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Valorization of hazelnut husk as a carbon source for L-DOPA production with *Corynebacterium glutamicum*

Beril Pakalın [a], Eldin Kurpejović [a], Gülsüm Merve Bastem [a], Nihat Alpagu Sayar [a], Volker F. Wendisch [b], Berna Sarıyar Akbulut [a,*,1]

[a] Department of Bioengineering, Marmara University, Kadıköy, Istanbul 34722, Turkey
[b] Genetics of Prokaryotes, Faculty of Biology and Center for Biotechnology (CeBiTec), Bielefeld University, 33615, Bielefeld, Germany

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ABSTRACT

Lignocellulosics are abundant carbon sources in nature, therefore they gain increasing attention in biotechnology for production of value-added chemicals. In this proof-of-concept study highlighting the techno-economic potential of L-3,4-dihydroxyphenylalanine (L-DOPA) production from lignocellulosic agro-waste hazelnut husk, first an exploratory model-based optimization was used to release the sugar content of the husk whilst maximizing sugar concentration. The sugar hydrolysate was then evaluated as a feedstock for production using an engineered *Corynebacterium glutamicum* strain. Optimal treatment conditions were found by Kriging model as 12.5% husk load with 3.8% acid for 50 min at 121 °C, which gave a hydrolysate of 27.67 g L^{-1} reducing sugar with a yield of 11.07 g sugar/100 g husk. When this hydrolysate was used as the sole carbon source during fermentative production, 20 ± 0.4 mg L^{-1} of L-DOPA was produced. When cells grown on husk hydrolysate were used for whole-cell biotransformation, a titer of 82 ± 1 mg L^{-1} of L-DOPA was obtained. From an economical point of view, the titer obtained during fermentative production would allow production of L-DOPA from hazelnut husk right around the breakeven point. However, further engineering of the strains and optimization of bioprocess conditions, would make it possible to bring the revenues to higher levels.

1. Introduction

Progress made in novel metabolic engineering tools has made it now possible to produce a wide range of different molecules from commodity to specialty chemicals using microbial routes [1–3]. In these processes, renewable feedstocks are replacing their conventional alternatives with increasing awareness of sustainability [4,5]. Biomass, especially that is sidestreams/wastes of agricultural activity is one such renewable feedstock with a steady and abundant supply as a result of rapid increase in volume and types of agricultural activities. Agricultural biomass may take the form of residual stalks, straw, leaves, roots, husk, nut or seed shells, and waste wood. These lignocellulosic materials primarily contain polysaccharides and the aromatic polymer lignin as the non-carbohydrate component. Lignin is a complex structure made from three hydroxycinnamyl alcohols with different degrees of methylation. The carbohydrate polymers and lignin intertwine to form a complex and recalcitrant matrix, the lignocellulosic structure, which commonly contains 30–50 % cellulose, 15–35 % hemicellulose [6], and 15–40 % lignin [7].

The focus here will be on hazelnut husk as biomass. Under normal weather conditions Turkey produces around 600,000 MT (metric tons) of hazelnut annually, which is approximately 75 % of the total production worldwide [8], yielding waste husk in half that amount. As other agricultural wastes, hazelnut husk is commonly discarded through burning and not utilized for any other processes. This lignocellulosic material is of cellulose, hemicellulose and a high percentage of lignin polymers with very small amounts of ash, pigment and protein [9]. At the core are the organized cellulose macro-fibers, which are enveloped by the lignin. Hemicellulose molecules form a dispersed network between the lignin and cellulose portions. Different sugars (e.g. glucose, mannose, galactose, xylose, arabinose, etc.) are the monomeric components of cellulose and hemicellulose, thus, making lignocellulosic biomass attractive for microbial bioprocesses. However, the bonds and interactions between the polymers make lignocellulosic biomass

* Correspondence to: Department of Bioengineering, Marmara University, Göztepe Campus, Kadıköy, Istanbul 34722, Turkey.
E-mail address: berna.akbulut@marmara.edu.tr (B.S. Akbulut).
[1] Orcid:0000-0002-4455-1192.

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recalcitrant to degradation [10]. To this end, in addition to a number of new industrial technologies, conventional physical, chemical, physico-chemical, and biological pretreatment & hydrolysis methods and their combinations, have been used to hydrolyze lignocellulosic biomass [11–13].

In the current work, quick, exploratory model-based optimization [14,15] was pursued to release the sugar content of hazelnut husk whilst maximizing the final sugar concentration in the hydrolysate. Such an exploratory optimization approach is useful to identify the potential of the feedstock within a defined design space. Because of their easier application to hazelnut husk, dilute acid treatment, enzymatic hydrolysis and their combined applications have been evaluated. Then, *Corynebacterium glutamicum* cells with the ability to consume the released glucose and xylose were cultivated in a medium with hazelnut husk sugar hydrolysate as the sole carbon source for the production of 3, 4-dihydroxyphenyl-L-alanine (L-DOPA). *C. glutamicum* is the industrial workhorse of white biotechnology. Indeed, it is used in the million tons per year production of amino acids. On the other hand, L-DOPA, the drug used primarily for the treatment of Parkinson's disease, is an amino acid used; thus, *C. glutamicum* might be attractive for L-DOPA production as the market size of this drug rapidly expands with the increase in the number of elderly people suffering from the Parkinson's disease [16]. Finally, a preliminary techno-economic analysis has been performed based on average annual hazelnut husk available in Turkey.

2. Materials and methods

2.1. Source of hazelnut husk and its preparation for treatment

Hazelnut husk was obtained from Giresun, in the Black Sea region of Turkey. It was oven dried at 55 °C and stored at room temperature in plastic containers. Hazelnut husk was ground with 1 L Waring commercial blender and particles with sizes smaller than 5 mm were obtained.

2.2. Dilute acid treatment

For acid treatment, the total volume was adjusted to 100 mL and experiments were carried out in 250-mL Schott bottles. Ground hazelnut husk was briefly mixed with sulfuric acid solutions with concentrations ranging from 1 % to 5 % (w/w). Husk loading varied from 3.5 to 12.5 g. Hazelnut husk in the acid solution was autoclaved at 121 °C for different periods from 15 to 60 min. The conditions were selected based on the protocols reported by the National Renewable Energy Laboratory (NREL) [17].

