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Research Article

By-products from the essential oil industry – hydrothermal and enzymatic hydrolysis for obtaining low-molecular carbohydrates

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Abstract
Solid industrial post-distillation rose (*Rosa damascena*) – RD, and lavender (*Lavandula angustifolia*) – L, residues were subjected to enzymatic and hydrothermal/enzymatic pretreatments aiming at increase of released low-molecular carbohydrates and obtaining of residual biomass, suitable for adsorption of pollutants. Enzymatic pretreatment with Novozymes Viscoferm® combined with preliminary ultra-sonication of residues for 1440 min led to release of 11.27±0.19% and 13.11±0.21% sugars (expressed as D-glucose), respectively. After enzymatic hydrolysis the composition of RD and L was: non-cellulosic polysaccharides (NCP) (7.79±0.15 and 9.86±0.16 $g.100^{-1}$ g biomass, respectively), uronic acids (3.54±0.12 and 2.28±0.10 $g.100^{-1}$ g biomass, respectively), cellulose (12.16±0.21 and 15.87±0.22 $g.100^{-1}$ g biomass, respectively), and lignin (45.48±0.14 and 49.79±0.18 $g.100^{-1}$ g biomass, respectively). The enzymatic treatment lead to two-fold decrease of the amount of cellulose and increase of lignin in the solid residues. The hydrothermal pretreatment was performed at 140 and 160°C for 1 h. The following enzymatic hydrolysis led to increase of the released sugars: 13.65±0.29% and 15.95±0.18% for RD and L, respectively. After hydrothermal and enzymatic hydrolysis the composition of RD and L was: NCP (5.22±0.17 and 7.16±0.19 $g.100^{-1}$ g biomass), uronic acids (1.67±0.15 and 1.08±0.09 $g.100^{-1}$ g biomass), cellulose (8.04±0.16 and 9.87±0.19 $g.100^{-1}$ g biomass), and lignin (55.61±0.24 and 57.92±0.17 $g.100^{-1}$ g biomass), respectively.

Keywords
rose (*Rosa damascena*), lavender (*Lavandula angustifolia*), hydrothermal pretreatment, enzymatic hydrolysis

Abbreviations
RD – *Rosa damascena* Mill.; L – *Lavandula angustifolia* Mill.; NCP – non-cellulosic polysaccharides

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Introduction

Agricultural and food industry wastes and by-products are the main sources of cellulose, hemicellulose and starch, which can be converted into low-molecular compounds, such as oligo- and monosaccharides, using various chemical and biological methods. These low-molecular compounds can be used as raw materials in the food, cosmetic and pharmaceutical industries, as well as in the production of intermediates and the development of biomaterials in the chemical and biotechnology industries (Cardenas-Toro et al. 2014). This approach for biomass (complex carbohydrates) transformation is in the foundation of the last years introduced biorefinery concept for replacement of oil for energy and different chemicals production (Cherubini 2010). Lignocellulosic biomass could mainly be obtained in a reasonable and economically viable ways from lumber mill residues, agricultural and food industry wastes, energy crops by-products, municipal solid residues, and organic-based waste, generated by the industry (Gupta and Demirbas 2010). By-products obtained from the processing of essential oil crops are a cheap, easily accessible, biodegradable and renewable starting material for the production of low-molecular hydrolysates rich in carbohydrates (oligo- and monosaccharides), which can be used as precursors for the production of valuable biopolymers (Wojcieszak and Itabaiana 2020). There are two main ways to degrade the cellulose and hemicellulose matrix – chemically (acid hydrolysis) and by using enzymes. The chemical method uses reagents that pose a threat to the environment, and the purification of the resulting hydrolysates is also associated with neutralization of the resulting highly acidic solutions. The use of biotechnological methods is associated with working at lower temperatures and in a moderate pH, which leads to more environmentally friendly conditions. One of the main problems of enzymatic hydrolysis is that plant matrices are resistant and the direct application of enzymes leads to a low degree of conversion. This requires that the raw materials be pretreated in order to facilitate the access of enzyme preparations to the biopolymers. Such treatments can be chemical in nature (which again leads to environmental problems), mechanical processing, sonication, etc. The search for new, alternative and environmentally friendly methods brings to the fore a method that involves the use of water heated above its boiling point – hydrothermal treatment (Martin et al. 2022). It is known that this technology has been used as a "green" variant of extraction with organic solvents, but this treatment also leads to hydrolysis of biopolymers in the cell walls (mainly polysaccharides) and thus increases the amount of reducing carbohydrates (Cardenas-Toro et al. 2014; Wojcieszak and Itabaiana 2020). Water, when subjected at higher temperatures and pressures, changes significantly its physicochemical properties and participates as a reagent for chemical depolimerization of biopolymers building the plant matrix. These parameters change lead to a reduced water dielectric constant, therefore increasing organic substances solubility, increase of charged products which intensifies acid-base reactions, and increase of water density and substances solubility (Cardenas-Toro et al. 2014). Pretreatment and enzymatic depolymerization, followed by microbial fermentation of low-molecular carbohydrates, are steps from the biorefinery sugar-derivatization pathway, aiming at production of bio-based fuels and additional products from biomass (Wyman and Dale 2015), but to the best of our knowledge no data could be found for hydrothermal treatment/ pretreatment of essential oil industry solid wastes. This led us, in addition to pretreatments with water and water-ethanol solutions, as options for preliminary handling of the raw materials, to the use of hydrothermal treatment of rose and lavender post-distillation solid biomass, as an innovative and environmentally friendly method for processing plant residues for obtaining of low-molecular carbohydrates.

