to 6.5 with NaOH/HCl. Pure glycerol (≥ 98%) was added to the medium as sole carbon source (25 g/L).

Per liter of distilled water, the cultivation medium included: 0.2 mol MES (2-(N-morpholino)ethanesulfonic acid), 0.53 g NH₄Cl, 0.123 g MgSO₄·7H₂O, 0.017 g CaSO₄·2H₂O, 0.01 g FeSO₄·7H₂O, 4 g yeast extract, 1 mL of a 1 g/L resazurin solution, 0.2786 g L-cysteine·HCl·H₂O, and 2 mL trace element solution. Per liter of distilled water, the trace element solution (DSMZ medium 144, 5 times concentrated) contained: 64 g nitrilotriacetic acid, 1 g FeCl₂·4H₂O, 0.5 g MnCl₂·4H₂O, 0.85 g CoCl₂·6H₂O, 0.5 g CaCl₂·2H₂O, 0.5 g ZnCl₂, 0.1268 g CuCl₂·2H₂O, 0.05 g H₃BO₃, 0.05 g Na₂MoO₄·2H₂O, 0.13 g NiCl₂·6H₂O, 5 g NaCl, and 0.1 g Na₂SeO₃·5H₂O. The pH value of the trace element solution was adjusted to 6.5 with NaOH/HCl. Pure glycerol (≥ 98%) was added to the medium as sole carbon source (50 g/L).

The pH value of the complete cultivation medium was adjusted to 7.2. The upstream processes as well as the fermentations were conducted under anaerobic conditions. The cultivations were carried out in duplicate. Growths of *C. diolis* DSM 15410 using 4 g/L yeast extract as the reference

condition were compared to rapeseed meal (RM) and Distillers' Dried Grains with Solubles (DDGS) hydrolysates. For all investigations with hydrolysates, the total nitrogen content was comparable to 4 g/L yeast extract.

Optical density was measured at 605 nm with a T80 UV/VIS Spectrophotometer (PG Instruments Limited, UK).

Results and Discussion

Reference and Basic Digestion

A digestion at 110 °C for 24 h in 3 M sulfuric acid served as reference. Hereby, a high hydrolysis degree of the protein-rich agricultural rapeseed meal (RM) and Distillers' Dried Grains with Solubles (DDGS) is achievable [8, 11]. Comparable to the usage of 3 M sulfuric acid the digestion was performed with 6 M sodium hydroxide at 110 °C for 24 h as well (Fig. 1).

Using 6 M NaOH as the reactant for the chemical hydrolysis, the degree of digestion was increased by 8% for DDGS and for RM by 5% compared to 3 M sulfuric acid. This digestion under reference condition pointed out that a higher amino nitrogen concentration could be achieved with the agricultural residue rapeseed meal. This is plausible due to the higher total nitrogen content of RM compared to DDGS. Therefore, the agricultural residue RM was selected for further investigations.

In addition to the spectrophotometric determination of free amino nitrogen (AN), amino acid profiles of both hydrolysate variants were determined for a better comparison of the basic and acidic digestion (Fig. 2). Depending on the hydrolysis methods, different amino acid profiles are generated. The digestion with sulfuric acid destroyed the aromatic amino acid tryptophan, but also partly tyrosine and glutamic acid. On the other hand, threonine and partly serine were destroyed during the digestion with NaOH. These findings agree with information in the literature that, among other, tryptophan is completely destroyed in acidic digestions and serine and threonine in alkaline digestions [27, 28].

The spectrophotometric determination of free amino nitrogen with ninhydrin also showed different levels depending on the digestion method and residue. Due to the increased degree of digestion with NaOH, higher concentrations of the majority of the amino acids were determined in the basic digested hydrolysates. Overall, both hydrolysis methods showed great potential for the structural breakdown of the protein fraction in the tested agricultural residues.

Beside the different free amino acid contents, a noticeable difference is given through the salt content of the acid and basic digested hydrolysates. After hydrolysis with sulfuric acid, these hydrolysates were neutralized with calcium hydroxide to remove the sulfate, and the precipitated

Fig. 1 Amino nitrogen content and degree of digestion of Distillers' Dried Grains with Solubles (DDGS) and rapeseed meal (RM) under reference digestions conditions: 110 °C, 24 h and 3 M H₂SO₄ or 6 M NaOH

