ENZYMATIC HYDROLYSIS OPTIMIZATION OF PRETREATED MUNICIPAL SOLID WASTE USING A MIXTURE OF ENZYMES PRODUCED BY *ASPERGILLUS NIGER*

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This study aimed to check the potential of wheat bran and biodegradable municipal solid waste enzyme cocktail to produce reducing sugar of high yield from the pretreated substrate. The enzyme cocktails were produced from *Aspergillus niger* fermentation using wheat bran and biodegradable municipal solid waste as carbon sources. The results showed that these enzyme cocktails had β-glucosidase and endoglucanase activities. The maximum yields of reducing sugar, *i.e.*, 87% and 83%, were achieved at 10% substrate concentration, 50 °C temperature, and 36 h fermentation time using wheat bran and biodegradable municipal solid waste enzyme cocktail, respectively. Moreover, a comparison of batch and fed-batch processes of enzymatic hydrolysis at a high loading of substrate dose showed that, in the fed-batch process, an increase in the yield of reducing sugar (83%) was achieved, as compared to the batch process (73%) using WB enzyme cocktail.

Keywords: *Aspergillus niger*, enzymatic hydrolysis, reducing sugar, optimization

INTRODUCTION

Solid waste (SW) generation is one of the world's fastest growing environmental issues and it is increasing due to population growth, urbanization, and industrialization.[1,2] In Pakistan, similarly to other developing countries, improper waste management creates serious environmental issues. About 60-70% of solid waste is collected and a major portion of the collected solid waste is either open dumped or open burned, causing serious health issues to the general population.[3] Major practices of solid waste management are composting and landfilling, which cause pollution of surface and groundwater, as well as the emission of foul smells.[4] Furthermore, thermochemical technologies, *i.e.*, pyrolysis and incineration, cause the generation of CO_2 and CO that negate the environmental benefit of energy production by thermochemical technologies using municipal solid waste (MSW).[5-7] Thus, the best practice is to utilize MSW as a resource to produce biofuels by biochemical conversion to provide alternatives to fossil fuels and render MSW management sustainable.

The biodegradable MSW (BMSW) contains starch, carbohydrates, fat, protein, and lignocellulosic materials.[8] Biofuel production from BMSW and lignocellulosic biomass waste material is gaining high interest due to its many advantages, such as low-cost and excessively available biomass that does not pose any competition to food feedstocks.[9] At a commercial scale, the production of biofuel requires an efficient pretreatment method development, hydrolysis for a higher yield of reducing sugar (RS) within a short incubation period, and fermentation of RS into biofuel.[10]

Enzymatic hydrolysis used to produce RS provides a great potential for the improvement of the economic viability of the biofuel production process. In this process, the combined action of three types of enzymes: β-glucosidase, cellobiohydrolases and endoglucanases, is required to produce RS from pretreated substrate. During this process, firstly the endoglucanases attack cellulose chains to expose their reducing and non-reducing ends, then the

cellobiohydrolases release cellobiose units by acting on the reducing and non-reducing ends, and afterward, β-glucosidase converts these into glucose.[11,12]

For commercial biofuel production, the cost of enzymes in the enzymatic hydrolysis process is the main challenge.[13] According to research, 70% of enzyme cost can be saved by the production of an enzyme on-site, as compared to commercial enzymes.[14] Thus, because of the high cost, it is inadvisable to use commercial enzymes. Generally, the process cost can be reduced significantly by the development of an inexpensive method for the production of highly concentrated enzymes. At the commercial level, microorganisms used for the application of enzymes in hydrolysis should favor productivity, activity, and be resistant to product inhibition.[15] Enzymes used for hydrolysis have been produced mostly by the *Trichoderma* genus, which secretes cellobiohydrolases, and endoglucanases in large amounts, but the secretion of β-glucosidase is very low, which results in incomplete hydrolysis. *Aspergillus* is another enzyme-producing fungus that secretes large amounts of β-glucosidase and is used to replace commercial enzymes.[16] Moreover, at the industrial level, *Aspergillus niger* is highly appreciated because it can ferment proteins and produce a combination of enzymes used for the enzymatic hydrolysis of biomass.[15] To achieve the maximum yield of RS from the pretreated substrate, an enzyme cocktail must have a variety of enzymes in sufficient amounts. However, a variety of enzymes, such as α-amylase, xylanases, carboxymethyl cellulase, β-glucosidase, cellobiohydrolases, endoglucanases and pectinases, can be produced extracellularly by the fungal strain (*Aspergillus niger*) itself, so that it helps in the digestion of cellulose, which is an insoluble substrate.[17,18] Various lignocellulose materials, such as bagasse, wheat bran, rice husk, rice bran, BMSW *etc*., can be used as a substrate for those enzyme productions. While wheat bran (WB) has been reported as the most suitable substrate due to its abundant cello-oligo saccharides that increase various extracellular activities, in order to get the maximum yield of RS from enzymatic hydrolysis, optimization of process parameters is very important. In the literature, various process optimization techniques have been reported, but Box Benkhen Design (BBD)-based response surface methodology (RSM) is one of the best techniques. Hamid *et al.*[19] reported 31 g/L RS after hydrolysis optimization of dilute acid pretreated date seeds using BBD-RSM.

In a previous study, 167.755 g/L and 159.141 g/L of RS concentration was achieved after the pretreatment of BMSW using toilet cleaner A_{TC} (acidic in nature) and washing detergent B_{WD} (basic in nature), respectively. Then, after the A_{TC} pretreatment, 237.83 ±11.028 g/L of RS concentration was achieved after 36 h of enzymatic hydrolysis.[20] The objective of this study has been to increase the yield of RS by optimizing the process parameters of enzymatic hydrolysis, such as substrate concentration, reaction time, and temperature, so that the maximum yield of biofuel can be achieved after fermentation of these RS. This study also aims to make the enzymatic hydrolysis process cost-effective by producing enzymes in a laboratory, instead of using commercial enzymes. For this purpose, an enzyme cocktail was produced in our laboratory using WB and BMSW as carbon sources by an environmentally friendly fungus, *Aspergillus niger*, under liquid submerged fermentation and applied for the enzymatic hydrolysis of chemically pretreated substrate.

EXPERIMENTAL

Materials

MSW was sampled from the Lakhodair landfill site, Lahore, Pakistan. BMSW was manually separated from non-biodegradable material, was dried and ground, as described earlier.[20] Briefly, after the segregation of the sample, it was dried at 50 °C temperature in the oven, ground to achieve 0.45 mm-1 mm particle size and was stored at 4 °C temperature for further use. WB was purchased from a local marketplace. The composition of WB (%) includes (60-75) total carbohydrates, (8.1-12.7) moisture, (9.6-18.6) proteins, (3.9-8.1) ash, (33.4-63) dietary fiber, and (9.1-38.9) starch.[21] Also, BMSW was collected from a landfill site and its composition was described earlier.[20] WB and BMSW were stored at 4 °C in a dark area. All reagents and chemicals used in this research were of analytical grade.