Solid husk was filtered out using a pre-weighed filter paper. The liquid hydrolysate was collected for sugar analysis. The volume of the liquid hydrolysate was recorded. The final hydrolysate volume was always less than the initial volume probably due to the swollen husk particles. The remaining solid residue was left to oven dry at 55 °C and its dry weight was recorded. Dry pretreated husk residues were stored at room temperature for further enzymatic hydrolysis.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated hazelnut husk was carried out in 12.5 mL 50 mM sodium citrate buffer of pH 6.0 for 1–7 days in a rotating shaker at 55 °C and 100 rpm. Cellulase from *Trichoderma reesei* (Sigma-Aldrich, USA) with an activity of 700 endoglucanase units (EGU)/g and a density of 1.2 g mL^{-1} was used. The enzyme was reported to have optimal conditions for hydrolysis at 50–60 °C and pH of 4.5–6 [18]. Dried pretreated husk load ranged from 1.25 to 2.5 g and 1–2.5 mL enzyme (840 EGU-2100 EGU) were added to the reaction mixture. The conditions were selected based on the protocols reported by NREL [17].

2.4. Quantification of reducing sugars

Total reducing sugars obtained from the hazelnut husk hydrolysate were detected and quantified using 3,5-dinitrosalicylic acid (DNS) method [19]. When necessary, the pH of the pretreated sample was adjusted to 7 with 10 M NaOH prior to analysis.

2.5. Quantification of lignin component in hazelnut husk

To determine the dry matter, 1–1.5 g of dried ground hazelnut husks were placed on aluminum sample plates and analyzed on a Moisture analyzer DBS 60–3 (Kern-Sohn) on gentle drying mode. Dry matter was recorded as a percentage value.

To determine the organic matter and ash content, 50 mL porcelain crucibles were incubated at 80 °C for 1 h and then placed into a desiccator until their temperature was lowered to room temperature. Crucible weights were recorded. Approximately 10 g of dried ground hazelnut husk was placed into crucibles. The amount of hazelnut husk added was recorded up to 4 decimals places. Crucibles were then placed in a muffle furnace (Nüve MF 106) and heated for 2 h at 550 °C. At the end of the program, crucibles were placed in a desiccator until they reached room temperature and ash content was weighed. Percent ash in the sample was calculated and subtracted from 100 to obtain the organic matter percentage.

To determine the acid insoluble lignin, 200 mg of dried ground hazelnut husk was mixed with 15 mL of H_2SO_4 and 6 mL of dH_2O to and stirred at 200 rpm for 1 h. Then, the solution was transferred to a 2 L Erlenmeyer flask and 367 mL dH_20 was added diluting to a final acid concentration of 3% H_2SO_4. Flask was autoclaved for 1 h at 121 °C. Then the flasks were cooled to room temperature and vacuum filtered. For filtration, 47-mm glass microfiber filter paper (Grade 50 C) was incubated at 80 °C in an oven for 1 h and then in a desiccator. Their weights were measured. After the hydrolysis solution was filtered through, approximately 400 mL of dH_2O was used for washing. Filter paper with acid insoluble particulates was dried with a moisture analyzer and weighed. From the net value of undissolved particulates within 200 mg, lignin insoluble content was calculated. All analyses were done in triplicates.

2.6. Design of experiments

An inscribed central composite design with 3 factors and 5 levels has been applied for the design of both pretreatment and enzymatic hydrolysis experiments using MATLAB's central composite design (ccdesign) function (MATLAB & Simulink R2022b, The MathWorks, Inc., Natick, Massachusetts, United States). Total number of experiments was 24 with 10 center points. 10 repetitions allowed for a more uniform estimation of prediction variance.

For the acid treatment experiments, three independent variables (factors) were: hazelnut husk load, acid concentration and time (Table 1). The upper limit for the husk load has been selected based on the maximum possible husk that could be resuspended in the given reaction volume. The upper limit for the acid concentration has been selected to minimize inhibitor formation.

For the enzymatic hydrolysis, three independent variables were: pretreated husk, enzyme amount, and time (Table 2). The upper limit for the husk load has again been selected based on the maximum possible husk that could be resuspended in the given reaction volume. Maximum

Table 1
Factors and levels of experimental design for acid hydrolysis.

Factors	Levels				
Time, min	15	24	37.5	50	60
Husk load, %	3.5	5.3	8	10.7	12.5
Acid, %	1	1.8	3	4.2	5

Table 2
Factors and levels of experimental design for enzymatic hydrolysis.

Factors	Levels				
Time, day	1	2.2	4	5.8	7
Pretreated husk load, %	10	12	15.2	18	20
Enzyme, mL	1	1.3	1.75	2.2	2.5

enzyme volume was selected based on preliminary experiments. The higher amounts did not make a significant difference in the total amount of reducing sugar recovered. Maximum time was selected based on NREL's protocol for enzymatic saccharification of lignocellulosic biomass [17].

2.7. Modelling and optimization

Deterministic low-order polynomial modelling (LOP) and stochastic Kriging are two modelling techniques used in this work to fit experimental data. LOP is often used as a part of response surface methodology (RSM) while Kriging has been shown to be a preferable alternative to LOP for complex biological systems under certain conditions [20]. For LOP, MATLAB's fitlm function has been used. The response (dependent variable) for both acid treatment and enzymatic hydrolysis was total reducing sugar concentration. For Kriging, MATLAB's fitrgp function has been used. A quadratic model with a pure quadratic basis function and a rational quadratic kernel function was employed.

For numerical optimization using both LOP and Kriging models, MATLAB's global optimization toolbox has been used. As the main optimization function, fmincon (minimization under constraint) with interior-point algorithm has been utilized. Fmincon finds the minimum of nonlinear and multivariable functions as the interior-point handles both large, and small problems. The objective function of both optimization problems was the maximization of final total reducing sugar concentration.

2.8. Strains, plasmids, and growth media

All strains and plasmids used in this study are summarized in Table 3. *C. glutamicum* cells were routinely grown aerobically at 30 °C in brain heart infusion broth (BHI) for multiplication and maintenance. For the analysis of microbial growth and L-DOPA production, CGXII defined medium was used [21]. When needed, growth medium was supplemented with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG), 25 μg mL^{-1} kanamycin and/or 100 μg mL^{-1}spectinomycin.

