Italian Journal of Food Science, 2024; 36(2): 231–245

Statistical optimization for comparative hydrolysis and fermentation for hemicellulosic ethanolgenesis

Asma Chaudhary[1]*, Ayesha Aihetasham[2], Smavia Younas[1], Nimra Basheer[1], Nageen Hussain[3], Sumaira Naz[4], Tariq Aziz[5], Thamer H. Albekairi[6]

[1]Department of Zoology, Division of Science and Technology, University of Education, Township, Lahore, Pakistan; [2]Institute of Zoology, University of the Punjab, Lahore, Pakistan; [3]Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan; [4]Department of Biochemistry University of Malakand Chakdara Dir Lower; [5]Laboratory of Animal Health Food Hygiene and Quality, University of Ioannina, Arta, Greece; [6]Department of Pharmacology and Toxicology College of Pharmacy King Saud University Riyadh Saudi Arabia

***Correspondence Authors:** Asma Chaudhary, Department of Zoology, Division of Science and Technology, University of Education, Township Block C, Phase 1, Johar Town, Lahore, Pakistan, Email: asma.ch@ue.edu.pk; Nageen Hussain, Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan, Email: nageen.mmg@pu.edu.pk

Received: 26 January 2024; Accepted: 6 May 2024; Published: 1 June 2024

ORIGINAL ARTICLE

Abstract

The concept of 'Energy from waste' is one of the most focused areas of work to find a solution for controlling trash and combat energy crises. In Pakistan and other agricultural countries, because of their substantial use during the summer, watermelon peels as fruit waste are usually thrown out as a trash. This study supported the management of huge quantities of waste to value-added products at a commercial scale. The current study aims to select and subject xylanolytic and ethanologenic *Bacillus cereus* XG2 for water melon peels valorization appropriately with comparison of three hydrolysis techniques. The study will be helpful for selection of economical and environmentally beneficial valorization strategies. For ethanalogenesis, separate hydrolysis and fermentation (SHF) protocols with Saccharomyces cerevisiae K7 and Metchnikowia cibodasensis Y34 were used. For hydrolysis, three different saccharification approaches, viz. dilute sulfuric acid, enzymatic hydrolysis (using *Bacillus cereus* XG2 xylanases), and combined acidic and enzymatic hydrolysis, were adopted. Two statistical models, Placket-Burman (hydrolysis) and Central composite design (ethanologenesis) were used. In untreated watermelon waste (WW), reducing sugar, total lipids, total carbohydrates, and protein contents were calculated as 16.70±0.05 g/L, 3.20±0.02 g/L, 28.7±0.04 g/L, and 3.70±0.03 g/L, respectively. Similarly, the lignin (15.51±0.22%), hemicellulose (17.20±2.30%). and cellulose (52.26±0.33%) contents were also analyzed. Based on the significance of the Plackett–Burman model for enzymatic saccharification, the released reducing sugars as well as total sugars were 21.62±0.01 g/L and 43.30±1.55 g/L, respectively, and enzymatic hydrolyzate was adopted for further fermentation experiments. By CCD model, the highest ethanol yield calculated for yeast *Metchnikowia cibodasensis* Y34 was 0.4±0.04 g/g of fermentable sugars at 32.5°C with 50% enzymatic hydrolysate of WW by incubating for 8 days. It was suggested that SHF could be a beneficial approach to increase the conversion of hemicellulose to fermentable sugars to produce bioethanol on a large scale.

Keywords: *Bacillus cereus*; ethanol production; fruit waste; separate saccharification and fermentation; xylanases

Introduction

Pakistan is a developing state with a sharp increase in population density, and countering serious economic and energy crises. Pakistan's requirement for energy is rising day by day, with the latest estimated demand of 84 million tons of oil equivalent (MTOE). For the time being, the usage of fossil fuels has been controlling Pakistan's energy zone. However, indigenous fossil fuel reserves are being consumed sharply and are not able to cope with increasing energy requirements. Therefore, to fulfill its rising energy demands, the country is required to find alternative energy resources. Biomass is one of the substitutes with wide capability to help Pakistan overcome its ever-growing energy demands (Ullah *et al.,* 2023; Narjis *et al.,* 2023; Aziz *et al.,* 2023; Shah *et al*., 2023; Khan *et al.*, 2022).