Microorganism and enzyme production

Aspergillus niger FCBP-0198 was used to produce enzyme cocktails using different substrates (as carbon sources), such as WB and BMSW, by liquid submerged fermentation. *Aspergillus niger* was grown on Potato Dextrose Agar (PDA) medium, as described earlier.[20] Briefly, it was grown at 30 °C for 7 days in an incubator and recollected in 50% glycerol solution.

Enzyme activity method

Enzyme activities of different enzymes present in the enzyme cocktails were measured using the following methods. To determine the endoglucanase activity, the supernatant of the enzyme solution was centrifuged in a centrifuge (80-2 electric centrifuge) for 10 min at 10,000 rpm to get a clear solution. 1% of carboxymethyl cellulose (CMC) was prepared in 0.05 mM sodium acetate buffer (pH 5) and then it was used as a substrate to measure the cellulase enzyme activity. 1 mL of enzyme solution was mixed with 1 mL of substrate solution and incubated at 50 °C for 30 min. After that, the mixture was autoclaved to stop the reaction and the amount of RS released during the hydrolysis was measured by the DNS procedure. The enzyme activity, denoted as IU/mL, is defined as the amount of enzyme required to release 1 µmol of the product (RS) per minute.[15]

The total cellulase Filter Paper Activity (FPase) was determined using Whatman filter paper weighing about 50 mg. The filter paper was dipped in a mixture containing 0.5 mL enzyme solution and 1 mL of sodium acetate buffer with 0.05M and 4.8 pH for 1 h. To stop the reaction, 3 mL of DNS reagent was added to it, and after proper mixing of the solution, it was boiled in a water bath for 5 min. After cooling, 20 mL of distilled water was added to the solution to dilute it. The amount of RS was measured by the DNS method at 540 nm.[22]

The assay of *β*-glucosidase was done using 10 mM 4-nitrophenyl *β*-d-glucopyranoside (pNPG) as the substrate in 50 mM sodium acetate buffer (pH 5). The mixture of enzyme (0.5 mL) and substrate (0.5 mL) was incubated at 50 °C for 10 min. Afterward, 2 mL Na_2CO_3 (0.2 M) was added to stop the reaction and the β-glucosidase activity was measured at an absorbance of 400 nm.[15]

Enzymatic hydrolysis

The previously acid pretreated BMSW[20] was hydrolyzed in a 100 mL Erlenmeyer flask using two enzyme cocktails (WB cellulolytic enzyme cocktail and BMSW cellulolytic enzyme cocktail), with an enzyme loading of 22.76 U and 19.23 U β-glucosidase per gram of substrate, 6.43 U and 8.95 U endoglucanase per gram of substrate, and 3.01 FPU and 2.74 FPU total cellulase per gram of substrate in WB and BMSW cellulolytic enzyme cocktail, respectively. The parameters of hydrolysis were optimized through response surface methodology (RSM) to get the highest yield of RS. The substrate in 0.050 M sodium citrate buffer at pH 4.8, with initial solid loading specified as per the BBD-RSM, was added to the flasks and incubated for the specified time. After that, the samples were immediately centrifuged at 10,000 rpm for 10 min to obtain the clear supernatant for RS analysis and then autoclaved to stop the reaction. RS concentration of the hydrolysate was determined according to Miller's methods, by a T80+ UV Spectrophotometer (PG Instruments, UK), using 3,5-dinitro-salicylic acid.[23]

Optimal response surface model design and statistical analysis

BBD-based RSM in Minitab software (Version 19.0) was used to optimize and analyze the enzymatic hydrolysis parameters. Ranges of three independent parameters deployed on a single-factor experiment were optimized and analyzed applying BBD. The ranges of the optimization parameters were the following: time (24–48 h), temperature (45–55 °C), and substrate concentration (8–12%, w/v). The ranges and levels of enzymatic hydrolysis parameters (substrate concentration, temperature, and time) are shown in Table 1. Sharma *et al.*[24] studied the same variables for the enzymatic hydrolysis of sorghum straw. Fifteen runs were created with three independent factors, and RS yield was taken as the response. All runs were performed in triplicates and mean values were reported.

Equation 1 shows the second-order polynomial model applied in the regression analysis:

$$Y = b_0 + \sum_{i=1}^{3}(b_1 X_i) + \sum_{n=1}^{\infty}(b_2 X_i^2) + \sum \sum (b_3 X_i X_j) \quad (1)$$

where Y is RS yield (dependent factor); X_i and X_j are independent factors; b_o is a constant, b_1 is the linear coefficient, b_2 is the quadratic coefficient, and b_3 is the interaction coefficient. Regression coefficients were obtained by using experimental values in the quadratic model. The coefficient of determination (R^2), lack of fit, and F-test attained from the ANOVA analysis were used to check the model efficiency. Response surface plots and regression analysis were generated to decide the individual and interactive effects of independent factors on the yield of RS. Model validation was established by comparing the actual *vs* predicted values of the optimized model.

Table 1
Variables and ranges of parameters used in BBD-RSM

Variables and ranges	Lower (-1)	Medium (0)	High (+1)
Substrate concentration: X1 (%)	8	10	12
Time: X2 (h)	24	36	48
Temperature: X3 (°C)	45	50	55

Batch and fed-batch enzymatic hydrolysis

For the batch process, 25% solid content with WB and BMSW cellulolytic enzyme cocktails was taken in a 250 mL Erlenmeyer flask and then incubated at 50 °C for 48 h. Meanwhile, for the fed-batch experiment, initially 8 g substrate was added to a 250 mL Erlenmeyer flask with the same enzyme concentration used in the batch process and was placed in an incubator for 12 h at 50 °C. Thereafter, the flask was fed with 5 g substrate and placed for 12 h and after 12 h, another 2 g of substrate was added and then incubated for 24 h. After the completion of the batch and fed-batch processes, all the samples were centrifuged for 15 min at 10,000 rpm and the yield of RS was measured using the DNS method.

RESULTS AND DISCUSSION

Production of enzyme cocktail

The enzyme cocktail produced from *Aspergillus niger* using two carbon sources (WB and BMSW) had β-glucosidase activities of 22.76 ± 0.41 U/mL and 19.23 ± 0.38 U/mL in WB and BMSW cellulolytic enzyme cocktails, respectively. Total cellulase activity using WB as carbon source was 3.01 ± 0.23 FPU/mL, while using BMSW as carbon source, the enzyme activity was 2.74 ± 0.10 FPU/mL. Moreover, endoglucanase activities of 6.43 ± 0.21 U/mL and 8.95 ± 0.28 U/mL were observed using WB and BMSW cellulolytic enzyme cocktail, respectively. BMSW is rich in protein, cellulose, hemicelluloses, fat, starch and minerals, promoting efficient production and development of the enzyme cocktail. WB is also a good source of carbohydrates required for the growth of enzymes. Thus, WB and BMSW both have a good potential as a carbon source for enzyme cocktail production by *Aspergillus niger*. Endoglucanase activity was comparatively higher in the BMSW enzyme cocktail than in the WB enzyme cocktail. Meanwhile, β-glucosidase activity was higher in the WB enzyme cocktail, as compared to the BMSW enzyme cocktail. Malik *et al.*[25] produced enzyme cocktails from *Aspergillus niger* using the organic fraction of MSW (OFMSW) and WB as a carbon source, and reported 50 and 40.6% change of the RS after enzymatic hydrolysis using WB and OFMSW enzyme cocktail, respectively. These results confirm the potential for WB and OFMSW as a carbon source to produce enzyme cocktails.