2.9. Bacterial growth using husk hydrolysate

Wild-type *C. glutamicum* or CgXyl cells grown in 5 mL BHI for 8 h in a rotary shaker at 200 rpm were used to inoculate 25 mL of standard CGXII medium with 4 % glucose in 250-mL baffled flasks. One liter CGXII medium was prepared by mixing 900 mL sterile basic medium with 100 mL sterile carbon source. Basic medium contained per liter: 20 g $(NH_4)_2SO_4$, 5 g urea, 1 g KH_2PO_4, 1 g K_2HPO_4, and 42 g 3-morpholinopropanesulfonic acid (MOPS). pH was adjusted to 7 with 4 M NaOH. Concentrations of trace elements were as follows: 0.25 g L^{-1} $MgSO_4{\cdot}7H_2O$, 10 mg L^{-1} $CaCl_2$, 10 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 10 mg L^{-1} $MnSO_4{\cdot}H_2O$, 1 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.2 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 0.02 mg L^{-1} $NiCl_2{\cdot}6H_2O$, 0.2 mg L^{-1} biotin, and 30 mg L^{-1} protocatechuic acid. All trace elements solutions were sterilized separately using 0.22 μm filters and stored at – 20 °C, and added to the main culture media before use. Cells were incubated overnight at 30 °C and 150 rpm.

This overnight culture was used to inoculate the 50 mL CGXII main culture, in 500-mL baffled flasks, with 2.6 % glucose, 2.6 % xylose, or husk hydrolysate containing an equivalent amount of reducing sugars (2.6 %). This amount was adjusted based on the maximum possible sugar concentration that could be obtained under the conditions optimized for extraction of sugars from hazelnut husk. Commonly for 50 mL medium, 40 mL husk hydrolysate was mixed with 10 mL concentrated CGXII medium. Heterologous expression of the xylose utilization genes was induced by adding 1 mM IPTG 90 min after inoculation. Bacterial growth was monitored by measuring absorbance at 600 nm.

Table 3
Strains and plasmids used in this study.

	Description	Source
Plasmids		
pEKEx2-tyr_{Rs}	pEKEx2 harboring tyrosinase gene from *Ralstonia solanacearum*	[22]
pEKEx3-$xylA_{Xc}xylB_{Cg}$	pEKEx3 harboring *xylA* from *Xanthomonas campestris* and *xylB* from *C. glutamicum*	[23]
Strains		
C. glutamicum	Wild-type strain ATCC 13032	ATCC
CgXyl	*C. glutamicum* carrying the plasmid pEKEx3-$xylA_{Xc}xylB_{Cg}$	This work
CgEKV-II	*C. glutamicum* carrying the plasmids pEKEx3-$xylA_{Xc}xylB_{Cg}$ and pEKEx2-tyr_{Rs}	[22]

2.10. L-DOPA production using husk hydrolysate

The ability of *C. glutamicum* to produce L-DOPA when grown on husk hydrolysate as the sole carbon source was tested. For this, CgEKV-II cells constructed to utilize xylose and synthesize tyrosinase [22] were used.

For in vivo L-DOPA synthesis, 50 mL CGXII medium with 0.4 mM $CuSO_4$, 0.2 mM thymol, 1 g L^{-1} L-tyrosine (L-Tyr), and husk hydrolysate (2.6% final reducing sugar concentration) was inoculated with overnight culture of CgEKV-II cells in CGXII medium. Heterologous expression of the xylose utilization genes and tyrosinase for L-DOPA production were induced simultaneously with 1 mM IPTG 90 min after inoculation. Cells grown for 48 h were precipitated with centrifugation at 10,000 rpm for 10 min at room temperature and the cell-free supernatants were used for L-DOPA detection and quantification using HPLC.

For whole-cell biotransformation, 50 mL CGXII medium with husk hydrolysate as the sole carbon source (2.6% final reducing sugar concentration) was inoculated with an overnight culture of CgEKV-II cells in CGXII medium. Cells grown for 48 h were collected by centrifugation at 10,000 rpm for 10 min at 4 °C, washed twice, and then resuspended in 3 mL sterile distilled water. To 15-mL falcon tubes containing 10 mL of sterile distilled water with 1 g L^{-1} L-Tyr, different amounts of resuspended cells (2–40 mg of DCW) were added. The tubes were incubated at 30 °C from 72 to 144 h. Cell-free supernatants were analyzed for L-DOPA every 24 h.

2.11. Detection and quantification of L-DOPA

Detection and quantification of L-DOPA was carried out using high-performance liquid chromatography (HPLC), as explained previously [22]. An Agilent 1100 system with C18 Zorbax column (250 ×4.6 mm, 5 μm) and a UV detector at 280 nm was used. 0.1 N acetic acid–methanol (10:1) mobile phase with a flow rate adjusted to 1.2 mL min^{-1} for 10 min at 30 °C was used. Injection volume of the cell free supernatants was 20 μL. Standard L-DOPA (0.01–1.5 mg mL^{-1}) was prepared in HPLC-grade water and the retention time obtained was ~ 2.7 min

2.12. Calculations for techno-economic analysis of L-DOPA production

The techno-economic analysis has been performed based on average annual hazelnut husk available in Turkey and the amount of hazelnut husk needed for one liter of production. L-DOPA titer calculated for fermentative production was used. Average process development costs were taken from Yang et al. [24] and operational costs for such a facility were taken from Yang et al. [24] and Boulamanti and Moya [25].

3. Results

3.1. Dilute acid treatment of hazelnut husk

The results obtained from dilute acid treatment of the hazelnut husk as detailed in materials and methods are presented in Table 2. In the second, third, and fourth columns of Table 4 the values (levels) of the selected factors determined with central composite design are given.

Under the selected conditions, the reducing sugar concentration in the husk hydrolysate varied between 6.48 and 16.73 g L^{-1}, while sugar yield varied between 4.13% and 10.61% (g reducing sugar/g husk). The mean with the standard deviation for the ten center point replicates (Exps. 1–10) was 11.97 ± 0.53 g L^{-1}. In general, increasing husk loading or acid percentage or duration of the pretreatment while keeping the other two variables constant, increased obtained reducing sugar concentration. The highest concentration was achieved when the maximum allowable amount of husk was used (experiment 14). Overall, lower acid concentration and husk loading yielded lower concentration in the hydrolysate. However, with lower husk load reducing sugar yield tended to be higher, e.g. with 3.5 g husk it was 10.61%. However, despite this high yield, the final reducing sugar concentration under this condition was too low to be used for bacterial growth. For this reason, the objective of the optimization was selected as the final concentration but not the yield.