Materials and Methods

Materials. The rose (*Rosa damascena*) and lavender (*Lavandula angustifolia*) biomasses, by-products resulted from industrial steam-water and steam distillation, respectively, were obtained from distillery of Mirkovo (Sofia district, Bulgaria, 2023). For enzymatic hydrolysis, commercial combined enzyme preparation Novozymes Viscoferm® (Novanesis, Novozymes Switzerland AG, Aesch) were used, which were kindly provided by Essentika (Katunitsa village, Plovdiv, Bulgaria).

Methods. The residues were roughly chopped using garden shredder and then finely milled with a

laboratory milling machine (the main fraction particle size was between 100 μm and 250 μm).

Ultrasonic treatment of the residues was performed at two operating ultrasonic frequencies: 35 kHz (using an ultrasonic bath Siel UST 5.7-150 (Gabrovo, Bulgaria), power 300 W) and 40 kHz (using an ultrasonic bath IsoLab Laborgeräte GmbH (Germany) model 621.05.003, power 120 W). The sonication time was one hour and the temperature 40°C.

Laboratory installation used for hydrothermal treatment of waste raw materials according to (Nenov 2005) was provided by Innosolv Ltd. (Plovdiv, Bulgaria), where the treatments themselves were carried out. After preliminary experiments, two temperature regimens were chosen: at 140 and 160°C. The remaining parameters were: duration of 1 h, use of deionized water and 100 g starting material. After completion of the treatment, filtration was performed through a metal mesh with a pore size of 100 μm, the solid residue was dried at 60°C, and the liquid phase was frozen until further used and analyzed.

The protein quantities were determined by the Kjeldahl method (MultiKjel K-365, Büchi, Switzerland) and 6.25 nitrogen multiplication factor. The polyuronide content (PUC) and degree of the esterification (DE) of the biomasses were determined as described by Marovska and Slavov (2023). The dietary fibers analyses were performed with K-TDFR-100A (Megazyme, Ireland) according to the instruction from the manufacturer provided with the kit. The low-molecular carbohydrates released by enzymatic and hydrothermal treatments were determined according to Lever (1972). The cellulose, non-cellulosic polysaccharides, lignin and pectic substances before and after enzymatic/hydrothermal treatments were determined according to Pla et al. (2012).

The experimental data (three replications) are presented as mean value ± standard deviation. For analysis one-way ANOVA test (Tukey's post hoc test; $p < 0.05$) was used with Microsoft Excel 2013 (with XL Toolbox NG module installed).

Results and Discussion

Preliminary characteristics of the RD and L residues. The protein amount in the RD biomass was 10.45±0.55% and in L residue was slightly lower – 9.33 ± 0.34% (Table 1). The L biomass (6.17±0.38%) had almost two times higher mineral content than RD (3.57±0.36%). A possible explanation for these observations might be the type of extraction of essential oil – the lavender fresh stems and leaves were steam distilled (meaning lower amount of water in the distillation vessel), while rose flowers were steam-water distilled and in this process water is added in the distillation still (rose: water = 1: 4 approximately) which could remove part of the inorganic salts. Besides, for obtaining rose oil only the rose flowers (hand-picked) were used and for lavender oil the whole plant (harvested mechanically) is steam distilled.