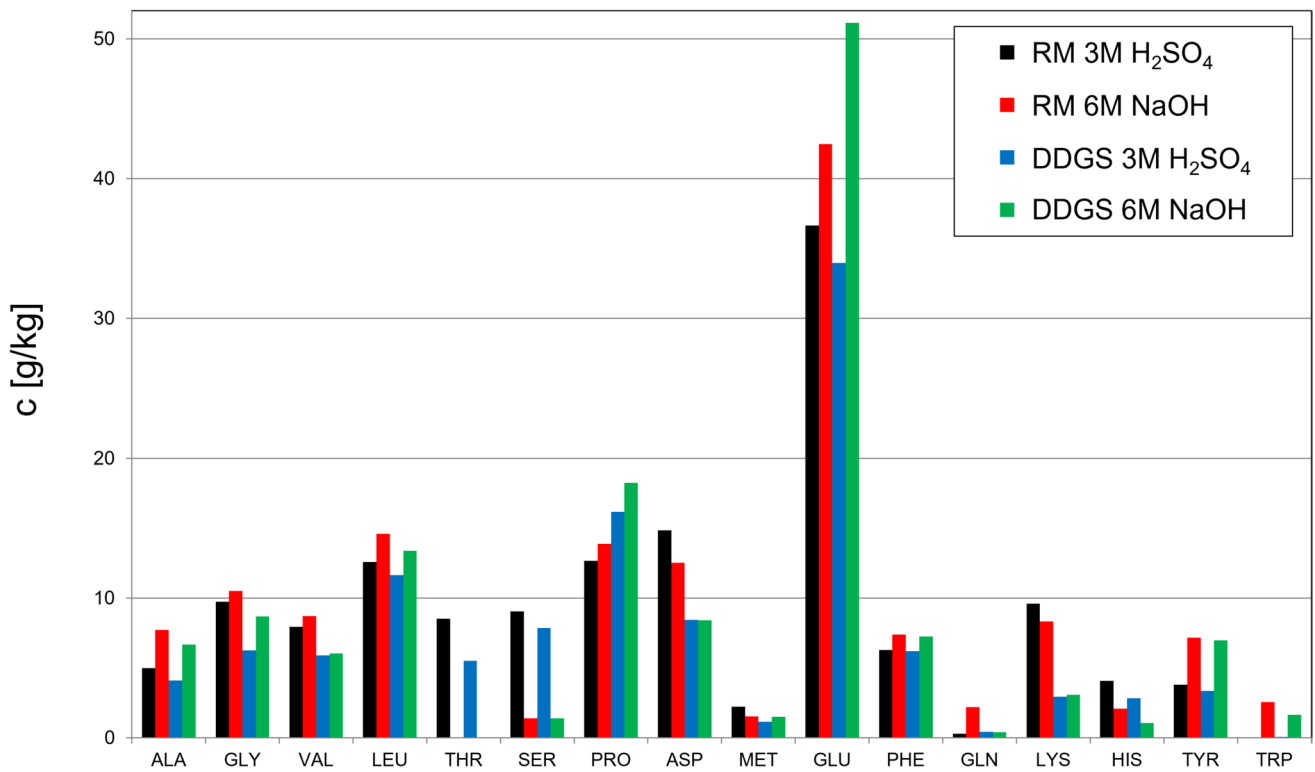
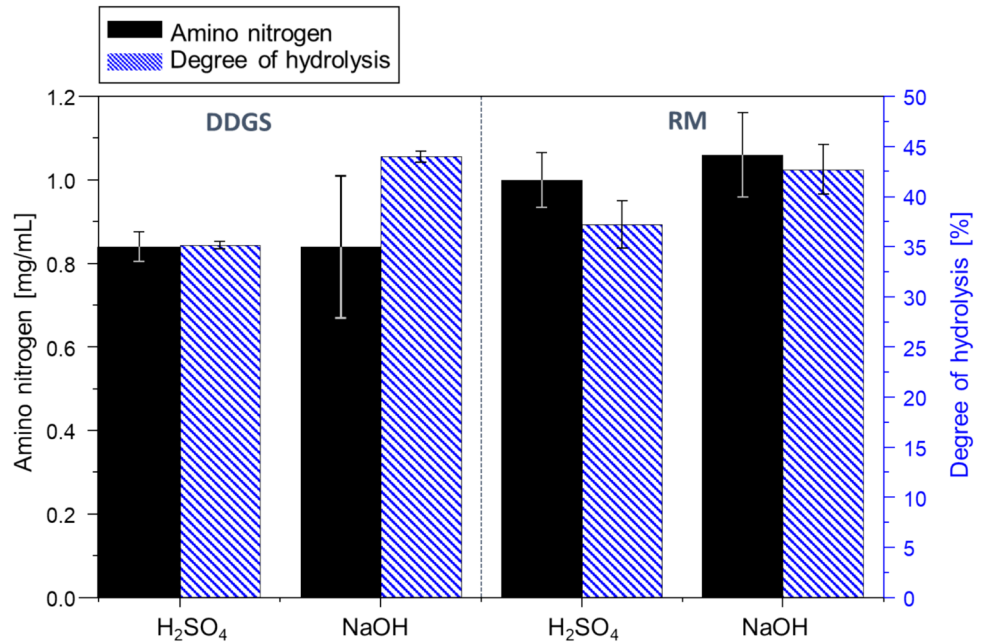