Optimum values of the selected factors were determined with two different models using the results displayed in Table 2 (Table 5). The R^2 values obtained as 0.86 and 0.90 for Kriging and LOP, respectively, have shown that both models successfully represent the dataset used to build the models. The ANOVA table is given as Supplementary material (Table S1) and the response surface plots are given as Supplementary materials (Fig. S1-S3).

The predicted sugar concentration by the models were close to each other; however, the experimentally measured concentrations under the conditions determined by the Kriging model were higher. Furthermore, the experimental conditions were slightly milder with Kriging. Thus, a treatment with 3.8 % acid for 50 min using a 12.5% husk load was selected as the optimum condition. The difference between predicted and measured reducing sugar concentrations can be attributed to uncontrolled external factors such as husk batch or shelving times of samples between experiments. However, the overall behavior and optimal trends of the system are well explained by both models as indicated by R^2 values which are satisfactory-enough for the exploratory nature of the optimization procedure to be used in preliminary techno-economic analysis.

Table 4
Reducing sugars obtained under different acid pretreatment conditions.

Exp.	Time (min)	% Husk load (w/v)	Acid % (w/w)	Reducing sugar (g L^{-1})	Yield (g sugar/100 g husk)
1	37.5	8	3	11.98	7.49
2	37.5	8	3	12.51	7.82
3	37.5	8	3	11.49	7.18
4	37.5	8	3	11.71	7.32
5	37.5	8	3	11.74	7.34
6	37.5	8	3	12.18	7.61
7	37.5	8	3	11.23	7.02
8	37.5	8	3	12.74	7.96
9	37.5	8	3	11.50	7.19
10	37.5	8	3	12.64	7.90
11	37.5	8	5	12.96	8.10
12	37.5	8	1	7.37	4.61
13	37.5	3.5	3	7.43	10.61
14	37.5	12.5	3	16.73	6.69
15	50	5.3	4.2	8.09	7.63
16	50	5.3	1.8	7.96	7.51
17	50	10.7	4.2	15.41	7.20
18	50	10.7	1.8	11.71	5.47
19	24	10.7	4.2	12.26	5.73
20	24	10.7	1.8	8.83	4.13
21	24	5.3	4.2	8.04	7.58
22	24	5.3	1.8	6.48	6.11
23	15	8	3	10.82	6.76
24	60	8	3	12.54	7.84
25	37.5	8	3	13.89	8.68

Table 5
Optimum values of variables of dilute acid treatment determined with two different models.

Model	Time (min)	% Husk load (w/v)	Acid % (w/w)	Reducing sugar (g L^{-1}) predicted	measured	Yield (g sugar/100 g husk)
Kriging	50	12.5	3.8	16.1	27.67	11.07
LOP	60	12.5	4.3	18.3	22.24	8.90

3.2. Enzymatic hydrolysis of hazelnut husk

Preliminary experiments with excess cellulase enzyme were carried out using (i) untreated hazel husk and (ii) husk residues that remained after acid treatments. The results have shown that final reducing sugar concentration obtained using the husk residues from acid treatment was approximately twice of that obtained from untreated husk. As expected, acid treatment has probably increased the surface area and the porosity of the lignocellulosic biomass for the subsequent enzymatic hydrolysis.

Next, experiments to determine the reaction temperature and pH were carried out. Based on the reported optimum values of the enzyme used, three different temperatures as 50, 55, and 60 and three different pH as 4.5, 5, and 6 were tested for the hydrolysis reaction. Reducing sugar obtained was slightly higher at 55 °C and there was no substantial effect of the selected pH values on hydrolysis. Therefore, the subsequent modelling part for enzymatic hydrolysis was carried out at the constant temperature and pH of 55 and 6.0, respectively.

A similar exploratory optimization strategy has been used to find the optimum conditions for enzymatic hydrolysis of the husk residues obtained from acid treatment. The results obtained from enzymatic hydrolysis are presented in Table 6. In the second, third, and fourth

Table 6
Reducing sugars obtained under different enzymatic hydrolysis conditions.

Exp.	Time (days)	% Husk* load (w/v)	Enzyme (mL)	Reducing sugar (g L^{-1})	Yield (g sugar/100 g husk*)
1	2.2	12	1.3	5.80	0.9
2	2.2	12	2.2	6.82	1.1
3	2.2	18	1.3	5.78	0.9
4	2.2	18	2.2	7.23	1.1
5	5.8	12	1.3	7.05	1.1
6	5.8	12	2.2	7.23	1.1
7	5.8	18	1.3	7.44	1.2
8	5.8	18	2.2	8.59	1.3
9	1	15.2	1.75	5.89	0.9
10	7	15.2	1.75	7.32	1.1
11	4	10	1.75	6.06	1.0
12	4	20	1.75	9.00	1.4
13	4	15.2	1	9.44	1.5
14	4	15.2	2.5	7.05	1.1
15	4	15.2	1.75	7.21	1.1
16	4	15.2	1.75	7.09	1.1
17	4	15.2	1.75	7.05	1.1
18	4	15.2	1.75	7.92	1.2
19	4	15.2	1.75	7.14	1.1
20	4	15.2	1.75	6.84	1.1
21	4	15.2	1.75	7.44	1.2
22	4	15.2	1.75	7.64	1.2
23	4	15.2	1.75	7.02	1.1
24	4	15.2	1.75	6.91	1.1

* Acid pretreated husk, dried.

columns of Table 4 given are the values of the selected factors determined with central composite design.

Under the selected conditions, in general, a positive correlation between increasing reaction times, husk loads, and enzyme amounts and the final reducing sugar concentrations was observed. However, there were a couple of exceptions to this. In experiments 1 and 3, as husk load increased, there was no significant change in yield. On the other hand, when experiments 13 and 14 were compared, it was seen that, despite an increase in enzyme load, yield dropped considerably. These anomalous observations may be attributed to the uncontrolled external factors as mentioned above as well as the possibility of auto-inhibitory effects of the enzyme at elevated concentrations.