Table 2
Actual and predicted responses for reducing sugar yield from enzymatic hydrolysis of pretreated BMSW

Runs	X1	X2	X3	Reducing sugar (%)					
				WB enzyme cocktail			BMSW enzyme cocktail		
				Actual	Predicted	Error	Actual	Predicted	Error
1	12	36	45	58	59	-1	57	54	3
2	8	36	45	64	61	3	60	59	1
3	10	36	50	85	86	-1	81	81	0
4	8	48	50	78	81	-3	78	78	0
5	10	24	45	55	59	-4	50	53	-3
6	12	24	50	63	61	2	55	54	1
7	12	36	55	47	50	-3	50	51	-1
8	12	48	50	67	68	-1	67	69	-2
9	8	24	50	70	71	-1	69	67	2
10	10	48	45	75	76	-1	70	71	-1
11	10	36	50	84	86	-2	80	81	-1
12	10	36	50	87	86	1	83	81	2
13	10	24	55	68	68	0	62	61	1
14	8	36	55	70	71	-1	65	68	-3
15	10	48	55	69	67	2	73	69	4

Optimization of enzymatic hydrolysis using BBD

For this work, the conditions of enzymatic hydrolysis using WB and BMSW enzyme cocktails were optimized using BBD-RSM. The experimental results were used to obtain a quadratic model consisting of fifteen runs with three replicates and analyzed by ANOVA and regression analysis. BBD-RSM was performed for investigating the interactive effects of the independent variables: substrate concentration (X1), time (X2), and temperature (X3) on RS yield obtained from enzymatic hydrolysis using WB and BMSW enzyme cocktail. Actual and

predicted values of the dependent variable (RS yield) are presented in Table 2. The errors associated with RS yield are shown in Table 2, demonstrating that all experimental values are very close to predicted values. Furthermore, the RS yields using WB enzyme cocktail and BMSW enzyme cocktail were in the ranges of 47%-87% and 50%-83%, respectively.

Table 3
ANOVA and regression analysis of RS after enzymatic hydrolysis of pretreated BMSW

Source	Degree of freedom	Sum of squares	Mean squares	F-Value	P-Value	
WB Enzyme cocktail from *Aspergillus niger*						
Model	9	1745.36	193.93	18.94	0.002	Significant
Linear	3	394.22	131.41	12.83	0.009	
Substrate concentration	1	265.22	265.22	25.9	0.004	
Time	1	128.46	128.46	12.55	0.017	
Temperature	1	0.54	0.54	0.05	0.827	
Square	3	1191.97	397.32	38.8	0.001	
Substrate concentration*Substrate concentration	1	492.03	492.03	48.05	0.001	
Time*Time	1	70.29	70.29	6.86	0.047	
Temperature*Temperature	1	763.79	763.79	74.59	0.000	
2-Way Interaction	3	159.17	53.06	5.18	0.054	
Substrate concentration*Time	1	3.49	3.50	0.34	0.584	
Substrate concentration*Temperature	1	76.38	76.38	7.46	0.041	
Time*Temperature	1	79.30	79.30	7.74	0.039	
Residual Error	5	51.20	10.24			
Lack-of-Fit	3	47.21	15.74	7.88	0.115	Non-significant
Pure Error	2	3.99	2.00			
Total	14	1796.56				
R^2	97.15%					
Adjusted R^2	92.02%					
BMSW Enzyme cocktail from *Aspergillus niger*						
Model	9	1626.31	180.70	15.62	0.004	Significant
Linear	3	585.29	195.10	16.87	0.005	
Substrate concentration	1	223.85	223.85	19.35	0.007	
Time	1	340.32	340.32	29.42	0.003	
Temperature	1	21.13	21.13	1.83	0.235	
Square	3	981.38	327.13	28.28	0.001	
Substrate concentration*Substrate concentration	1	361.48	361.48	31.25	0.003	
Time*Time	1	63.42	63.42	5.48	0.066	
Temperature*Temperature	1	666.85	666.85	57.65	0.001	
2-Way Interaction	3	59.64	19.88	1.72	0.278	
Substrate concentration*Time	1	3.39	3.39	0.29	0.611	
Substrate concentration*Temperature	1	36.00	36.00	3.11	0.138	
Time*Temperature	1	20.25	20.25	1.75	0.243	
Residual Error	5	57.84	11.57			
Lack-of-Fit	3	53.17	17.72	7.6	0.119	Non-significant
Pure Error	2	4.67	2.33			
Total	14	1684.15				
R^2	96.57%					
Adjusted R^2	90.38%					

The second-order polynomial model for RS yield of BMSW obtained from the experimental results of enzymatic hydrolysis using WB and BMSW enzyme cocktail provided in Table 2 are expressed in Equations 2 and 3, respectively. The coded equation expresses the relative effect of the process parameters by comparing the coefficients of these parameters. Moreover, the quadratic equation of the actual factors-based can be used to predict the responses for actual levels of each independent factor. The positive and negative signs in the quadratic equations (Eqs. 2 and 3) exhibit the synergistic and antagonistic effects of the process parameters, respectively, on the enzymatic hydrolysis.

$$Y1 = -2032 + (78.1 * X1) + (6.62 * X2) + (64.62 * X3) - (2.886 * X1^2) - (0.0303 * X2^2) - (0.5753 * X3^2) - (0.0389 * X1 * X2) - (0.437 * X1 * X3) - (0.0742 * X2 * X3) \quad (2)$$

$$Y2 = -1706 + (60.4 * X1) + (4.11 * X2) + (58.43 * X3) - (2.474 * X1^2) - (0.0288 * X2^2) - (0.5376 * X3^2) + (0.0384 * X1 * X2) - (0.300 * X1 * X3) - (0.0375 * X2 * X3) \quad (3)$$

F-test was performed to check the statistical significance of the second order polynomial model for the dependent variable (RS), while regression analysis was carried out to check the fitness of the models. The respective models represent the experimental values with the coefficient of determination (R^2) of RS production from enzymatic hydrolysis of the pretreated substrate using WB and BMSW enzyme cocktails, as shown in Table 3. As for the WB enzyme cocktail, the R^2 value (97.15%) was comparable with adjusted R^2 (92.02%), while for the BMSW enzyme cocktail, the R^2 value was 96.57% with adjusted R^2 of 90.38%, so it may be said that maximum variation in the data was possible by these models. Table 3 shows the ANOVA results for the response of RS yield obtained from enzymatic hydrolysis using WB and BMSW enzyme cocktails. A statistically significant multiple regression relationship is observed between the dependent and independent factors. The adequacy of the respective model to fit the actual experimental work is determined using the lack of fit test and for the lack of fit test, F-value will be insignificant if the p-value > 0.05, indicating the fit of experimental values for dependent variables.[26]