Fig. 2 Free amino acids of Distillers' Dried Grains with Solubles (DDGS) and rapeseed meal (RM) hydrolysates under reference digestions conditions: 110 °C, 24 h and 3 M H₂SO₄ or 6 M NaOH. ALA—Alanine, GLY—Glycine, VAL—Valine, LEU—Leucine, THR—

Threonine, SER—Serine, PRO—Proline, ASP—Aspartic acid, MET—Methionine, GLU—Glutamic acid, PHE—Phenylalanine, GLN—Glutamine, LYS—Lysine, HIS—Histidine, TYR—Tyrosine, TRP—Tryptophan

calcium sulfate and other solids were separated off by centrifugation. Therefore, the salt content caused by the chemical hydrolysis with sulfuric acid is relatively low. In

contrast, the basic digestion was neutralized with hydrochloric acid and thus a high concentration of sodium chloride is given in the basic digested hydrolysate. Using 6 M

NaOH a concentration of about 35 g/L NaCl occurs. To reduce the content of dissolved Na-salts, the use of another neutralizing acid would not have helped at this point, since the Na-salts of common acids are all easily soluble as well as of other bases, e.g., KOH, which are strong enough to enable hydrolysis.

In order to evaluate the potential of acidic and basic hydrolysates, growth tests with microorganism were carried out. For this purpose, the complex media components used were reduced and replaced by rapeseed meal and DDGS hydrolysates in the cultivation of *L. casei*, *L. reuteri*, as well as *C. diolis*. Using the hydrolysates digested with 6 M NaOH, the growths of the microorganisms were almost completely inhibited. Using the sulfuric acid digested hydrolysates in combination with the applied sulfate removal as CaSO₄ nearly no negative effect on the growth was observed (Table 1: data exemplarily shown for *C. diolis*).

To reduce the salt content of NaCl of the basic digestion, the influence of the NaOH molarity on the degree of digestion was proofed and 3 M, 1.8 M and 0.6 M NaOH were used. Compared to 6 M NaOH a similar amino nitrogen content of 1.12 mg/mL and a degree of digestion of 42% were achieved with 3 M NaOH. In contrast, the degree of digestion with 1.8 M and 0.6 M NaOH decreased by 16% and by 80%, respectively. By halving the molarity to 3 M NaOH, the same digestion effect was achieved and at the same time the salt load that was introduced into the bioprocess was reduced. This basic hydrolysate, using 3 M NaOH, was also used for growth experiments for *L. casei* and again led to an inhibition of growth. Thus, the digestions with NaOH obviously contain impurities that lead to growth inhibition. Another reason that negatively affects the growth of the microorganisms or the product formation could be the racemization of the amino acids due to the digestion using 6 M NaOH. Racemisation of amino acids in alkaline hydrolysis is well known as well

as the fact that many D-amino acids are metabolized to a lesser extent than the protein bound L-amino acids [29].

For further hydrolysis of the agricultural raw material following conditions were set as reference: rapeseed meal (RM), 110 °C, 24 h and 3 M H₂SO₄.

Optimising the Acid Hydrolysis

To optimize the hydrolysis of the agricultural raw material rapeseed meal with sulfuric acid, digestions were carried out between 110 and 160 °C and between 1 and 24 h. In addition, the molarity of the used sulfuric acid was varied between 0.1 and 3 M and finally the free amino nitrogen (AN) and the degree of digestion were determined.

The severity correlation (severity factor—R₀) was chosen for better comparison (Fig. 3). This factor is used in the literature for the pretreatment of lignocellulose hydrolysis and correlates to temperature and digestion time [30, 31]. The severity factors are calculated using the following equation:

$$R_0 = \log \left(t \cdot e^{\frac{\theta-100}{14.75}} \right)$$

R₀ severity factor.

t duration of hydrolysis [min].

θ temperature of hydrolysis [°C].

The conditions expressed as severity factor R₀ are harsher at higher R₀ values than at lower values. Thus, with increasing R₀ and increasing molarity of the sulfuric acid, the released amino nitrogen content increased. After graphical evaluation, the slope flattens off at R₀ ≈ 3.2 for 3 M H₂SO₄ and R₀ ≈ 3.7 for 1 M H₂SO₄ indicating no significant further hydrolysis at even higher R₀ values. The molarity of the sulfuric acid has a clear influence on the hydrolysis (Fig. 3). With a log (R₀) value of 4.04, 0.12 mg/mL of free amino nitrogen was measured with 0.1 M H₂SO₄, which corresponded to a degree of digestion of 5%. With 0.5 M H₂SO₄, the degree of digestion was tripled, which corresponds to