The reducing sugar concentrations varied between 5.78 and 9.44 g L^{-1}, while sugar yield using pretreated husk varied between 0.9 % and 1.5 %. The mean with the standard deviation for the ten center point replicates (Exps. 15–24) was 7.23 ± 0.34 g L^{-1}. Contrary to expectations, the effect of enzymatic hydrolysis was not found to be very effective on releasing the sugar content of hazelnut husk. The sugar yield from this treatment remained only ~ 1 %.

Optimum values of the selected factors were again determined with two different models using the results displayed in Table 6 (Table 7). The R^2 values obtained as 0.91 and 0.58 for Kriging and RSM, respectively, have shown that Kriging represented the dataset more successfully. The ANOVA table is given as Supplementary Table S2. The optimum conditions and the concentrations predicted by the two models were very similar and there was no significant improvement on neither sugar concentration nor yield under optimized conditions.

3.3. Overall sugar yield from hazelnut husk

A detailed summary of the hazelnut husk treatment under optimized conditions is presented in Table 8. As it can be seen, approximately 12 % of the weight of hazelnut husk was released as reducing sugars with dilute acid treatment. The husk residues that remained after this treatment was 62.4 % of the initial husk weight. While the initial reaction volume was 100 mL, the final reaction volume was only 55 mL, due to swelling of the husk during treatment. On the other hand, when 2.5 g of the acid treated husk was subjected to enzymatic treatment, approximately 1.3 % of this mass was released as reducing sugars.

Table 9 summarizes the total reducing sugar yield from 12.5 g hazelnut husk. The presented values show that significant amount of sugar was already released during dilute acid treatment. Sugar release from the acid treated husk after enzymatic treatment was very low, i.e, less than 1%. This is not significant enough considering the cost and the amount of enzyme used. One possible reason for this might be the high lignin content of hazelnut husk. Dilute acid treatment reduces the recalcitrance due to the lignin rendering the additional effect of enzymatic hydrolysis relatively insignificant. Therefore, in further experiments to evaluate the husk hydrolysate as a feed stock, fermentable sugars obtained from acid treatment have been used.

3.4. Composition of hazelnut husk

A rough compositional analysis of 100 g of hazelnut husk has been performed and the results are summarized in Table 10. The presented data represent approximate values since soluble lignins and proteins were not included in the analysis. Considering that 1–15 % of a lignocellulosic feed stock may constitute proteins [26], the reported values will change only slightly. Overall, the obtained values show that approximately 83 % of hazelnut husk is organic matter, which constitutes the lignin and the carbohydrates excluding acid-soluble lignins and proteins. Carbohydrates, which are mainly cellulose and hemicellulose make together approximately 43 % of the hazelnut husk.

Table 7
Optimum values of variables in enzymatic hydrolysis determined with two different models.

Model	Time (day)	% Husk* load (w/v)	Enzyme volume (mL)	Reducing sugar (g L^{-1}) predicted	measured	% Yield (g sugar/g husk*)
Kriging	5.35	20	1	8.10	9.04	1.4
LOP	6.88	20	1	8.36	9.35	1.5

* Acid pretreated husk.

3.5. Evaluation of husk sugar hydrolysate as a feedstock

The obtained husk hydrolysate was further used as a renewable carbon source for cultivation of *C. glutamicum* cells. Glucose is the preferred carbon source for *C. glutamicum* therefore, all cells were expected to utilize glucose. Here, wild-type *C. glutamicum* strain was used as the reference strain. However, these cells are unable to utilize xylose, one of the fermentable sugars abundant in the husk hydrolysate. Therefore, to evaluate husk hydrolysate as a carbon source, the used *C. glutamicum* cells, CgEKV-II and CgXyl, harbored the pEKEx3-$xylA_{Xc}$-$xylB_{Cg}$ plasmid that carry genes required for xylose utilization.

Growth results obtained are displayed in Fig. 1.

As demonstrated previously [23], *C. glutamicum* wild-type and CgXyl cells displayed pretty similar growth characteristics when grown on glucose. Cell growth ceases when the carbohydrates are depleted in the medium. When xylose was used as the sole carbon source for CgXyl cells that are capable of utilizing this sugar, lag phase was longer and growth was slower, while the final biomass concentration was similar to that obtained with glucose. When growth media with husk hydrolysate was used as the carbon source, growth rate and final biomass concentration were lower with wild-type cells when compared to CgXyl cells. This suggested that while the wild type strain only grew with the glucose fraction of the husk hydrolysate, CgXyl utilized both glucose and xylose present in the husk hydrolysate. CgXyl does not display diauxic growth, since it can consume glucose and xylose simultaneously [27]. Here, it should be underlined that DNS method measures the total reducing sugar content of the husk hydrolysate, which is expected to contain significant amounts of different reducing sugars apart from xylose and glucose [28].

Unfortunately, *C. glutamicum* is unable to utilize arabinose [29] and galactose [30]. Therefore, although the sugar contents were adjusted to 2.6%, while growth media with xylose and glucose contained this exact amount, the utilizable sugars in the growth media with husk hydrolysate would be expected to be lower. This likely explains the lower final biomass concentration for growth with the husk hydrolysate.

3.6. L-DOPA production by fermentation using husk hydrolysate

Production of L-DOPA from tyrosine using glucose/xylose mixtures (Fig. 2) has recently been described [22]. To this end, L-DOPA production from husk hydrolysate as a renewable carbon source was evaluated using the previously developed strain CgEKV-II and the optimized conditions with 0.4 mM copper ions and 0.2 mM thymol in the fermentation broth.

After 24 h of fermentation, no L-DOPA was detectable in the cell-free supernatants. This was expected since OD_{600} of the cells was hardly above 10 after 24 h when husk hydrolysate was used as the carbon source (see Fig. 1). However, after 48 h L-DOPA titer was 17 ± 1 mg L^{-1} as the OD_{600} increased to 11 ± 1. After 72 h, L-DOPA titer was 20 ± 0.4 mg L^{-1}, as OD_{600} reached 23 ± 1. There was no further improvement after this point. Considering that the amount of the carbon source was lower and that the exact amount of each sugar in the hydrolysate was unknown, this titer should be acceptable when compared with 74 ± 3 mg L^{-1} obtained with the same cells using 4% glucose [22].