Lignocellulosic biomass (LCB) chiefly comprises three polymers: cellulose, hemicellulose, and lignin. These three polymers are linked to each other in a heterogenous form to distinct degrees and with differing constitutions based on the type, species, and even origin of biomass. The comparative profusion of cellulose, hemicellulose, and lignin are important factors to dictate optimum energy (Bajpai, 2016). Hemicellulose is the second most available renewable biomass and is regarded as 25–35% of LCB (Kumar *et al.*, 2008).

The main obstacle to the stable usage of LCB as a substrate for bio-based fuels is the complex processing of LCB because of its complex structure to decompose into fermentable C5 and C6 sugars on account of recalcitrance (Guerriero *et al.*, 2016). Debilitating the recalcitrance needs a mixture of thermal, chemical, enzymatic, and microbial pretreatment processes, leading to high financial inputs (Alvira *et al.*, 2010). Circumstantially, pretreatment is a crucial step designated to distort recalcitrant structure in LCB, shatter lignin bonding, and decrease degrees of cross-linking of cellulose and hemicellulose held within it (Chen *et al.*, 2017; Loow *et al.*, 2015).

Hard as well as annual supply of wood comprises xylan (hemicellulose) as the second highest polysaccharide available in nature. Hemicellulose is as abundant as cellulose and accounts for roughly one-third of the sustainable organic carbon reserves of the earth (Kamble and Jadhav, 2012). Owing to its complexities and variety, the complete breakdown of xylan needs several working enzymes, known as xylanases. Bacteria and fungi are equipped with the xylanolytic system. Different habitats, such as marines (Annamalai *et al.*, 2009), thermal springs (Bouacem *et al.*, 2014), soda lakes (Huang *et al.*, 2015), and Antarctic environments (Bradner *et al.*, 1999), possess xylanolytic microorganisms. Various *Bacillus* species produce large amounts of extracellular enzymes to ferment a variety of substrates over a range of pH and temperature values, making them the most useful hosts for the industrial production of many improved novel products (Rashid and Sohail, 2021).

Various methods are studied for the best saccharification/hydrolysis of xylan into monosaccharides, especially xylose. Nowadays, hydrolysis is done by acids, alkalis, peroxides, high temperatures, vapor, microwave, and ionic liquids. Hydrolysis by dilute acid is considered an effective way to make hemicellulose susceptible to subsequent hydrolytic enzymes. Therefore, for optimum breakdown of hemicelluloses, harmonious action of acids and enzymes is required (Azhar *et al.*, 2015; Isikgor and Becer, 2015).

For this purpose, available xylanases are potentially beneficial enzymes for breaking the xylosidic bonding of xylan-rich LCB. Xylanases are excessively obtained from microorganisms for multiple industrial/commercial purposes. In recent times, the maximum industrial focus has been on xylanases for production of bioenergy, wood pulp bioleaching, food and beverages manufacturing, animal diet, production of chemical and pharmaceutical goods, etc. (Chaudhury *et al.*, 2023). Focusing on the global energy crisis, it is utmost importance to convert biofuels, such as bioethanol, into energy system. In this regard, the production of ethanol via microbial fermentation using LCB, such as fruit wastes, can be considered as the most favorable and economical means in agricultural and developing countries like Pakistan. Today, ethanol is considered one of the most efficient liquid biofuels, capable of substituting depleting ordinary fuels (Saleem *et al.*, 2020).

Various bacterial species are known to harbor influential xylanases for the transformation of hemicellulose into xylose. A few examples include *B. halodurans*, *B. subtilis, Thermomonospora fusca* and *B. amyloliquefaciens* (Li *et al.,* 2023; Banka *et al.*, 2014; Chakdar *et al.*, 2016; Chaudhary *et al.*, 2023; Thomas *et al.*, 2014). Industrial xylanases removed from these microbes during different bioprocesses yield highly value-added products. However, utilizing commercially feasible enzymes for these production processes leads to huge input costs.

In this context, the current study aimed to explore the potential of xylanase degrading bacterial isolates for processing of locally disposed of watermelon waste (WW). Pakistan is the 18th largest producer of watermelon, with 2.41 million tons of annual cultivation and around 540,000 metric tons of fruit waste, which requires proper elimination as well as utilization. Watermelon waste, if managed and utilized properly as a raw material for

fermenting bioethanol, helps to lessen environmental pollution and earn economic benefits (Alex *et al.*, 2017; Kassim *et al.*, 2022).