The statistical significance of the model was examined using the F-test. The F-value for the models using WB and BMSW enzyme cocktails was 18.94 and 15.62, with a p-value of 0.002 and 0.004 ($p < 0.05$), respectively. The higher F-values of the models (Eqs. 2, 3) with lower p-values indicate the significance of the respective models. Meanwhile, the F-values for the lack of fit were 7.88 and 7.60 using WB and BMSW enzyme cocktails, respectively (Table 3), indicating that it was insignificant relative to the pure error. Conversely, if the p-value ($p < 0.05$) is significant for lack of fit, then to fit the results a more complex model would be required.[26] The p-value for lack of fit was 0.115 and 0.119 for WB and BMSW enzyme cocktail, respectively, *i.e*., not significant, indicating the good fit of the quadratic models. In addition, the R^2 values were 0.9715 and 0.9657 for enzymatic hydrolysis using WB and BMSW enzyme cocktail, respectively, which are very close to 1 and help to determine the strength the of the respective model.

3-D response surface plots of the model help visualizing the effects of a parameter among the other parameters. To understand the effect of two independent parameters on the dependent parameter, (response) 3D plots were constructed. These 3-D plots graphically evaluate the effects of independent factors on the RS yield by keeping the other parameters constant.[35]

Figures 1 and 2 show the interaction between substrate concentration (X1) and time (X2), substrate concentration (X1) and temperature (X3), time (X2) and temperature (X3) on RS yield after enzymatic hydrolysis using WB and BMSW enzyme cocktail, respectively. Substrate concentration is an important parameter in enzymatic hydrolysis process. Figure 1a shows that an increase in substrate concentration resulted in an increase in RS and at 10% substrate concentration, maximum RS yield was achieved and after that it started decreasing. High substrate concentration causes inhibition of substrate to enzymes, and it may also hinder homogenous mixing of substrate and enzymes.[27] Moreover, high solid loading may cause end-product diffusion and limitation of enzyme access to the substrate.[28] Therefore, beyond 10% substrate concentration, the RS yield starts decreasing gradually.

Enzymes released from *Aspergillus niger* are temperature-dependent and work efficiently at specific temperatures. An increase in temperature with substrate concentration caused an increase in RS yield, but maximum RS yield was achieved at

50 °C and 10 % substrate concentration, while with a further rise in temperature, it started decreasing continuously (Fig. 1b). In addition, at high temperature, the enzyme activity becomes slow, which causes the reduction in RS yield.

The time of enzymatic hydrolysis is another important factor. The effect of temperature and time (Fig. 1c) shows that an increase in time with temperature resulted in a gradual increase in RS yield and for 36 h at 50 °C maximum yields were achieved. However, after 36 h of incubation time, it started decreasing. The decrease in the RS yield is caused by the inactivation of the enzyme after optimum hydrolysis time. Figure 2 shows the significant interaction of the independent factors on the RS yield after enzymatic hydrolysis using the BMSW enzyme cocktail. The effect of time and substrate concentration on RS yield is presented in Figure 2a. Maximum RS yield was achieved at 10% solid loading, and after increasing the substrate concentration, it starts decreasing due to hindrance of homogeneous mixing of enzymes to the substrate. Figure 2b presents the interaction of the substrate concentration and time, which indicates that with the rise in temperature, RS concentration increases, and at 50 °C, maximum RS yield was achieved, thus confirming that 50 °C is the optimum temperature for enzymes. Similarly, the interaction of temperature and time (Fig. 2c) shows that the maximum yield of RS was achieved at 36 h and after that the enzymes become inactive.

2-D contour plots were generated by a regression model to visualize and explain the effect of temperature, substrate concentration and time on RS yield (Figs. 3 and 4). The shapes of the contours give the visualized impact of independent variables on the dependent variable. Contour plots of oval shape show a significant impact, as well as strong interactions of different factors. However, circular-shaped contour lines show insignificant interaction of two factors.[29] Figure 3 (a-c) shows contour lines of oval shapes for RS yield, indicating a strong interaction of independent variables, *i.e.*, time and substrate concentration (Fig. 3a), temperature and time (Fig. 3b), and temperature and substrate concentration (Fig. 3c), on the RS yield. In addition, Figure 4 (a-c) also shows the strong interaction between independent parameters on the response *i.e.*, RS yield. At substrate concentration (10%), time (6 h), and temperature (50 °C), the maximum RS yield achieved after enzymatic hydrolysis using WB and BMSW enzyme cocktails was 87% and 83%, respectively. Figure 5 (a-b) presents the analysis of predicted *vs* experimental (actual) values of RS yield, and all the values of the RS yield are within the vicinity of the regression line. Figure 5a reflects that there is good agreement between the predicted values of RS yield (%) and its experimental values. The higher R^2 value (0.9715) of the regression model (Eq. 2) indicates that the predicted and the actual data values have been well explained by the model. Moreover, for enzymatic hydrolysis using BMSW enzyme cocktail, the quadratic model presented in Equation 3 has a high R^2 value (0.9657), presenting good agreement between the predicted and actual results (Fig. 5b).

Verification of optimum conditions for enzymatic hydrolysis

The predictive capability of the respective model is verified using the response optimizer in Minitab software, and to get the maximum yield of RS, the optimum condition of parameters, *i.e.*, substrate concentration, time and temperature, were defined using the optimization plot in RSM. Figure 6 represents the optimum conditions for enzymatic hydrolysis to achieve maximum response yield. Figure 6a shows the maximized responses against the optimum parameters, depicting substrate concentration of 9.5%, time of 41.9 h, and temperature of 49.8 °C for enzymatic hydrolysis using the WB enzyme cocktail for a maximum RS yield of 87.3%. Meanwhile, by using the BMSW enzyme cocktail for hydrolysis, the optimum conditions were the following: substrate concentration of 9.5%, time of 45.1 h, and temperature of 50.2 °C for a maximum RS yield of 84.4% (Fig. 6b). To verify these results, experiments of enzymatic hydrolysis were performed under these conditions and RS yields of 85.8% and 82.4%, using WB and BMSW enzyme cocktails, respectively, were achieved, which were very close to the predicted values. This demonstrates that the respective models fitted well.