Table 8
Sugar yield from hazelnut husk after treatment under optimized conditions.

Method	Initial dry husk weight (g)	Initial reaction volume (mL)	Final reaction volume (mL)	Sugar in hydrolysate (g L^{-1})	Dry husk weight after treatment (g)
Dilute acid treatment	12.5	100	55	27.7	7.8
Enzymatic hydrolysis	2.5*	12.5	3.9	8.4	1.3

* Weight of acid pretreated husk

Table 9
Summary of sugar yield from 12.5 g hazelnut husk.

	Total sugar in hydrolysate (g)	% Yield (g sugar/ g husk)
Dilute acid treatment	1.5	12.0
Enzymatic hydrolysis	0.1	0.8
Total	*1.6*	*12.8*

Table 10
Approximate composition of 100 g hazelnut husk.

Description	Amount (g)
Water	10.3
Dry material	89.7
Inorganic matter (e.g. ash)	6.6
Organic matter	
Lignin	40.0
Carbohydrates	43.1
Total	100

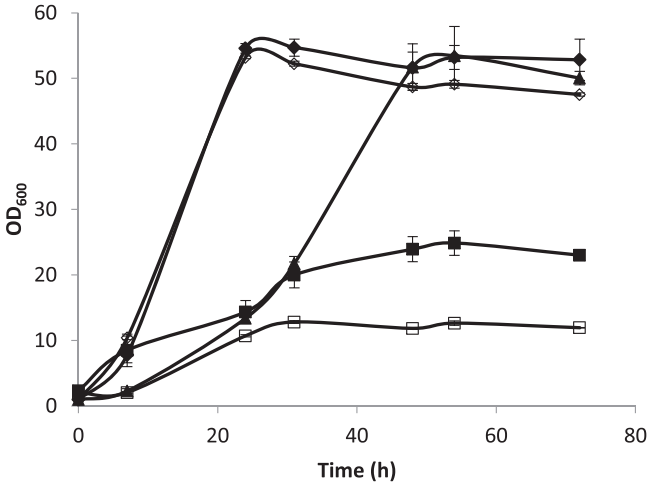


Fig. 1. Bacterial growth on glucose, xylose, and husk. *C. glutamicum* wild-type was grown on glucose (empty diamonds) and husk (empty squares). Strain CgXyl was grown on glucose (black diamonds), xylose (black triangles), and husk (black squares).

3.7. L-DOPA production via whole-cell biotransformation

As the final attempt to use husk hydrolysate as a renewable carbon source, CgEKV-II cells grown on husk were used for whole-cell biotransformation of L-Tyr to L-DOPA. The final OD_{600} of the cells grown on husk for 48 h to be used for the reaction was 17 ± 0.12.

For the biotransformation reaction, 2–24 mg DCW CgEKV-II cells were tested. Preliminary results have shown that when using 2 or 4 mg DCW, L-Tyr titer was pretty similar but yield dropped with more cells. With a further increase of the cell concentration in the reaction, titer slightly increased but yield per mg DCW CgEKV-II cell was dramatically low (results not shown). Therefore, whole-cell biotransformation was carried out with 2 mg DCW.

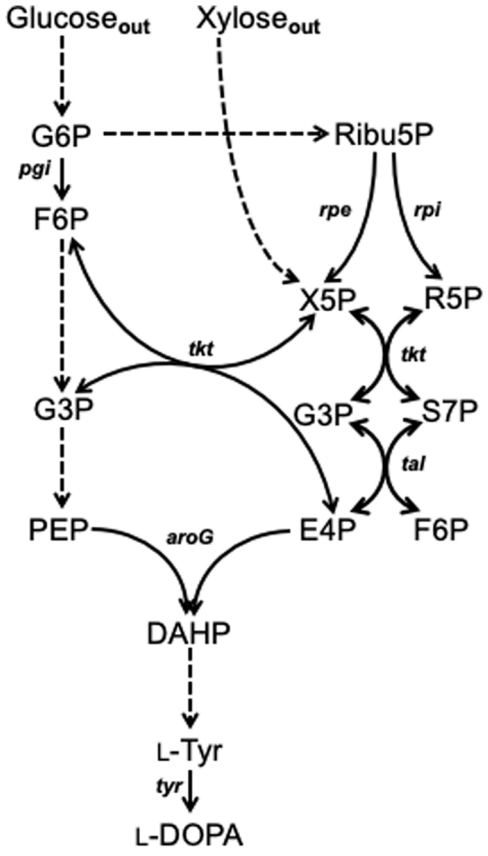


Fig. 2. Pathway from glucose/xylose to L-DOPA. Glucose and xylose in the hydrolysate are first directed to glycolysis and/or pentose phosphate pathway. In these pathways the two metabolic precursors (PEP and E4P) of L-Tyr are obtained. Following the condensation of PEP and E4P, which initiates shikimate pathway, L-Tyr is synthesized via the branched aromatic amino acid metabolic pathway. Finally, L-Tyr is converted to L-DOPA with the action of the tyrosinase. G6P: glucose-6-phosphate, F6P: fructose-6-phospate, G3P: glyceraldehyde 3-phosphate, PEP: phosphoenolpyruvate, Ribu5P: ribulose-5-phosphate, X5P: xylulose-5-phosphate, R5P: ribose-5-phosphate, S7P: sedoheptulose-7-phosphate, E4P: erythrose-4-phosphate, DAHP: 3-deoxy-7-phosphoheptulonate, *glk*: glucokinase, *pgi*: glucose-6-phosphate isomerase. *tkt*: transketolase, *tal*: transaldolase, *rpe*: ribulose-phosphate 3-epimerase, *rpi*: ribose-5-phosphate isomerase, *aroG*: DAHP synthase, *tyr*: tyrosinase.

The presence of copper ions enhances tyrosinase activity, thus, accelerating the conversion of L-Tyr to L-DOPA. However, the major disadvantage of the presence of copper is the fact that it leads to the formation of L-DOPA oxidation products, which can make purification processes challenging. Furthermore, copper is a heavy metal and its presence makes the process less bio-friendly. For this reason, whole-cell biotransformation was pursued both in the presence and absence of copper.

L-DOPA measurements of samples taken every 24 h from the reactions which were allowed to proceed for 144 h are presented in Fig. 3.