Table 1 Growths of *Clostridium diolis* DSM 15410 using 4 g/L yeast extract (Reference) compared to rapeseed meal (RM) and Distillers' Dried Grains with Solubles (DDGS) hydrolysates (Hydrolysis were

performed under reference digestions conditions: 110 °C, 24 h and 3 M H₂SO₄ or 6 M NaOH, afterwards neutralization to pH 7.2 and 7.3). Values are normalized: OD at 605 nm at Time_i / Time_{end}

Time	Reference	RM 3 M H ₂ SO ₄	RM 6 M NaOH	DDGS 3 M H ₂ SO ₄	DDGS 6 M NaOH
[h]	OD _{ti/tend}	OD _{ti/tend}	OD _{ti/tend}	OD _{ti/tend}	OD _{ti/tend}
0	0.03	0.04	1.07	0.07	1.05
4	0.05	0.07	1.01	0.08	0.97
6	0.10	0.11	1.00	0.16	0.97
8	0.26	0.23	1.00	0.40	0.97
10	0.57	0.56	1.03	0.82	1.00
12	1.00	1.04	1.02	1.09	1.00
24	1.00	1.00	1.00	1.00	1.00

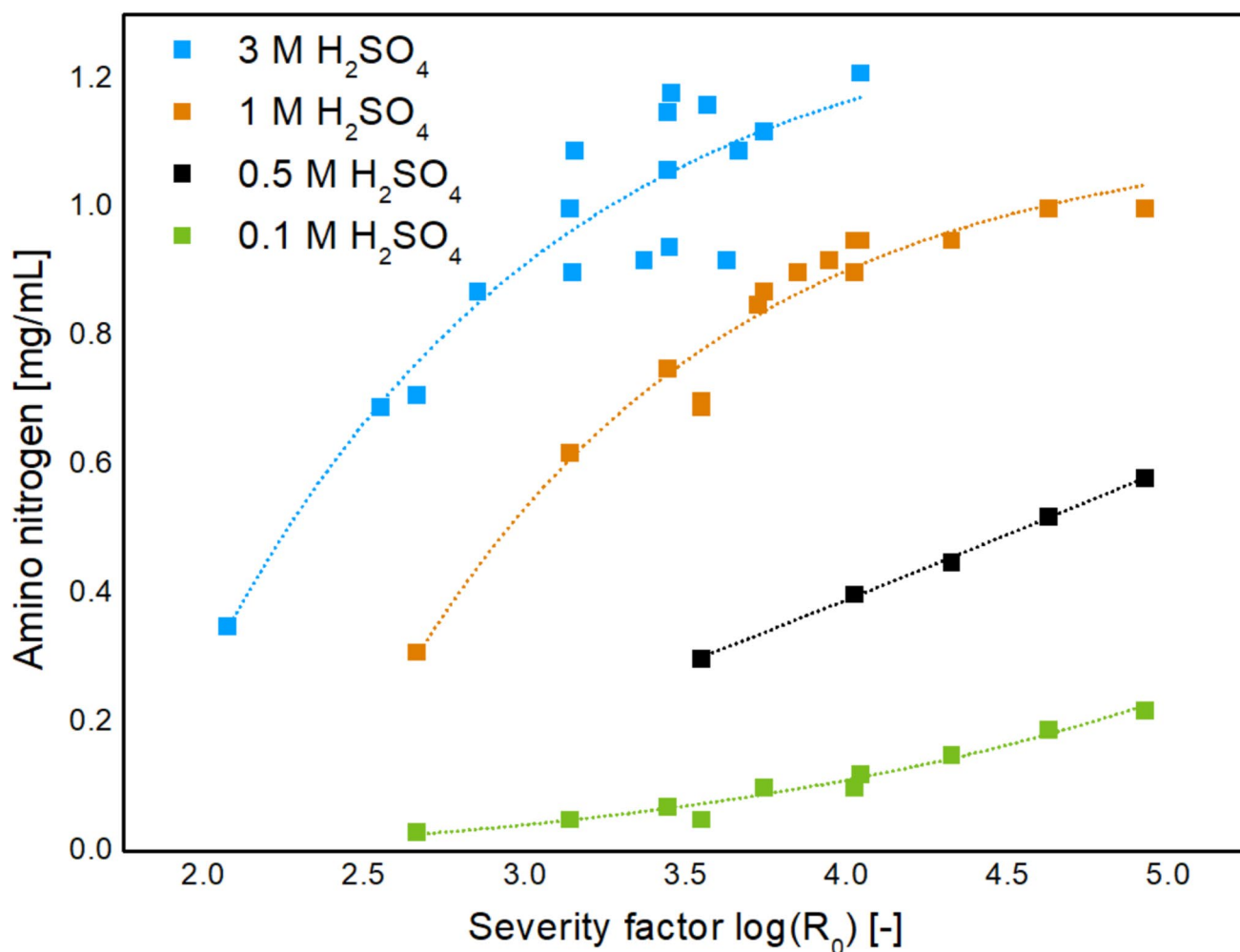


Fig. 3 Effect of hydrolysis temperature (110–160 °C), hydrolysis time (1–24 h) and molarity (0.1 M–3 M) of the sulfuric acid on the ammonium nitrogen content during the hydrolysis of rapeseed meal