The development of the bioeconomy encouraged advances in conventional methods for converting cellulose to ethanol. In order to apply process technology at an industrial level, hemicellulosic stream should be assimilated equally with celluloses for ethanol conversion to cut the cost of waste management. Therefore, screening special strains of bacteria and yeast for the fermentation of both pentose and hexose, as well as the selection of a better saccharification technique, is required to accomplish process economization in a consolidated bioprocess.

After the biomass hydrolysis of recruiting monosugar (i.e., xylose), next step is the conversion of these fermentable sugars into ethanol. In this regard, various yeast species have shown remarkable ethanologenic potential. However, yeast in its native form rarely shows combined xylanolytic and ethanologenic properties. Prospectively, the present investigation drives for the appropriate selection and subjection of xylanolytic and ethanologenic microbes via separate hydrolysis and fermentation by utilizing watermelon peels. It is expected that once the above-mentioned approaches are established, biowaste valorization into value-added products, such as ethanol, could be intensified in a cost-competitive and eco-friendly manner.

Materials and Methods

The most tasty and affordable fruit consumed in Pakistan throughout the summer season is watermelon. Watermelon waste was used as a source of raw LCB for the study. It was obtained from local market and processed after proper washing with water and drying at 60°C. A fine particle size of 1 mm was screened by grinding and sieving. Dried WW substrate was stored in airtight jars. For analysis of protein and sugar contents, extraction with distilled water (10%) was done; for extraction of lipids, ethanol (10%) was used. Phenol–sulfuric acid (PSA), Zollner and Kirsch c*olorimetric* method, 3,5-dinitrosalicylic acid (DNS), and Lowery assay protocol were followed for the determination of total sugar (carbohydrates), lipids, reducing sugar (RS), and total protein contents (Dubois *et al.*, 1956; Lowry *et al.*, 1951; Miller, 1959; Zöllner and Kirsch, 1962). The protocol suggested by Association of Official Analytical Chemists (AOAC, 2012) was followed to determine the moisture content of peels. Hemicellulose, lignin, and cellulose contents and extractives were determined using the approach followed by Lin *et al.* (2010), with some modifications.

Microbes used for the study

Bacillus cereus XG2 (OM 970803) with a xylanolytic potential of 0.226±0.011 µmol/min/mL was used for enzymatic saccharification (Chaudhary *et al.*, 2023). Two yeast isolates *Saccharomyces cereviceae* K7 and *Metschnikowia cibodasenis* Y34 were obtained from the author's microbiology laboratory to carry out fermentation experiments (Chaudhary and Karita, 2017). *Saccharomyces cerevisiae* K7, provided by the Brewing Society of Japan (Tokyo, Japan), served as a standard yeast strain. *Metchnikowia cibodasensis* Y34 was isolated from Abelia flower; it has the capacity to produce ethanol (1.80±0.05%) through ethanologenic processes (Chaudhary and Karita, 2017).

Placket–Burmen (PB) design for screening of saccharification parameters

For a comparative study of WW hydrolysis, three treatments, viz. enzymatic, diluted sulfuric acid, and combined modality (acidic followed by enzymatic), were used. For screening of hydrolysis parameters, PB designs were used, where main effects were confounded with two-factor interactions. PB tool was used to determine significant elements and complete preliminary screening and evaluation of experimental parameters. The tool helped in the screening of irrelevant variables to prevent the accumulation and processing of ample data. In all, 12 runs of PB design dealt with multiple parameters for three different treatments.

Parameters for acidic saccharification were 1:10 WW, temperature of 50–100°C, 30–60-min hydrolysis time, and 2–6% H_2SO_4 concentration. To prepare crude enzyme for enzymatic hydrolysis, a neutral basal medium (%) consisting of 0.1-g yeast extract, 0.05-g $Na_3C_6H_5O_7$, 0.2-g potassium dihydrogen phosphate, and 0.01-g $MgSO_4$ was prepared. It was then incubated for 72 h at a temperature of 37°C (Bai *et al.*, 2012). Acetate buffer (0.2 M) with WW was used as a substrate buffer (Abu-Gharbia *et al.*, 2018).

Enzymatic hydrolytic parameters were as follows: hydrolysis duration of 1–5 days, temperature of 55–65°C, acetate buffer, 80–90%, enzyme load of 9.17–18.97 µmol, buffer pH of 6–9, and WW 5–10 g. Sulfuric acid hydrolysate was prepared for combined treatment by following the saccharification conditions computed by the software for optimum responses (Table 1): temperature range of 55–65°C, enzyme dosage of 9.17–18.97 µmol, duration of hydrolysis of 1–5 days, acid hydrolysate volume of 50–75 mL, acetate buffer volume of 25–50 mL, and buffer pH of 6–9. Experimental responses in three PB designs were conducted to investigate reducing and total sugars (g/L). Released sugars from WW

Table 1. Compositional analysis of untreated watermelon peels waste (WW).