In the absence of copper ions, biotransformation using the CgEKV-II cells was slower. It took 144 h to reach the maximum L-DOPA titer of 98 ± 2 mg L^{-1}. In the presence of copper ions, L-DOPA titer was already 82 ± 1 mg L^{-1} at the end of 72 h and it remained essentially constant at around 100 mg L^{-1} after 96 h.

3.8. Preliminary techno-economic assessment for fermentative L-DOPA production

One of the ultimate objectives of this research project was to demonstrate the techno-economic feasibility of producing the value-added product L-DOPA from the lignocellulosic agro-waste hazelnut husk. Therefore, a preliminary techno-economic assessment of this potential was necessary. At this very early stage of process design, where most process-related details are yet unavailable or vague, back-of-the-envelope level projections with analogy to similar processes were used to guide the necessary preliminary assessment. Based on the scale and characteristics of production, one needs to calculate two economic metrics using available technical data (e.g. titer and yield) from performed experiments. These two economic metrics are the costs associated with and the revenue that can be generated from such a process. Costs include process development (including R&D and facility construction) and annual operating costs. The cost structure for the preliminary techno-economic assessment is tabulated in Table 11.

Considering that on average 300,000 MT of hazelnut husk is available in Turkey annually, the total scale of fermentation can be assumed to have a volume of 1430 mL (million liters). This value is based on the fact that 100 mL reaction volume was necessary to be used with reducing sugars obtained from 21 g of hazelnut husk, as presented above. 1430 mL of fermentation would allow the production of a total of 28.6 MT of L-DOPA. This projection is based on the achieved titer of 20 mg L-DOPA L^{-1} in fermentative production, as found above.

The production process would require a pretreatment step prior to fermentation. The effect on yield of this pretreatment step was reflected in the final L-DOPA titer used above. Following fermentation, three main steps of downstream processing were projected. An initial cell removal step such as filtration or centrifugation would be proceeded by a product recovery step such as extraction and a purification step of chromatography or crystallization [32]. If 90 % yield from each step could be achieved, the overall loss would amount to 30% during downstream processing. This production scheme would deliver 28.6 MT *0.70 = 20.02 MT of final product. Priced at 14 €/g (5 g L-DOPA is 70.00 €, Sigma: D9628), ~ 20 MT of L-DOPA would generate 280 M € of revenue annually. This scale of production would constitute approximately 7 % of the total global annual L-DOPA production [16], which can be considered a reasonable market share for such a manufacturing facility.

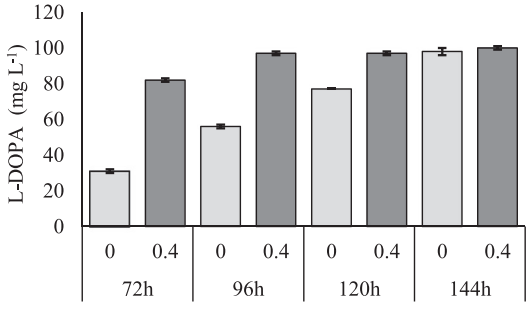
The annual revenue must cover process development, feedstock and operational costs. Hazelnut husk is traditionally burnt on the fields. Therefore, the major raw material associated costs would be related to collection and delivery, which could be assumed to be on the order of 40 € per MT [31] and to amount to a total of 12 M € annually. In addition, L-Tyr is a substrate required specifically for this production at a concentration of 1 g L^{-1}, as given in Section 2.10. Considering the total annual fermentation volume of 1430 mL, L-Tyr cost would be ~ 23 M € per year (1 kg L-Tyr is 16.00 €, AliBaba: 60-18-4). Process development costs would include the necessary R&D and total capital investment for facility construction. Yang et al. [24] has reported an average of ~ 400 M € for total capital investment for a range of bioproducts produced from cellulosic biomass. Therefore, a comparable cost could be expected for the current process. If process development costs with the addition of R&D expenditures were kept to an average of 500 M €, which would be distributed over an estimated 20-year operational period, the annual burden would be around 25 M €. Operational costs for such a facility could be assumed to be around 200 M € per year based on the values reported by Yang et al. [24] and Boulamanti and Moya [25]. In total, the annual costs for production of 28.6 MT of L-DOPA would add up to 260 M €, comparable to the achievable revenues (~ 280 M€). Therefore, it would be reasonable to say that the production of the value-added product, L-DOPA, from the lignocellulosic agro-waste hazelnut husk offers techno-economic potential to be considered even at this early phase of process development.

4. Discussion

With their high cellulose and hemicellulose contents, lignocellulosic agricultural sidestreams (corn stover, wheat straw, sugar bagasse, rice straw, cotton stalks, etc.) and forestry products (softwoods or hardwoods) emerge as cheap sources for microbial growth and production. Furthermore, their utilization avoids the displacement of food crops and the issues related to the deforestation, reducing the negative impacts on the environment. Unfortunately, the recalcitrant nature of lignocellulosic materials to processing is a major challenge, thus, optimization of pretreatment methods to release fermentable sugars remains to be an important issue to reduce costs and improve feasibility. This work focused on the optimal extraction of fermentable sugars from hazelnut husk to be used as a renewable carbon source for the production of speciality chemicals by *C. glutamicum*.

On average, lignocellulosic material contains, 30–50 % cellulose and 15–35 % hemicellulose [6]; however, the quantity of the sugar that can be extracted from lignocellulosic biomass varies considerably depending on the biomass, on the type of crop species and the extraction method used [33] Here, among the leading treatment techniques, dilute acid treatment, which is applicable on large scale, has been used to extract of fermentable sugars from hazelnut husk, which is known to have a high lignin content.

Optimum conditions found for acid treatment in this study are comparable to the conditions for acid pretreatments of different lignocellulosic biomass. While Sindhu et al. [34] reported optimum conditions for Indian bamboo as 15 % (w/w) biomass loading, 5 % sulfuric acid and 30 min, Aguilar et al. [35] reported 2 % sulfuric acid (w/w),



Time (h)	Copper ions [mM] 0 mM	0.4 mM
72	31±1	82±1
96	56±1	97±1
120	77±0	97±1
144	98±2	100±1

Fig. 3. L-DOPA production by whole-cell biotransformation of 1 g L^{-1} L-Tyr using 2 mg DCW CgEKV-II in a 10-mL reaction. The reaction mixtures contained 4 mM ascorbic acid to prevent L-DOPA oxidation.