Table 1. Characteristics of RD and L biomass

Residue	Protein, %	Ash, %	Moisture, %	DE, %	PUC, %
RD	10.45^{a}±0.55	3.57^{b}±0.36	11.54^{a}±0.26	75.18^{a}±1.56	9.26^{a}±0.41
L	9.33^{a}±0.34	6.17^{a}±0.38	8.78^{b}±0.29	70.85^{b}±1.68	8.19^{a}±0.47

*DE: degree of esterification; PUC: polyuronide content. Results are presented as the mean of three measurements; $^{a, b}$ Different letters in columns indicate statistically different values (Tuckey's HSD test, $p<0.05$)

The polyuronide content, usually related to presence of acidic type polysaccharides in the plant matrix, for both biomasses were similar (no significant difference) – 8.19±0.47% for L and 9.26±0.41% for RD. Comparing the data of steam distilled lavender residue from the same location but harvested in 2022 the protein content is similar (statistically not different) but the polyuronide content is higher with 19% and the DE is lower around 10% (Marovska et al. 2024). Slavov et al. (2024) reported for a steam-water distilled wastes from essential rose oil industry (2020 crop) twice as higher ash content (8.74±0.36%), higher protein content (13.05±0.38%) and comparable polyuronide

content: 8.89±0.14%. These results suggest that although variability of the amount of some the substances in the wastes from different years exists the pectic substances (polyuronides) remains generally in similar ranges.

Enzymatic hydrolysis of RD and L residue. Based on the data from the manufacturer of the enzymatic preparation, several preliminary experiments were carried out with the starting materials, varying the time, temperature, pH of the medium and the amount of enzymes used (Table 2). Whether the conditions for hydrolysis of the plant biomass were suitable was judged mainly by the released low-molecular carbohydrates (expressed as glucose quantity) by the amount of remaining solid mass (after filtering the solution and drying), as well as by the analyses of the solid mass for the content of cellulose, lignin, hemicelluloses and pectic substances (Pla et al. 2012).

The results of the experiments showed that increasing time, an increase in the amount of glucose in the liquid phase was observed (Table 3). In general, the other parameters had a lesser effect than the hydrolysis time. It was established that above 50°C hydrolysis proceeds at approximately the same rate, and for this reason, also in order to save energy, this temperature was chosen as the optimal one from an ecological point of view for further experiments. The results of the experiments on the pH of the medium showed that the optimum was achieved at 5.5, which gave us reason to use further this value. When using 150 µL of enzyme preparation, optimal conditions for hydrolysis were established, which showed us that in the following experiments we can use similar amounts, recalculated to the initial sample amount used.

Table 2. Values of the parameters time, temperature, pH of the medium and amount of enzyme preparation used (initial amount of RD or L 2 g; hydromodule 1: 20; shaking speed 100 min^{-1})

Parameter / Value	Time, min	Temperature, °C	pH	Amount enzyme, µL
X1	30	40	4	25
X2	60	50	4.5	50
X3	120	60	5	100
X4	360	70	5.5	150
X5	1440	80	6	200

Table 3. Influence of time on the degree of hydrolysis and the production of glucose as the main monosaccharide (temperature 50°C, pH of the medium 5.5 (0.5 M citrate buffer), amount of enzyme preparation used 150 µL, starting amount of RD and L biomass 2 g; hydromodule 1:20; agitation speed 100 min^{-1})

Time, min	RD	L
	Amount of glucose released, %	
30	0.49^{a}±0.20	0.53^{a}±0.15
60	1.92^{b}±0.08	2.51^{a}±0.09
120	2.24^{b}±0.08	2.99^{a}±0.11
360	6.71^{b}±0.20	7.51^{a}±0.15
1440	11.27^{b}±0.19	13.11^{a}±0.21

$^{a, b}$ – different letters indicate statistically different results in the rows (Tuckey's HSD test; $p<0.05$); results are presented as the mean of three measurements

After 24 h, no significant increase in the amount of glucose was observed, and for this reason we established 24 hours as the optimal duration of hydrolysis. The results of the enzymatic hydrolysis showed that the amount of glucose obtained from L was 14% higher at the end of the incubation period. This can be explained by the higher content of cellulose and hemicelluloses in the lavender residue, since it consists mainly of the stems of the plant, due to the technological method of harvesting

(mechanized harvesting for lavender and hand picking of flowers for roses). At the same time, RD is richer in pectin polysaccharides. Table 4 presents the composition of the raw materials (solid biomass) before and after enzymatic hydrolysis.