Parameters	Contents
Proteins (g/L)	3.7 ± 0.03
Lipids (g/L)	3.2 ± 0.02
Carbohydrates (g/L)	28.7 ± 0.04
Reducing sugars (g/L)	16.7 ± 0.05
Hemicellulose (%)	17.20 ± 2.30
Weight loss (%)	15.03 ± 0.50
Lignin (%)	15.51 ± 0.22
Cellulose (%)	52.26 ± 0.33
Moisture (%)	0.74 ± 0.05

All values represent mean of three replicates ± standard error of mean (SEM).

were determined statistically using PB tools and the highest theoretical reducing sugars were calculated. Conditions for predicted theoretical reducing sugars were validated by carrying out hydrolysis experiment.

Optimization of fermentation parameters by central composite design (CCD)

Among three hydrolysis techniques, enzymatic hydrolyzate of WW with screened conditions was selected for the fermentation experiment. CCD tool was used to optimize fermentation parameters. The Design Expert software (ver. 8.0; Stat-Ease Inc., Minneapolis, MN, US) was employed to design PB and CCD models. A 20-run experiment was designed by CCD with three parameters. Three factors for fermentation were 25–40°C with 1–15 days of incubation period, and ratio of hydrolyzate and minimal medium (25:75 to 75:25). Three responses, viz. ethanol assay, ethanol yield, and yeast growth were analyzed in the experiment. Yeast inoculum was prepared in MYG (Malt extract-Yeast extract-Glucose) medium having composition (%) as Malt extract 0.3 g, Yeast extract 0.3 g and glucose 1.0 g. Minimal medium (g/L) comprised the following: 0.7 g of yeast extract, 0.09 g of magnesium sulfate heptahydrate, 0.3 g of KH_2PO_4, 0.042 mg of zinc chloride, 0.27 of $(NH_4)_2SO_4$, 0.155 of citric acid, 0.7 of sodium citrate, and 0.035 of calcium chloride (Camelia et al., 2010). Biochemical analysis (g/L) of ethanol and reducing sugars was done following DNS and potassium dichromate protocols (Bennett *et al.*, 1971; Miller, 1959). Ethanol yield (g/g) was calculated by dividing ethanol contents (g/L) by the sugar consumed (g/L). Optimized point prediction of parameters with the highest ethanol yield was computed by CCD statistical tools. By performing fermentation experiment, selected optimum conditions were validated and actual yield was computed. Three-dimensional (3D) graphs were plotted to elucidate interconnection of factors on responses.

Results

Biochemical compositional analysis of watermelon waste

The biochemical compositional analysis of WW is presented in Table 1. In WW (without pretreatment), following contents were calculated: reducing sugars 16.7±0.05, total lipid 3.2±0.02, total sugars (TS) 28.7±0.04, and total proteins 3.7±0.03. The calculated cellulosic contents (52.26±0.33) were computed by subtracting the sum of weight loss (15.03±0.5), hemicellulose (17.20±2.30), and lignin (15.51±0.22) from 100.

Placket–Burman design to screen saccharification conditions for different pretreatments

The data for different responses of acidic hydrolysis are presented in Table 2. The highest amount of total sugars and reducing sugars released were 39.82±2.75 g/L and 29.46±0.01 g/L, respectively, at 100°C with 6% diluted H_2SO_4 and 10-g WW treated for 60 min. For enzymatic hydrolysis of WW, reducing sugars (21.62±0.01 g/L) and total sugars (43.3±1.55 g/L) were released in 5 days with an enzyme dose of 9.17 µmole/mL/min. Other parameters were buffer of 80 mL, pH of 9, and temperature of 55°C, as shown in Table 3. The most favorable conditions for optimum combined (acidic+enzymatic) hydrolysis were as follows: hydrolysis time: 5 days, bacterial xylanase: 9.17 µmole/mL/min, buffer: 50 mL with pH of 6, acid hydrolysate at 65°C: 75 mL, with respective optimum reducing sugars and total sugars as 22.30±0.02 g/L and 41.20±1.15 g/L (Table 4).