0.42 mg/mL amino nitrogen. A further increase in sulfuric acid to 1 M showed a degree of digestion of 35% and 0.95 mg/mL AN (130 °C, 24 h) and 34% and 0.92 mg/mL AN (160 °C, 3 h). With a $\log(R_0)$ of 4.04 with 3 M sulfuric acid, the highest degree of digestion was achieved with 45% and 1.22 mg/mL free amino nitrogen. Due to restrictions of the reactor more severe conditions, in the form of higher temperatures and molarities, could not be performed.

Table 2 shows the variations of the digestions, which had a high amino nitrogen concentration of > 1.1 mg/mL compared to the reference hydrolysis (110 °C, 24 h, 3 M sulfuric acid). In addition, the digestion with 1 M H_2SO_4 was included in this list, since the cost of concentrated sulfuric acid makes the reactants a noteworthy cost factor compared to other operating materials. By increasing the temperature from 110 to 130 °C and reducing the hydrolysis time from 24 to 6 h, it was possible to achieve a similar amino nitrogen concentration with 3 M sulfuric acid with a constant $\log(R_0)$

Table 2 Content of amino nitrogen of digested rapeseed meal at varying temperature, time and sulfuric acid molarity; severity correlation (severity factor— R_0), amino nitrogen content [%] related to the reference (110 °C, 24 h, 3 M H_2SO_4)

$\log(R_0)$ [-]	Temperature [°C]	Time [h]	Molarity [M]	Amino nitrogen	
				[mg/mL]	[%]
3.45 (Reference)	110	24	3	1.17	100
4.02	130	24	3	1.22	104.6
3.44	130	6	3	1.14	97.1
4.32	160	6	1	0.95	81.3

value. The reference digestion (110 °C, 24 h) and the modified digestion variant (130 °C, 6 h) only differed by 2.9%. By increasing the digestion temperature to 130 °C and keeping the hydrolysis time constant, it was possible to increase the amino nitrogen concentration by 4.6%. In order to achieve