Table 4. Composition of RD and L biomass before and after enzymatic hydrolysis

Composition	Initial biomass		Residual solid mass after enzymatic hydrolysis	
	RD, g.100^{-1} g	L, g.100^{-1} g	RD, g.100^{-1} g	L, g.100^{-1} g
NCP	12.79^{b}±0.15	15.86^{a}±0.16	7.79^{d}±0.15	9.86^{c}±0.16
Uronic acids	10.54^{a}±0.12	8.28^{b}±0.10	3.54^{c}±0.12	2.28^{d}±0.10
Cellulose	25.16^{b}±0.21	30.87^{a}±0.22	12.16^{d}±0.21	15.87^{c}±0.22
Lignin	20.48^{d}±0.14	21.79^{c}±0.18	45.48^{b}±0.14	49.79^{a}±0.18

$^{a, b, c, d}$ – different letters indicate statistically different results in the rows (one-way ANOVA (Tuckey's HSD test; $p<0.05$)

Table 5. Enzymatic hydrolysis of hydrothermally treated (at 140 and 160°C) initial materials (temperature 50°C, pH of the medium 5.5 (0.5 M citrate buffer), amount of enzyme preparation used 7.5 mL, starting amount 100 g; hydromodule 1:20; agitation speed 100 min^{-1})

Sample		Sugars, %	Amount of solids (after drying), g	Amount of liquid phase, mL
RD 140°C	before hydrolysis	3.52^{g}±0.04	53.08^{a}±0.86	1430^{c}±3
	after hydrolysis	9.55^{d}±0.17		
RD 160°C	before hydrolysis	4.51^{f}±0.08	44.96^{b}±0.79	1680^{a}±4
	after hydrolysis	13.65^{b}±0.29		
L 140°C	before hydrolysis	4.24^{f}±0.08	52.11^{a}±0.91	1450^{b}±5
	after hydrolysis	11.45^{c}±0.13		
L 160°C	before hydrolysis	5.08^{e}±0.12	41.57^{c}±1.05	1690^{a}±5
	after hydrolysis	15.95^{a}±0.18		

$^{a, b, c, d, e, f, g}$ – different letters indicate statistically different results in the columns (one-way ANOVA (Tukey test; $p<0.05$).

Enzymatic hydrolysis of hydrothermally pretreated RD and L residue. The hydrothermally treated starting materials were further subjected to enzymatic hydrolysis, using the already specified and optimized process conditions. Since the hydrothermally treated raw materials were previously separated into a liquid and solid phase, a mixture of the two phases in a total volume of 2000 mL was used for the enzymatic hydrolysis experiment, in which the total amount of dry matter was 100 g. For each of the samples, the pH of the medium was measured before starting hydrolysis and, if necessary, the values were corrected to 5.5.

The results of the combined application of hydrothermal treatment and enzymatic hydrolysis show that there is a significant increase in the amount of glucose, which reaches 13.65±0.29% for RD hydrothermally pretreated at 160°C and 15.95±0.18% for the lavender biomass pretreated at the same temperature (Table 5). Du et al. (2010)

reported that, especially at higher temperature regimens, during hydrothermal pretreatment variety of sugar degradation, cyclisation and aromatization products are formed. Additionally, the sugars obtained could be transformed catalytically to various aromatic compounds (i.e. furanoic derivatives) which could serve as precursors for synthesis of bio-based polymers (Slavov et al. 2022).

At the same time, the analyses of the composition of the obtained solid residues after completion of the enzymatic hydrolysis and filtration of the samples (Table 6) show that the amounts of non-cellulosic polysaccharides, uronic acids (pectic polysaccharides) and cellulose decrease significantly, at the expense of an increase in the amount of lignin. Considering that previous experiments of Angelova et al. (2021) have shown that lignin-rich plant biomass can be successfully transformed using higher fungi into mycelium-based biocomposites, which is a prerequisite for using this residual mass for additional and waste-free utilization.

Table 6. Composition of RD and L biomass (g.100^{-1} g DW residue) after combined hydrothermal treatment and enzymatic hydrolysis

Composition	Residue after enzymatic hydrolysis		Residue after combined hydrothermal pretreatment and enzymatic hydrolysis			
			140°C		160°C	
	RD	L	RD	L	RD	L
NCP	7.79^{c}±0.15	9.86^{a}±0.16	6.19^{e}±0.21	8.51^{b}±0.16	5.22^{f}±0.17	7.16^{d}±0.19
Uronic acids	3.54^{a}±0.12	2.28^{b}±0.10	2.15^{b}±0.12	1.54^{c}±0.12	1.67^{c}±0.15	1.08^{d}±0.09
Cellulose	12.16^{b}±0.21	15.87^{a}±0.22	9.81^{c}±0.18	12.35^{b}±0.21	8.04^{d}±0.16	9.87^{c}±0.19
Lignin	45.48^{f}±0.14	49.79^{d}±0.18	48.99^{e}±0.22	51.63^{c}±0.23	55.61^{b}±0.24	57.92^{a}±0.17

$^{a, b, c, d, e, f}$ – different letters indicate statistically different results in the rows (one-way ANOVA, Tukey HSD test; $p<0.05$).