Statistical analysis of Placket–Burman model for different treatments

The data for ANOVA to analyze the fitness of model for acidic, enzymatic, and combined hydrolysis treatments are tabulated in Table 5. For reducing sugars released by acidic hydrolysis, PB model was nonsignificant with a carrying model F-value of 1.98, which occurred due to 95% chance and noise. Total sugars PB hydrolysis model is non-significant due to lower F-value (2.28) as related to signal to noise ratio. F value is the measure of the ratio between the variance among group (signal) and the variance within each group (noise). The F value provides a quantitative measure of the signal-to-noise ratio. The PB model for reducing sugars in enzymatic hydrolysis was significant due to an F-value of 6.50 with 94% chance. The PB total sugars model for enzymatic hydrolysis

Table 2. Different parameters and responses interpreted for acidic hydrolysis of watermelon peels waste (WW) by Plackett–Burman (PB) design.

Runs	A: Temp. (˚C)	B: Incubation time (min)	C: Acid conc. (%)	D: Peels (%)	Reducing sugars (g/L)	Total sugars (g/L)
1	50	60	6	5	0.75 ± 0.01	7.76 ± 0.03
2	50	30	6	10	27.42 ± 0.01	33.16 ± 3.84
3	100	60	6	5	5.45 ± 0.01	11.60 ± 1.34
4	50	30	2	5	15.23 ± 0.01	27.41 ± 0.69
5	50	60	2	10	2.79 ± 0.02	14.86 ± 0.92
6	100	30	2	10	26.62 ± 0.02	33.70 ± 1.99
7	100	60	6	10	29.46 ± 0.01	39.82 ± 2.75
8	50	30	6	10	21.10 ± 0.02	31.67 ± 0.66
9	100	60	6	5	28.87 ± 0.01	35.51 ± 2.37
10	100	30	2	10	13.18 ± 0.16	25.12 ± 0.62
11	100	60	2	5	11.00 ± 0.01	18.89 ± 0.20
12	50	30	2	5	0.13 ± 0.16	8.21 ± 0.02

All values represent mean of three replicates ± standard error of mean (SEM).

Table 3. Different parameters and responses interpreted for enzymatic hydrolysis of watermelon peels waste (WW).

Runs	A: Temp. (°C)	B: Incubation time (days)	C: Enzyme dose (µmole/mL/min)	D: Buffer conc. (mL)	E: Peels (g)	F: pH	Reducing sugar (g/L)	Total sugar (g/L)
1	65	5	9.17	90	10	6	18.38 ± 0.13	41.80 ± 0.71
2	65	5	9.17	90	5	6	8.82 ± 0.01	17.42 ± 0.02
3	65	1	18.34	80	5	6	17.32 ± 0.01	39.50 ± 0.12
4	55	5	9.17	80	5	9	21.62 ± 0.01	43.30 ± 1.55
5	55	1	9.17	90	10	9	13.13 ± 0.04	28.98 ± 0.07
6	55	1	18.34	90	10	6	15.60 ± 0.03	32.71 ± 0.03
7	65	1	18.34	90	5	9	12.19 ± 0.04	29.13 ± 0.10
8	65	5	18.34	80	10	9	16.70 ± 0.13	32.03 ± 0.20
9	55	5	18.34	90	5	9	4.62 ± 0.01	11.71 ± 0.06
10	65	1	9.17	80	10	9	9.54 ± 0.02	24.99 ± 0.01
11	55	5	18.34	80	10	6	10.02 ± 0.05	24.75 ± 0.01
12	55	1	9.17	80	5	6	3.70 ± 0.03	17.10 ± 0.009

was also significant with an F-value of 18.29 with 98.0% chance because of noise. As the combined treatment was studied, the model for reducing sugars was nonsignificant with an F-value of 2.07, with 63% chance occurring because of noise. Nonsignificant total sugars model values was indicated by 73% chance that was due to large occurring noise and an F-value of 1.57.