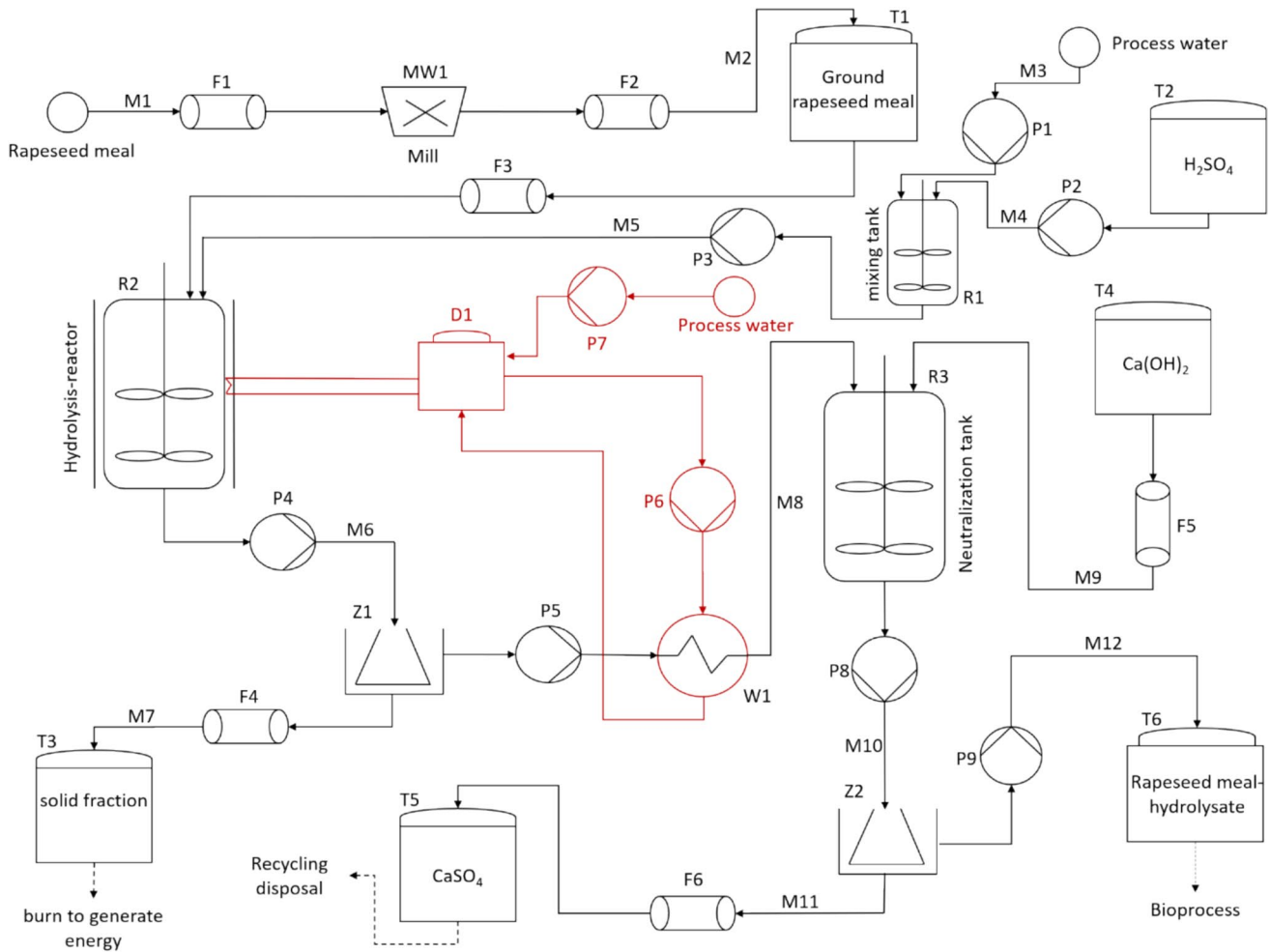


Fig. 4 Scheme of a process for the production of rapeseed meal hydrolysate, the process section for heating reactor R2 being shown in red. D steam generator, F conveyor, M mass flow, MW grinder, P pump, R reactor, T tank, W heat exchanger, Z centrifuge

Table 3 Investment costs for the construction of a plant for the production of rapeseed meal hydrolysate. Lump sum values are taken from a TEA by Tejayadi and Cheryan 1995 [34]

1. Investments	Reference (110 °C, 24 h, 3 M H ₂ SO ₄)	Variation (160 °C, 6 h, 1 M H ₂ SO ₄)
Grinding	278,478 €	278,478 €
Hydrolysis	2,122,129 €	2,959,009 €
Neutralisation	992,880 €	992,880 €
2. Other Investments of 1.		
Ducts	30% 1,018,046 €	1,269,110 €
Buildings	10% 339,349 €	423,037 €
Rural repairs	5% 169,674 €	211,518 €
3. Others of 1. and 2.		
Engineering, contracts, the unforeseen	27.5% 1,353,153 €	1,686,895 €
Sum	6,273,708 €	7,820,889 €

acceptable amino nitrogen concentrations with reduced sulfuric acid molarity, the hydrolysis temperature had to be raised to 160 °C. This increase of temperature by 50 °C resulted in 0.95 mg/mL, i.e. 81.3% of the AN concentration

using 1 M H₂SO₄ compared to the reference conditions with 3 M H₂SO₄.

Three of the four hydrolysis methods lead to similar amino nitrogen concentrations of 1.1–1.2 mg/mL, the fourth with the lowest R_0 factor still leads to almost 1 mg/mL

amino nitrogen. Nevertheless, all four methods can be used to digest protein-rich agricultural residues to a large extent. However, in order to keep the costs for complex media components as low as possible and the costs for concentrated sulfuric acid represent a noteworthy cost factor compared to other operating materials in the hydrolysis process, the combination of 1 M H_2SO_4 and 160 °C is as well considered for economic and resource efficiency reasons although slightly less amino nitrogen is released.

Technological and Economic Estimation of the Hydrolysis Process

The process was divided into three steps, grinding, hydrolysis and neutralization, and the system boundaries were set. The transport of the raw material as well as the storage, filling and transport of the rapeseed meal hydrolysate, the calcium sulfate and the treated rapeseed meal were not included. An annual operating time of 8,300 h and a hydrolysis reactor with a volume of 70 m³ and a filling volume of 80% were selected as the basis for the entire process. To compare the costs of commercially available yeast extract and rapeseed meal hydrolysate, the total nitrogen content (TN) of both substances were considered and the cost of rapeseed meal hydrolysate was calculated for the nitrogen content of one tonne of yeast extract. This scheme was filled with preliminary experimental data and, if not available, data from literature, respectively.

Process Outline

A technical analysis was first performed for the reference conditions for the hydrolysis of rapeseed meal (110 °C, 24 h, 3 M H_2SO_4).