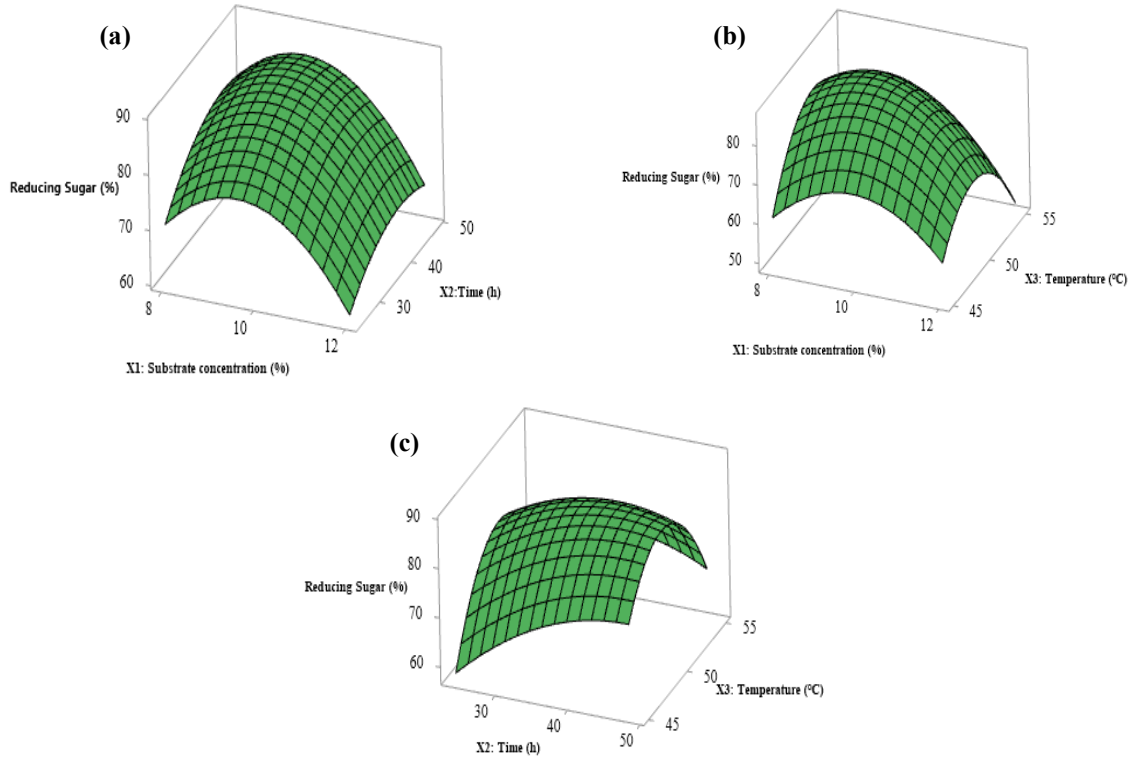

Figure 1: 3D response surface plots for reducing sugar yield after enzymatic hydrolysis using WB enzyme cocktail

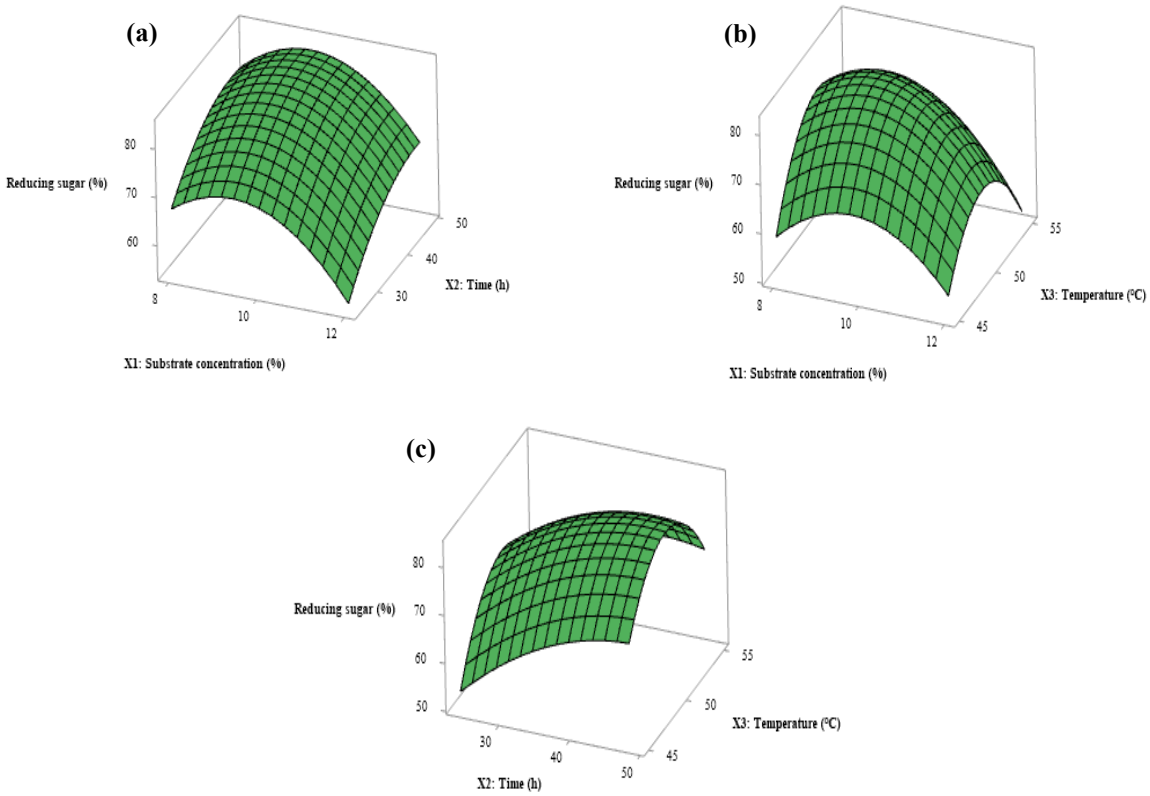

Figure 2: 3D response surface plots for reducing sugar yield after enzymatic hydrolysis using BMSW enzyme cocktail