Statistical data of calculated regression coefficients for three treatments are presented in Table 6. For acidic hydrolysis, 'pred R^2' of 0.979 and 'adj R^2' of 0.886 for reducing sugars coincided with each other. The value of 8.51 for adeq precision provided adequate signals to favor the model's navigation to design space. For total sugars, pred R^2 of 0.456 inferred better prediction of response than the current model. The value of 14.52 for adeq precision indicated better signal for model. Concerning enzymatic hydrolysis, R^2 = 0.920 and adj R^2 = 0.779 interpreted the significance of model for reducing sugars. The value of 6.25 for adeq precision navigated the design space by adequate signal. For total sugars response, the larger adeq precision value of 13.70 predicted the appropriateness of model. For reducing sugars response in combined treatment, overall means predicted better results due to a negative pred R^2 of -1.070 than the current model, implying that the overall means was a better predictor of response. The values of 0.630 for R^2 and 0.205 for adj R^2 implied the nonreliability of model. For total sugars response, R^2 = 0.733 and adj R^2 = 0.266 implied less reliability of model. Adeq precision

Table 4. Different parameters and responses interpreted for combined hydrolysis of watermelon peels waste (WW).

Runs	A: Temp. (˚C)	B: days	C: Enzyme dose (µmole/mL/min)	D: Buffer conc. (mL)	E: Acid hydrolyzate (mL)	F: pH	Reducing sugars (g/L)	Total sugars (g/L)
1	55	5	18.97	25	50	9	3.20 ± 0.01	17.30 ± 0.28
2	65	1	18.97	50	50	9	6.34 ± 0.01	26.80 ± 0.03
3	65	5	9.17	25	75	9	10.40 ± 0.03	29.00 ± 0.05
4	65	1	18.97	25	75	9	15.10 ± 0.01	27.30 ± 0.03
5	55	5	18.97	25	75	6	7.40 ± 0.01	25.20 ± 0.01
6	65	5	9.17	50	75	6	22.30 ± 0.02	41.20 ± 1.15
7	55	1	9.17	50	75	9	12.10 ± 0.10	30.50 ± 0.04
8	55	5	18.97	50	50	9	20.50 ± 0.07	37.70 ± 0.07
9	55	1	9.17	25	50	6	19.50 ± 0.03	34.10 ± 0.05
10	65	5	9.17	50	50	6	13.04 ± 0.02	32.40 ± 0.03
11	55	1	18.97	50	75	6	16.40 ± 0.03	29.70 ± 0.04
12	65	1	9.17	25	50	6	5.20 ± 0.02	28.30 ± 0.06

All values represent mean of three replicates ± standard error of mean (SEM).

Table 5. Analysis of variance for the responses with watermelon peels waste (WW) treatments using Plackett–Burman (PB) design.

Treatments	Responses	Source	Sum of squares	DF	Mean square	F value	p value
Acidic hydrolysis	Reducing sugars	Model	1382.21	9	153.58	1.98	0.51, not significant
		Residual	29.25	2	14.62		
		Core total	1411.46	11			
	Total sugars	Model	1403.61	10	140.36	2.28	0.22, not significant
		Residual	5.32	1	5.32		
		Core total	1408.93	11			
Enzymatic hydrolysis	Reducing sugars	Model	241.47	10	24.15	6.50	0.04, significant
		Residual	94.30	1	9.00		
		Core total	80.78	11			
	Total sugars	Model	682.68	10	68.27	18.29	0.02, significant
		Residual	426.14	1	426.14		
		Core total	1108.82	11			
Combined treatment	Reducing sugars	Model	418.65	10	41.86	2.07	0.50, not significant
		Residual	20.22	1	20.22		
		Core total	438.87	11			
	Total Sugars	Model	412.64	10	41.26	1.57	0.35, not significant
		Residual	0.11	1	0.11		
		Core total	412.75	11			

Table 6. Regression model for various responses with watermelon peels waste (WW) hydrolysis strategies using Plackett–Burman (PB) design.

Treatments	Responses	CV	Press	R^2	Adj R^2	Pred R^2	Adeq precision
Acidic hydrolysis	Reducing sugars	25.21	1052.98	0.979	0.886	0.254	8.51
	Total Sugars	9.62	766.08	0.996	0.959	0.456	14.52
Enzymatic hydrolysis	Reducing sugars	8.81	58.49	0.920	0.779	0.276	6.25
	Total Sugars	11.97	4127.47	0.979	0.926	0.678	13.70
Combined hydrolysis	Reducing sugars	82.63	1583.14	0.630	0.205	–1.070	4.20
	Total Sugars	32.35	4921.97	0.733	0.266	–1.401	4.22

values of 4.20 (for reducing sugars) and 4.22 (for total sugars) presented adequate signals.