The influence of preliminary ultrasonic treatment of the raw materials on the subsequent enzymatic hydrolysis and the amount of glucose obtained was also tested. In these experiments, no significant increase in glucose in the aqueous phase was observed (statistically indistinguishable results). These data showed that the plant matrix is resistant and higher powers of ultrasonic treatment might be required to break it. However, this inevitably leads to an increase in the energy input and makes this type of treatment, in this particular case, economically unprofitable. Better results were obtained combining hydrothermal pretreatment and enzymatic hydrolysis.

Conclusions

Combined enzymatic and hydrothermal pretreatment was used aiming at increase of released low-molecular carbohydrates. Solid industrial rose (*Rosa damascena*) – RD, and lavender (*Lavandula angustifolia*) – L residues were utilized as substrates in the present work. Enzymatic treatment with Novozymes Viscoferm® enzyme preparation combined with preliminary ultra-sonication of residues for 1440 min led to release of 11.27±0.19% and 13.11±0.21% low-molecular carbohydrates (expressed as D-glucose), respectively. After enzymatic hydrolysis the composition of RD and L was: NCP (7.79±0.15 and 9.86±0.16 g.100^{-1} g biomass, respectively), uronic acids (3.54±0.12 and 2.28±0.10 g.100^{-1} g biomass, respectively), cellulose (12.16±0.21 and 15.87±0.22 g.100^{-1} g biomass, respectively), and lignin (45.48±0.14 and 49.79±0.18 g.100^{-1} g biomass, respectively). The enzymatic treatment lead to twofold decrease of the amount of cellulose and more than twice increase of lignin in the solid residues. The hydrothermal pretreatment was performed at 140 and 160°C for 1 h. The following enzymatic hydrolysis led to increase of the released sugars: 13.65±0.29% and 15.95±0.18% for RD and L, respectively. After combined hydrothermal and

enzymatic hydrolysis the composition of RD and L was: NCP (5.22±0.17 and 7.16±0.19 $g.100^{-1}$ g biomass, respectively), uronic acids (1.67±0.15 and 1.08±0.09 $g.100^{-1}$ g biomass, respectively), cellulose (8.04±0.16 and 9.87±0.19 $g.100^{-1}$ g biomass, respectively), and lignin (55.61±0.24 and 57.92±0.17 $g.100^{-1}$ g biomass, respectively).
The study results suggest the RD and L industrial post-distillation biomass could be successfully utilized as starting material for combined hydrothermal and enzymatic hydrolysis and obtaining of low-molecular carbohydrates and lignin-rich biomass. Introduction of hydrothermal and enzymatic treatment has advantage compared to conventional chemical treatment, due to the mild pH medium, resulting in less hampers i.e. corrosion of the equipment. Additional plus is the enhanced selectivity regarding polysaccharides degradation, which simplifies the procedure. The remaining biomass could be further utilized as cheap and bioavailable bioadsorbent for pollutant removal and as substrate for higher fungi growth.

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Author Contributions

Conceptualization, A.S., P.G. and N.N.; methodology, A.S. and N.N.; formal analysis A.S., P.G. and N.N.; investigation, A.S., P.G. and N.N.; resources, A.S.; data curation, A.S., P.G. and N.N.; writing - original draft preparation, A.S.; writing – A.S., P.G. and N.N., review and editing, A.S.; visualization, A.S., P.G. and N.N.; supervision, A.S.; project administration, A.S.; funding acquisition, A.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The experimental protocol used in this study was designed in compliance with the guidelines of the European and Bulgarian legislation regarding the protection of animals used for experimental and other scientific purposes (Directive 2010/63; EC, 2010 - put into law in Bulgaria with Regulation 20/2012). The protocol was based on the permit for the use of animals in experiments No. 277 of the Bulgarian Food Safety Agency (Statement No. 193 of the Bulgarian Animal Ethics Committee, prot.No.18/02.07.2020, 2 July 2020).

Informed Consent Statement

Not applicable.

Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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