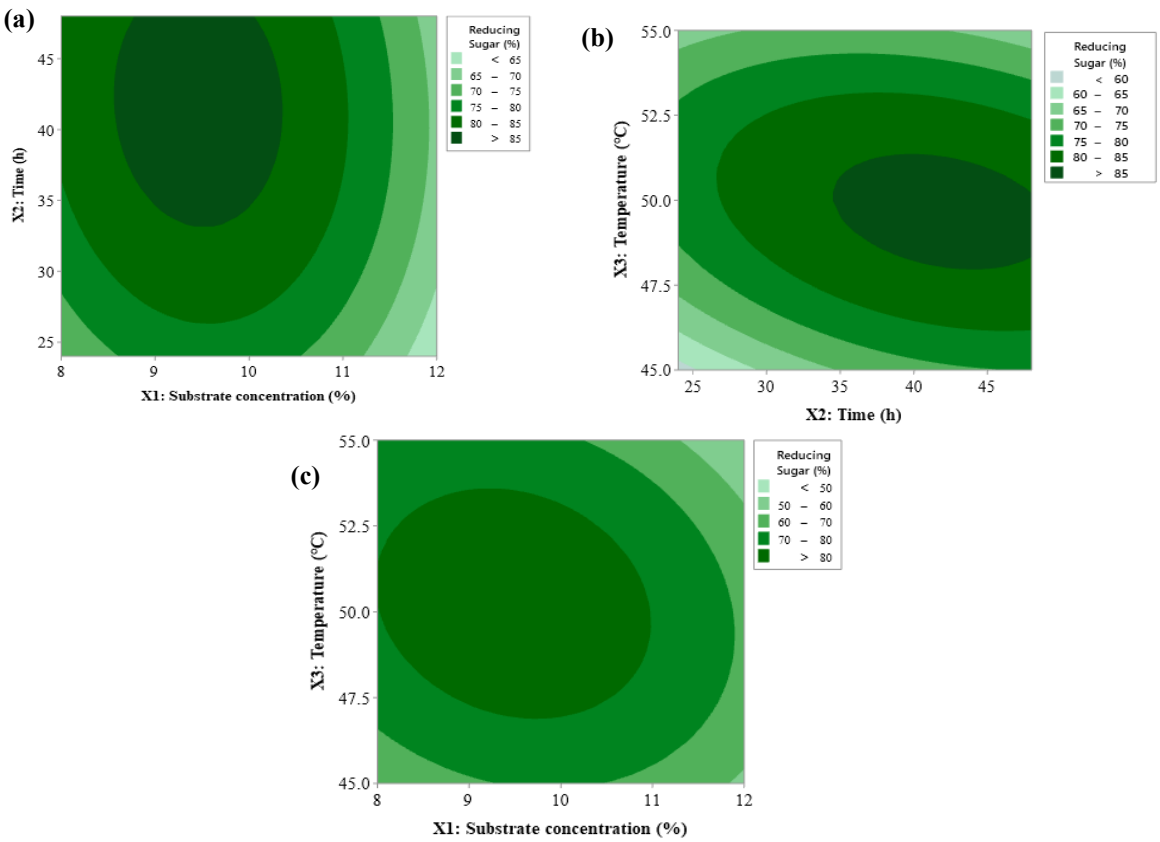

Figure 3: Contour plots for reducing sugar yield after enzymatic hydrolysis using WB enzyme cocktail

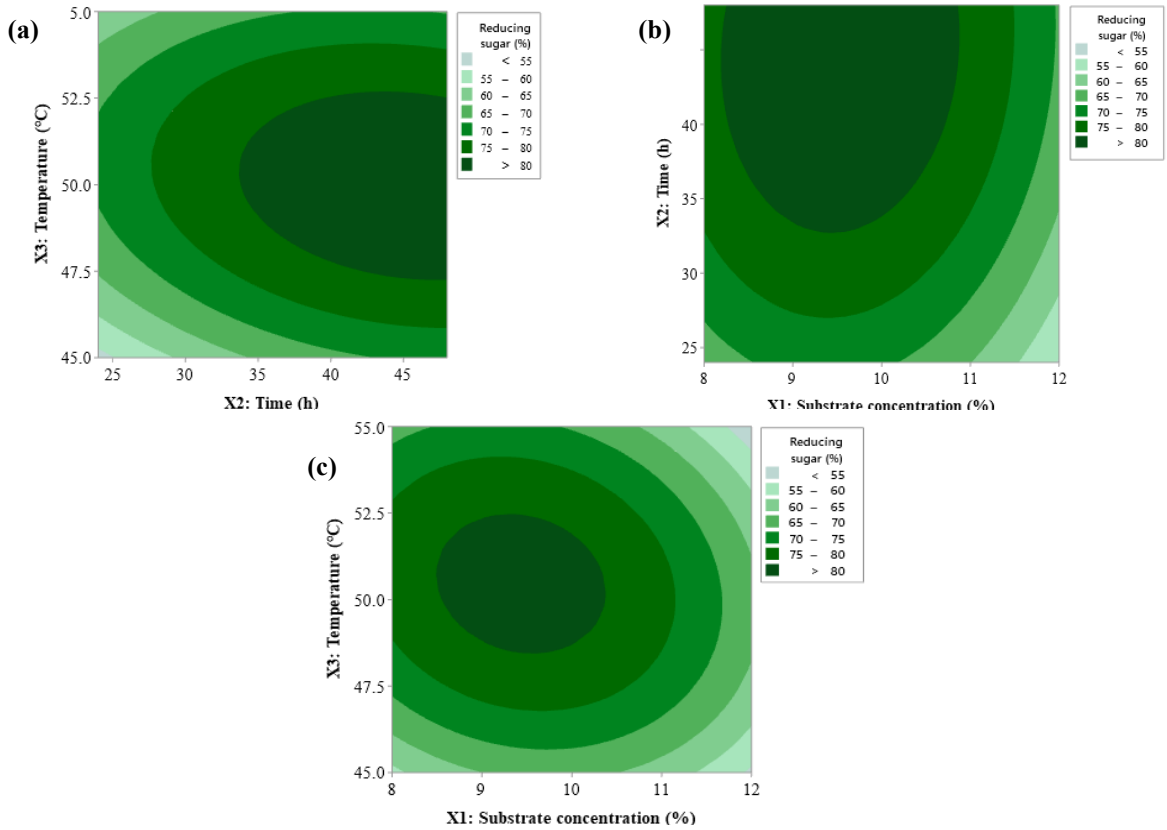

Figure 4: Contour plots for reducing sugar yield after enzymatic hydrolysis using BMSW enzyme cocktail

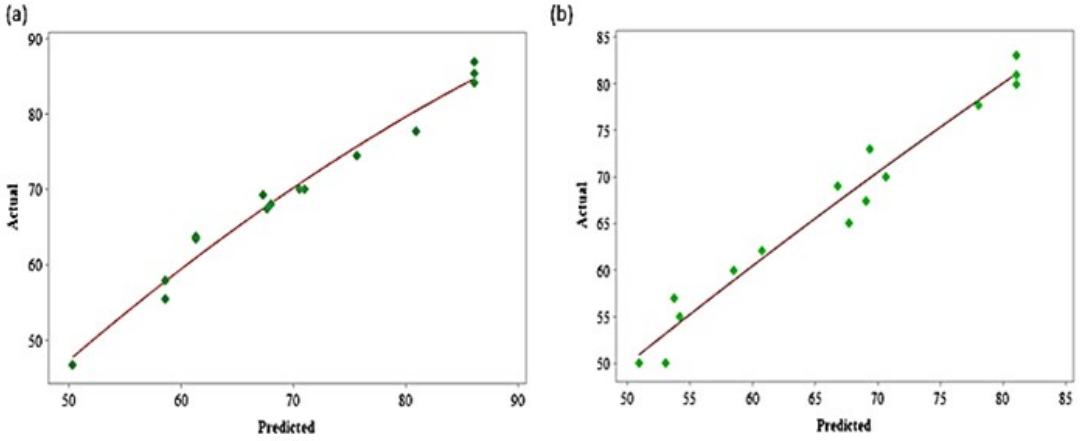

Figure 5: Predicted vs. actual plot of enzymatic hydrolysis using (a) WB enzyme cocktail and (b) BMSW enzyme cocktail

The results of RS released from different organic waste and enzymes are summarized and compared in Table 4. Lignocellulosic biomass is considered a cheap and easily available substrate for biofuel production, but municipal solid waste, which is abundantly available and a zero-cost substrate for biofuel production, has not been much explored. Table 4 clearly shows that biodegradable municipal solid waste has potential for biofuel production. The maximum RS yield (87%) released by the enzymatic hydrolysis of BMSW using WB enzyme cocktail was comparatively higher than those reported by Tsegaye *et al*.[30] (*i.e*., 62.09%) and Li *et al*.[31] (*i.e*., 72.80%) (Table 4).