Validation of predicted contents by PB model via experimentation

Table 7 shows the data for predicted and experimental responses. In acidic hydrolysis, the predicted values for total and reducing sugars were 39.37 g/L and 29.95 g/L, respectively, with 10% WW of 30 min at 100°C with 6% diluted sulfuric acid. Predicted values for both responses of enzymatic hydrolysis were 41.3 g/L and 26.96 g/L, respectively, under the incubation period of 5 day at 65°C with 9.17 µmole/min/mL enzyme load, 80 mL of acetate buffer at pH 6 and 5% WW.

Predicted values with combined treatment for reducing and total sugars were 25.38 g/L and 35.41 g/L, respectively, computed under the following conditions: 5-day incubation, 65°C temperature, 9.17 µmole/min/mL enzyme load, 25-mL acetate buffer, pH 9, and 50-mL acid hydrolyzate. The experimental values improved in case WW was subjected to different hydrolysis with predicted parameters.

Central composite design for optimization of fermentation parameters

The values showing ethanol yield and titer under different conditions of CCD are shown in Table 8. Both strains of yeast gave maximum yield at 32.5°C with 50-mL hydrolysate incubated for 8 days, where 0.37±0.03 g/g ethanol was yielded by *S. cereviseae* K7 and 0.40±0.04 g/g ethanol was yielded by *M. cibodasensis* Y34.

The ANOVA data are shown in Table 9. The model for ethanol yield was found significant with respective F-values and P-values of 5.07 and 0.009 for *S. cereviseae* K7 and 4.42 and 0.034 for *M. cibodasensis* Y34. The F-value of 2.52 for ethanol content showed the model's insignificance for standard yeast.

The statistical values of regression coefficients, CV (Coefficient of variation), and adequate precision are shown in Table 10. The variable of the models attributed up to 91% and the reliability of yield for standard yeast was indicated by R^2 = 0.82 and adj R^2 = 0.65 that was coincided with values of adequate precision (7.21) and CV (21.37). The values of CV and adequate precision suggested the model fit. In this study, the smaller CV values and adequate precision more than 4 suggested the good model fit due to smaller the residuals relative to the predicted value. Similarly, the experimental yeast indicated yield significance by R^2 (0.76), adj R^2 (0.53), adeq precision (6.25), and CV (22.41). For ethanol titer synthesized by *S. cereviseae* K7 standard yeast, the following values were observed: R^2 = 0.78, adj R^2 = 0.59, and adeq precision = 7.28. On the other hand, the experimental yeast presented the following values: R^2 = 0.69, adj R^2 = 0.42, and adeq precision = 6.47. These values interpreted less reliability of model for this response.

Presentation of variable interrelationship in the form of surface graphs

Figure 1 (*S. cereviseae* K7) and Figure 2 (*M. cibodasenis* Y34) show the interconnection of different parameters for response, that is, ethanol yield. 3D illustration was used to analyze the effect of all variables with both yeasts. In Figure 1A, with increase in incubation period, ethanol yield decreased slowly, while increase in hydrolyzate resulted in slight increase in response. Figure 1B shows a sharp increase with hydrolyzate and an infinitesimal increase in temperature. In Figure 1C, optimum response is observed for up to day 8, followed by a decreasing pattern. Temperature had no effect on incubation days.

Figure 2A presents a slight increase in yield with an increase in incubation period and hydrolyzate. In

Table 7. Validation of predicted parameter for watermelon peels waste (WW) hydrolysis using Plackett–Burman (PB) design.

Treatments	Responses	Predicted value (g/L)	Experimental value (g/L)	Residual	Error (%)
Acidic hydrolysis	Reducing sugars	29.95	30.46 ± 0.004	0.51	1.70
	Total Sugars	39.37	39.82 ± 0.75	0.45	1.14
Enzymatic hydrolysis	Reducing sugars	26.96	28.62 ± 0.01	1.66	6.15
	Total Sugars	41.30	42.30 ± 0.55	1.00	2.42
Combined hydrolysis	Reducing sugars	25.38	26.30 ± 0.02	0.92	3.62
	Total Sugars	35.41	37.20 ± 0.15	1.79	5.05

Residual = Experimental value – predicted value.
Error (%) = Residual/predicted value ×100.

Table 8. Central composite design (CCD) matrix representing optimized fermentation parameters for ethanol titer and yield responses.