To do this, a process scheme was designed as a basis for the calculation of the investment, material and energy costs (Fig. 4). The rapeseed meal is ground to a defined particle size (MW1) and stored until further use (T1). For the hydrolysis, the concentrated sulfuric acid (T2) is mixed with process water in a tank (R1) in advance, so that a concentration of 3 M H_2SO_4 is achieved. Then the 3 M H_2SO_4 and the ground rapeseed meal are pumped / transported into the hydrolysis reactor (R2) and heated to 110 °C under continuous stirring. The hydrolysis temperature of 110 °C is kept constant for 24 h using the steam generator (D1). The components of the crude hydrolysate (M6) are separated by a centrifuge (Z1). The solid fraction of the crude hydrolysate is stored in a tank (T3), the liquid fraction of the crude hydrolysate (M8) is cooled to room temperature by a heat exchanger (W1) and pumped into the neutralization tank (R3). By means of the heat exchanger, the water that is used to heat the hydrolysis reactor (R2) is heated from approx. 25

to 90 °C and is used for heating the next batch. The process for heating the reactor (R2) is shown in red in the process scheme. The liquid phase of the crude hydrolysate is mixed with $\text{Ca}(\text{OH})_2$ (T4) in a stirred reactor (R3) until a neutral pH value of approximately pH 6.6 has been reached. Afterwards this mixture is separated using a centrifuge (Z2). The solid fraction (CaSO_4) which precipitated is transported into a tank (T5). The final product, the liquid phase (M12), is the rapeseed meal hydrolysate, which is also stored in a tank (T6). The residual CaSO_4 is recycled for building materials and the treated rapeseed meal (solid fraction of the crude hydrolysate (T3)) can be burned to generate energy.

In a theoretically approach calculated on a dry matter base, 16.5 t of rapeseed meal could be converted into approx. 24 m³ of rapeseed meal hydrolysate, which corresponds to a total nitrogen content of 9.3 t of yeast extract. Based on this calculation, with an annual operating time of the designed plant of 8,300 h, hydrolysate could be produced with a total nitrogen concentration that corresponds approximately to the amount of 3,200 t of yeast extract.

Economic Estimation

An economic estimation was performed for the reference conditions for the hydrolysis of rapeseed meal (110 °C, 24 h, 3 M H_2SO_4).

For this economic analysis, the annual investment, material and energy costs for the plant shown in Fig. 4 based on dry matter with an operating time of 8,300 h were calculated. The investment costs for all equipment [32–34] were adjusted using the Chemical Engineering plant cost index [35]. Energy costs were estimated on the basis of the performance of all machines [36] and additional energy costs such as MRS technology, control center, lighting, buildings etc. were added with 30% of the calculated energy costs. Table 3 shows a summary of the investment costs and Table 4 shows the calculation of the annual running costs. It was assumed that the investment costs would be depreciated over 20 years, which would then amount to approximately 6.2 million € and an interest rate of 8%. The annual costs of the plant, the costs for materials and recycling of the CaSO_4 thus amount to approximately 3.36 million €, whereby approximately 8,400 m³ of rapeseed meal hydrolysate can be produced. In the economic analysis, the energy generation by incineration of the treated rapeseed meal, i.e. the residual rapeseed meal after hydrolysis, and the recycling of the CaSO_4 incurred for the construction industry were considered. Thus, based on the calculated mass flows, rapeseed meal hydrolysate with the same nitrogen content as a ton of yeast extract would cost 1,044 € (Table 5), whereas yeast extract has a price between 6,000–10,000 € per ton. Hence, a very

Table 4 Annual running costs of the rapeseed meal hydrolysate plant based on a depreciation of 20 years at 8% interest. Lump sum values are taken from a TEA by Tejayadi and Cheryan 1995 [34]

Cost type	% of Investment costs	Reference (110 °C, 24 h, 3 M H ₂ SO ₄)	Variation (160 °C, 6 h, 1 M H ₂ SO ₄)
Energy		138,153 €	416,716 €
Maintenance and cleaning	2%	125,474 €	156,418 €
Administration	0.5%	31,369 €	39,104 €
Insurance	1%	62,737 €	78,209 €
Unforeseen	0.75%	47,053 €	58,657 €
Depreciation		313,685 €	391,044 €
Imputed interest	8%	501,897 €	625,671 €
Staff		200,000 €	200,000 €
Rapeseed meal		1,150,818 €	3,948,232 €
H ₂ SO ₄		360,434 €	412,193 €
Process water		22,040 €	83,567 €
Ca(OH) ₂		212,724 €	129,015 €
Recycling CaSO ₄		195,432 €	118,527 €
Sum		3,361,816 €	6,657,353 €

Table 5 Comparison and distribution of the calculated costs for the production of rapeseed meal hydrolysate as a yeast extract-replacement based on the same nitrogen content