Also, it was comparable with those of other studies published by Allouache *et al*.[32] (*i.e*., 85.01%), Triwahyuni *et al*.[33] (*i.e*., 84.14%), by Ge *et al*.[34] (*i.e*., 80.7%) and Xu *et al*.[35] (*i.e*., 80%) (Table 4). However, the enzymes used in this study are produced in the lab by *Aspergillus niger*, while commercial enzymes are used in other previous studies.[33-35] The major drawback of this procedure is the high cost of commercial enzymes in the production process of biofuel from lignocellulosic biomass. Thus, this approach using lab produced enzymes makes the production of biofuel cost-effective, while also offering maximum yield of RS.

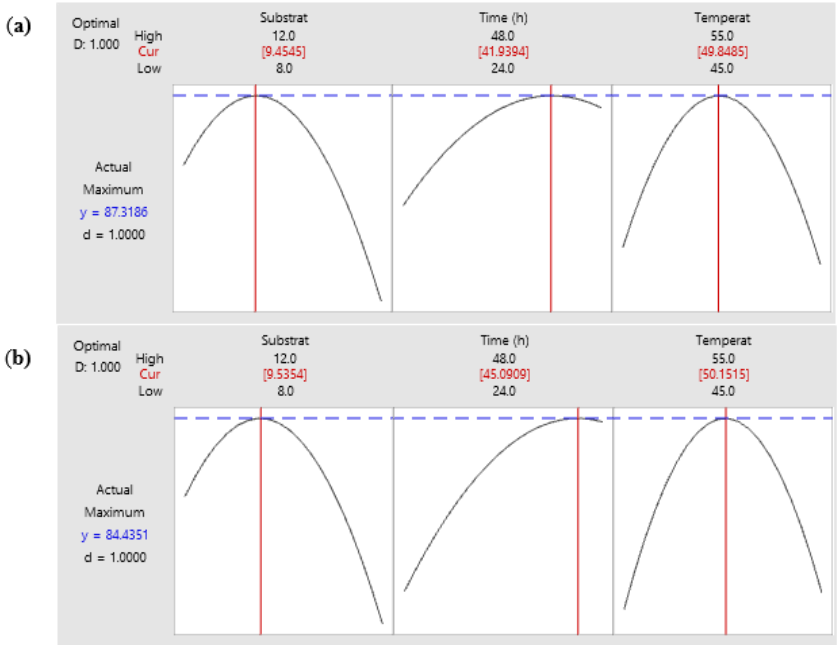

Figure 6: Response optimization of reducing sugar by enzymatic hydrolysis using (a) WB enzyme cocktail and (b) BMSW enzyme cocktail

Table 4
Comparison of RS yield released from different feedstock

Feedstock	RS yield (%)	Types of enzymes	Reference
Municipal solid waste	87 83	WB Enzyme cocktail from *Aspergillus niger* BMSW Enzyme cocktail from *Aspergillus niger*	This study
Ulva lactuca	85.01	Commercial grade cellulase from *Trichoderma reesei* Celluclast® 1.5 L	[32]
Oil palm empty fruit bunch	84.14	*Cellic® Ctec2* and *Cellic® Htec2 w*	[33]
Peanut shell	80.7	Novozym 22C cellulase enzyme	[34]
Sugarcane bagasse	80	Commercial enzyme Cellic CTec3	[35]
Municipal solid waste	72.80	Two commercial enzyme solutions *T. viride* and *T. reesei* ATCC26921	[37]
Rice straw	62.09	*Bacillus* sp. BMP01	[30]

Batch and fed-batch enzymatic hydrolysis

In this study, a comparison of batch and fed-batch enzymatic hydrolysis processes was made to get the maximum yield of RS from the pretreated substrate by previously optimized conditions. The results of the batch hydrolysis process showed that the RS yield of 73% and 69% was released after 36 h of hydrolysis time, using WB and BMSW enzyme cocktail, respectively. Also, for the fed-batch process, the maximum release of RS yield was 83% and 81%, for the same time (36 h) using WB and BMSW enzyme cocktail, respectively. In the case of the batch process, a lower RS yield was achieved, compared to the fed-batch hydrolysis, which can be explained by the high dose of the substrate (25%) and high viscosity of the medium. Sharma *et al.*[24] obtained the highest RS concentration of 285.3 mg/gds and 355.6 mg/gds after the batch and fed-batch enzymatic hydrolysis, respectively. Similarly, in another study, Karapatsia *et al.*[36] reported that 88.1% saccharification hydrolysis was achieved after the fed-batch process of acid pretreated substrate. So, the current research clearly shows the high solid loading fed-batch process can effectively be used for enzymatic hydrolysis enhancement.

CONCLUSION

In the current research, BMSW as a substrate showed great potential to produce RS after hydrolysis of the acid pretreated substrate using the WB and BMSW enzyme cocktail produced from *Aspergillus niger*. The results of this study showed that the maximum RS yield was achieved after enzymatic hydrolysis of the acid pretreated sample using WB enzyme cocktail produced from *Aspergillus niger* fermentation. The maximum yield of RS, *i.e.*, 87%, was obtained at substrate concentration of 10% and temperature of 50 °C, within 36 h incubation time using WB enzyme cocktail. Furthermore, a comparison of batch and fed-batch hydrolysis at a 25% substrate loading rate showed the highest yield of RS was achieved from the fed-batch process. Thus, this work may be effective in the development of efficient methods for enzymatic hydrolysis of the substrate (BMSW) for domestic and industrial biofuel production.