Table 11
Tabulated cost structure for preliminary techno-economic analysis.

Cost Item	Basis Used for Calculation	Annualized Cost
R&D Expenditure[a]	100 M€ [24]	5 M€
Capital Investment[a]	400 M€ [24]	20 M€
Operating Costs		
Transportation	40 €/MT [31]	12 M€
Pretreatment	12 % sugar yield from husk[b]	200 M€ + 23 M€ (L-tyr)
Fermentation	20 mg L-DOPA/L[b]	
Downstream Processing	70 % yield	

a: Annualized over an assumed 20-year operational period.
b: This work.

122 °C, and 24 min as the optimum conditions for 1 g sugar cane bagasse/10 g liquor on dry basis. In another study, the highest total sugar yield was achieved using lower sulfuric acid concentration of 0.75% (v/v) and a relatively longer treatment duration of 90 min at 121 °C [36]. A solid load of 10 % w/v of wheat stubble has been used in this work. An acid pretreatment optimization was also performed by [37] using hazelnut husk, where the conditions were 90 min, 4 % sulfuric acid and 5% biomass loading at 121 °C. Our optimum pretreatment duration and acid concentration were lower but our biomass loading was higher. Although their yield was slightly higher as 18 % their final sugar concentration in the hydrolysate was as low as 7 g L^{-1}. If this is directly to be used as a cultivation media, the maximum sugar concentration would be 0.7 %, a concentration way below the requirements for biotechnological processes. Another study with dilute acid pretreatment on hazelnut shells reported similar optimal conditions as 130 °C, 3.42 % sulfuric acid, and 31.7 min, even though the structure and composition of hazelnut husk and shells are different [38].

Interestingly, the findings of this work and the values reported are low when the total carbohydrate content of ~ 40 % is taken into account; however, this is not very surprising considering the recalcitrant nature of the husk. The findings reported by Surek and Buyukkileci [28] and Demirbaş [39] are consistent with the composition reported here. Demirbaş [39] has found that hazelnut kernel husk is composed of 29.6 % cellulose, 53 % lignin and 15. 7 % hemicellulose, which makes ~ 45 % carbohydrate content. Surek and Buyukkileci [28] have made a more detailed analysis and given the composition of hazelnut husk as 15.4 % cellulose, 25.9 % klason lignin, 5.8 % xylan, 2.6 % galactan, 1.7 % arabinan, 10.6 % uronic acids, 5 % ash, 24.6 % extractives and 8 % protein. In total, this yielded ~ 35 % total carbohydrate content.

The motivation of this work was to obtain fermentable sugars from a lignocellulosic agro-waste for microbial cultivation towards the production of value-added specialty chemicals. To this end *C. glutamicum* has been selected as the host microorganism. It is not only an important industrial microorganism, but with metabolic engineering approaches its capability has been broadened to simultaneously consume glucose and xylose [27], which constitutes approximately one third of the sugars in the lignocellulosic feedstock [40]. Furthermore, its ability to tolerate inhibitory by-products of hydrolysis steps such as phenolic compounds, furfurals and organic acids makes it even more advantageous in microbial growth [41].

Various studies have evaluated the potential of the production of precursors and value-added chemicals by *C. glutamicum* using green substrates, as the sole carbon source. Gopinath et al. [42] have reported that *C. glutamicum* grown in sulfuric acid hydrolysates of rice straw and wheat bran yielded 93 mM L-glutamate from either lignocellulosics hydrolysate. Rice straw hydrolysate was also successfully utilized for the production of 260 mg L^{-1} 5-amino valeric acid and 91 mg L^{-1} putrescine [43]. In another study, *C. glutamicum* GJ04, produced 61.7 g L^{-1} L-glutamate from acid pretreated wheat straw hydrolysate [44]. *C. glutamicum* CGS5, was able to grow on enzymatically hydrolyzed corn straw and produce 98.6 g L^{-1} succinate [45]. Lee et al. [46] used enzymatically hydrolyzed Canadian Ponderosa Pine to be simultaneously saccharified and co-fermented by a recombinant *C. glutamicum* strain. In another study, recombinant *C. glutamicum* cells were grown on ionic liquid pretreated sorghum hydrolysate and were able to produce (15 mg L^{-1}) of isopentenol [47]. Becker et al. [48] was able to engineer *C. glutamicum* cells to efficiently consume not only carbohydrates but also the aromatics obtained from hydrothermal conversion of lignin from pine to produce 12.5 mM *cis, cis*-muconic acid. Later, Mhatre et al. [49] demonstrated that corn stover biomass hydrolysates for simultaneous utilization of sugars and aromatics can be used for mixed-acid fermentation of lactate, succinate, and acetate. In another recent study, brown seaweed extract and brown seaweed hydrolysate from *Laminaria hyperborean* were used as green substrates to produce riboflavin with a titer of 1291.2 mg L^{-1} in cells engineered to consume mannitol and glucose [50]. A comparable L-DOPA titer was obtained with *C. glutamicum* cells using husk hydrolysate.

5. Conclusion

The results obtained in this study demonstrated that it is possible to extract fermentable sugars from hazelnut husk using dilute acid treatment, however this treatment was not sufficient to make this lignocellulosic material amenable to enzyme degradation. On the other hand, it was possible to use the obtained hazelnut husk sugar hydrolysate as the sole carbon source for the cultivation of industrial workhorse *C. glutamicum* and production of L-DOPA. As it stands from an economical point of view, the titers obtained in the laboratory experiments of this work would allow production right around the breakeven point. However, by engineering the microbial cells and optimizing bioprocess conditions, it would be possible to bring the revenues to higher levels. Such improvements are not uncommon in process development studies. Furthermore, utilization of the cells obtained after fermentation for whole-cell biotransformation would make the process even more profitable. Findings of this preliminary techno-economic analysis have motivated the ongoing work on the optimization of the detailed design of this bioprocess.

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CRediT authorship contribution statement

Berna Sarıyar Akbulut, Nihat Alpagu Sayar, and Eldin Kurpejović designed the study. Beril Pakalın, Eldin Kurpejović, and Gülsüm Merve Bastem carried out experimental work. Volker F. Wendisch reviewed the results and provided critical feedback for their discussion. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2022.108768.

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