	Reference (110 °C, 24 h, 3 M H ₂ SO ₄)	Variation (160 °C, 6 h, 1 M H ₂ SO ₄)
T [°C]	110	160
H ₂ SO ₄ [M]	3	1
t [h]	24	6
Annual costs of the plant [Mio. €]	~3,64	~6,66
Replacement of YE [t/a]	3219	8719
Price of YE-replacement [€/t]	1.044	783
Percentage of the annual costs [%]		
Raw materials	52	69
Recycling CaSO ₄	6	2
Energy	4	6
Maintenance and cleaning	4	2
Administration	1	1
Insurance	2	1
Unforeseen	1	1
Depreciation [20 a]	9	6
Interest rate	15	9
Staff	6	3

significant cost reduction for the nutrient source compared to yeast extract can already be achieved with the standard hydrolysis process setup.

According to our analysis, the raw materials have the greatest impact with 52% of the annual production costs of rapeseed meal hydrolysate (Table 5). The raw material costs consist of the costs for rapeseed meal (66%), sulfuric acid (21%), calcium hydroxide (12%) and process water (1%). A quarter of the costs for the production of the hydrolysate under standard conditions (110 °C, 24 h, 3 M H₂SO₄) is the repayment of the investment costs (imputed interest 15% and depreciation 9%). Costs of staff and recycling of the resulting calcium sulphate for the construction industry each account for 6% and energy, as well as the maintenance and cleaning of the system, each account for 4% of the hydrolysate costs. The remaining costs consist of administration, insurance and the unforeseen.

Technological and Economic Estimation of the Hydrolysis Variation

Different variations of the digestions were performed and compared to the reference hydrolysis with 110 °C, 24 h and 3 M H₂SO₄ (data not shown). The economic efficiency analysis of the digestion with 1 M H₂SO₄ will be provided here, because the cost of 128 € per ton of concentrated H₂SO₄ makes this reactant to a considerable cost factor compared to other operating materials. Further on, less Ca(OH)₂ will be needed for neutralization leading to a more resource efficient overall process.

In this approach, the concentration of the sulfuric acid was reduced to 1 M H₂SO₄, the temperature was raised to 160 °C and the hydrolysis duration was set to 6 h (Table 3, 4, and 5). The amino nitrogen yield of 81.3% (Table 2) compared to the reference was considered. The price per ton of yeast extract replacement could be reduced to 764 € under these hydrolysis conditions (Table 5). Due to the shortened hydrolysis time and thus increased throughput of hydrolysate, the filter belts used were extended to 50 m and the increased investment and electricity costs were taken into account (Table 3). Due to the reduction of the hydrolysis time an almost tripled higher production of the yeast extract-replacement of 8700 t/a is possible in the plant, despite the slightly lower yield compared to the reference conditions. Due to the considerably increased product quantity at only slightly higher investment costs, the price per produced ton drops. As the molarity of sulfuric acid is also reduced by 66%, the costs in the area of acquisition costs for sulfuric acid and recycling are reduced accordingly, unless the reduced yield of 81.3%. In the end, the price per ton of yeast extract replacement drops to 764 € due to all considered variations (Table 5). Thus, the cost of the hydrolysate is 7.6–12.7% of the original cost of the yeast extract with the same nitrogen content. In summary, rapeseed meal hydrolysate therefore represents a

significantly cheaper alternative compared to yeast extract. Further on it can be assumed that many other protein-rich agricultural besides the studied rapeseed meal and DDGS would lead to comparable cost-efficient nutrient sources for fermentation purposes if treated according to the processes described in this paper.

Conclusions

Total chemical hydrolysis enables extremely efficient use of the nutrient sources. The acid used and the subsequent neutralization with $\text{Ca}(\text{OH})_2$ result in additional costs, but these are low compared to the costs of yeast extract. In order to minimize the digestion and neutralization costs, the acid concentration used was successively reduced and the resulting degree of hydrolysis was determined based on the free amino nitrogen content.

The optimization of the hydrolysis of protein-rich agricultural raw materials shows, that the molarity of sulfuric acid has a significant influence. The amount of free amino nitrogen increases, with increasing molarity of the acid. In order to achieve acceptable amino nitrogen concentrations with reduced sulfuric acid molarity, the temperature was raised to 160 °C. This increase of 50 °C achieved 81.3% of the amino nitrogen concentration with 1 M sulfuric acid compared to using 3 M sulfuric acid. As a result of this optimization, the costs for the hydrolysate rapeseed meal are 7.6–12.7%, of the original costs of the yeast extract with the same nitrogen content.

In further studies, it has already been shown that this rapeseed meal hydrolysate was successfully used in growth experiments in bioprocesses for the production of L-lactic acid and 1,3-propanediol [37].

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Data Availability All data supporting this paper results are included in this document.

Code Availability Not applicable.

Declarations

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