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REFERENCES

[1] Z. M. A. Bundhoo, *J. Mater. Cycles Waste Manag.*, **20**, 1867 (2018), https://doi.org/10.1007/s10163-018-0728-3

[2] D. Q. Zhang, S. K. Tan and R. M. Gersberg, *J. Environ. Manage.*, **91**, 1623 (2010), https://doi.org/10.1016/j.jenvman.2010.03.012

[3] Waste Management. Pakistan - Waste Management 2019 13-10-2019 [accessed 2021 30-06-2021]; available from: https://www.trade.gov/knowledge-product/pakistan-waste-management

[4] Z. Zhao, R. Bian, F. Zhao and X. Chai, *Environ. Prog. Sustain. Energ.*, **39**, 13372 (2020), https://doi.org/10.1002/ep.13372

[5] K. Kang, N. B. Klinghoffer, I. ElGhamrawy and F. Berruti, *Renew. Sust. Energ. Rev.*, **149**, 111372 (2021), https://doi.org/10.1016/j.rser.2021.111372

[6] O. N. Uncu and D. Cekmecelioglu, *Waste Manag.*, **31**, 636 (2011), https://doi.org/10.1016/j.wasman.2010.12.007

[7] S. K. Karmee and C. S. K. Lin, *Sustain. Chem. Process.*, **2**, 1 (2014), https://doi.org/10.1186/s40508-014-0022-1

[8] S. Vieira, M. V. Barros, A. C. N. Sydney, C. M. Piekarski, A. C. D. Francisco *et al*., *Bioresour. Technol*., **299**, 122635 (2020), https://doi.org/10.1016/j.biortech.2019.122635
[9] R. Sindhu, P. Binod and A. Pandey, *Bioresour. Technol*., **199** 76 (2016), https://doi.org/10.1016/j.biortech.2015.08.030
[10] L. Canilha, A. K. Chandel, T. S. D. S. Milessi, F. A. F. Antunes, W. L. D. C. Freitas *et al*., *J. Biotechnol. Biomed*., **2012**, ID 989572 (2012), https://doi.org/10.1155/2012/989572
[11] S. Kumar and R. K. Sani, in "Biorefining of Biomass to Biofuels", edited by S. Kumar and R. K. Sani, Springer International Publishing, Cham, 2018, https://link.springer.com/book/10.1007/978-3-319-67678-4
[12] M. O. Daramola and A. O. Ayeni, in "Valorization of Biomass to Value-Added Commodities: Current Trends, Challenges, and Future Prospects", edited by M. O. Daramola and A. O. Ayeni, Springer, 2020, https://link.springer.com/book/10.1007/978-3-030-38032-8
[13] D. K. Marcuschamer, P. O. Popiel, B. A. Simmons and H. W. Blanch, *Biotechnol. Bioeng*., **109**, 1083 (2012), https://doi.org/10.1002/bit.24370
[14] O. Takimura, T. Yanagida, S. Fujimoto and T. Minowa, *J. Jpn. Pet*., **56**, 150 (2013), https://doi.org/10.1627/jpi.56.150
[15] M. I. I. Rodríguez, J. A. R. Sánchez, S. D. Moral, M. C. Santoyo and M. G. A. Uscanga, *Biotechnol. Appl. Biochem*., **69**, 198 (2022), https://doi.org/10.1002/bab.2097
[16] S. Khare and O. Prakash, *Curr. Devel. Biotechnol. Bioeng*., **158**, 380 (2017), https://doi.org/10.1016/j.jclepro.2017.05.040
[17] P. L. Hurst, J. Nielsen, P. A. Sullivan and M. G. Shepherd, *Biochem. J*., **165**, 33 (1977), https://doi.org/10.1042/bj1650033
[18] M. Hamza and S. Sayadi, *Int. J. Food Sci. Technol*., **50**, 1882 (2015), https://doi.org/10.1111/ijfs.12839
[19] H. S. H. B. Hamid and K. S. K. Ismail, *Biocatal. Agric. Biotechnol*., **24**, 101530 (2020), https://doi.org/10.1016/j.bcab.2020.101530
[20] N. Zahara, M. I. Jalees and M. U. Farooq, *Environ. Prog. Sustain. Energ*., **42**, 13966 (2022), https://doi.org/10.1002/ep.13966
[21] O. O. Onipe, A. I. Jideani and D. Beswa, *Int. J. Food Sci. Technol*., **50**, 2509 (2015), https://doi.org/10.1111/ijfs.12935
[22] B. V. D. Santos, P. O. Rodrigues, C. J. B. Albuquerque, D. Pasquini and M. A. Baffi, *Appl. Biochem. Biotechnol*., **189**, 37 (2019), https://doi.org/10.1007/s12010-019-02991-6
[23] G. L. Miller, *Anal. Chem*., **31**, 426 (1959), https://doi.org/10.1021/ac60147a030
[24] S. Sharma, A. Kuila and V. Sharma, *Clean Technol. Environ. Policy*, **19**, 1577 (2017), https://doi.org/10.1007/s10098-017-1346-9
[25] N. Mlaik, S. Khoufi, M. Hamza, M. A. Masmoudi and S. Sayadi, *Biomass Bioenerg*., **127**, 105286 (2019), https://doi.org/10.1016/j.biombioe.2019.105286
[26] J. K. Kim, B.-H. Um and T. H. Kim, *Korean J. Chem. Eng*., **29**, 209 (2012), https://doi.org/10.1007/s11814-011-0169-3
[27] A. I. Vavouraki, V. Volioti and M. E. Kornaros, *Waste Manage*., **34**, 167 (2014), https://doi.org/10.1016/j.wasman.2013.09.027
[28] A. I. Vavouraki, E. M. Angelis and M. Kornaros, *Waste Manage*., **33**, 740 (2013), https://doi.org/10.1016/j.wasman.2012.07.012
[29] Z. Zhou, D. L. Hu, W. M. Qiao, G. H. Chen, L. Y. Jiang *et al*., *Huanjing Kexue*, **35**, 2249 (2014), https://europepmc.org/article/med/25158503
[30] B. Tsegaye, C. Balomajumder and P. Roy, *Renew. Energ*., **148**, 923 (2020), https://doi.org/10.1016/j.renene.2019.10.176
[31] A. Li, B. Antizar-Ladislao and M. Khraisheh, *Bioprocess Biosyst. Eng*., **30**, 189 (2007), https://doi.org/10.1007/s00449-007-0114-3
[32] A. Allouache, A. Majda, A. Z. Toudert, A. Amrane and M. Ballesteros, *Cellulose Chem. Technol*., **55**, 629 (2021), https://doi.org/10.35812/CelluloseChemTechnol.2021.55.51
[33] E. Triwahyuni, A. K. Miftah, M. Muryanto, R. Maryana and Y. Sudiyanni, *Cellulose Chem. Technol*., **55**, 839 (2021), https://doi.org/10.35812/CelluloseChemTechnol.2021.55.71
[34] S. Ge, Y. Wu, W. Peng, C. Xia, C. Mei *et al*., *Chem. Eng*., **385**, 123949 (2020), https://doi.org/10.1016/j.cej.2019.123949
[35] C. Xu, J. Zhang, Y. Zhang, Y. Guo, H. Xu *et al*., *Bioresour. Technol*., **292**, 121993 (2019), https://doi.org/10.1016/j.biortech.2019.121993
[36] A. Karapatsia, I. Pappas, G. Penloglou, O. Kotrotsiou and C. Kiparissides, *Bioenerg. Res*., **10**, 225 (2017), https://doi.org/10.1007/s12155-016-9793-4
[37] A. Li, B. Antizar-Ladislao and M. Khraisheh, *Bioprocess Biosyst. Eng*., **30**, 189 (2007), https://doi.org/10.1007/s00449-007-0114-3