Factors				*Saccharomyces cerevisiae* K7		*Metchnikowia cibodasensis* Y34	
Runs	A: HDL (mL)	B: Time (days)	C: Temperature (°C)	Ethanol contents (g/L)	Ethanol yield (g/g)	Ethanol contents (g/L)	Ethanol yield (g/g)
1	50	8	45.1	2.55 ± 0.01	0.18 ± 0.01	4.5 ± 0.02	0.27 ± 0.04
2	25	1	40	2.14 ± 0.02	0.09 ± 0.02	0.41 ± 0.01	0.13 ± 0.03
3	75	1	40	3.78 ± 0.03	0.27 ± 0.01	4.38 ± 0.04	0.14 ± 0.03
4	25	15	40	1.06 ± 0.01	0.10 ± 0.01	2.90 ± 0.02	0.23 ± 0.02
5	75	15	25	2.17 ± 0.03	0.15 ± 0.01	2.4 ± 0.02	0.14 ± 0.03
6	7.95	8	32.5	2.04 ± 0.01	0.07 ± 0.01	3.0 ± 0.01	0.10 ± 0.01
7	75	1	25	2.32 ± 0.02	0.18 ± 0.01	3.6 ± 0.02	0.20 ± 0.01
8	50	8	32.5	2.60 ± 0.04	0.30 ± 0.03	3.84 ± 0.01	0.33 ± 0.03
9	25	15	25	2.23 ± 0.01	0.32 ± 0.01	3.20 ± 0.01	0.34 ± 0.04
10	50	8	19.8	4.51 ± 0.03	0.35 ± 0.02	4.50 ± 0.03	0.36 ± 0.02
11	50	19.7	32.5	3.53 ± 0.03	0.29 ± 0.01	3.60 ± 0.02	0.27 ± 0.04
12	50	8	32.5	2.08 ± 0.01	0.23 ± 0.02	2.50 ± 0.03	0.23 ± 0.03
13	50	–3.77	32.5	1.36 ± 0.03	0.05 ± 0.01	2.10 ± 0.01	0.10 ± 0.01
14	50	8	32.5	4.59 ± 0.01	0.37 ± 0.03	5.40 ± 0.01	0.40 ± 0.04
15	50	8	32.5	4.22 ± 0.01	0.35 ± 0.01	5.00 ± 0.02	0.39 ± 0.05
16	92.04	8	32.5	3.63 ± 0.03	0.36 ± 0.02	4.60 ± 0.02	0.38 ± 0.03
17	50	8	32.5	3.94 ± 0.04	0.35 ± 0.03	4.80 ± 0.02	0.37 ± 0.04
18	25	1	25	0.16 ± 0.01	0.13 ± 0.04	1.10 ± 0.01	0.11 ± 0.03
19	50	8	32.5	2.40 ± 0.01	0.2 ± 0.02	2.40 ± 0.01	0.21 ± 0.02
20	75	15	40	3.48 ± 0.01	0.30 ± 0.01	3.50 ± 0.02	0.31 ± 0.08

Table 9. Fitted quadratic regression model for various responses in fermentation of watermelon peels waste (WW).

Responses	Yeast isolates	Source	Sum of squares	DF	Mean of square	F value	P value
Ethanol yield	*S. cerevisiaea* K7	Model	0.16	9	0.017	5.07	0.009, significant
		Residual	0.03	10	0.003		
		Lack of fit	0.03	5	0.006	8.62	0.016, significant
		Pure error	0.04	5	0.001		
		Cor total	0.19	19			
	M. cibodasensis Y34	Model	0.13	9	0.014	4.42	0.034, significant
		Residual	0.04	10	0.004		
		Lack of fit	0.04	5	0.007	6.45	0.030, significant
		Pure error	0.06	5	0.001		
		Cor total	0.17	19			
Ethanol titer	*S. cerevisiaea* K7	Model	0.63	9	0.070	2.52	0.083, not significant
		Residual	0.28	10	0.028		
		Lack of fit	0.06	5	0.013	0.29	0.890, not significant
		Pure error	0.22	5	0.043		
		Cor total	0.91	19			
	M. cibodasensis Y34	Model	0.71	9	0.079	4.09	0.019, significant
		Residual	0.19	10	0.019		
		Lack of fit	0.16	5	0.031	4.23	0.069, significant
		Pure error	0.04	5	0.007		
		Cor total	0.90	19			

Table 10. Analysis of variance of responses in fermented hydrolyzate by yeast isolates.

Responses	Yeast isolates	CV	Press	R^2	Adj R^2	Pred R2	Adeq precision
Ethanol yield	*S. cerevisiaea* K7	21.37	0.26	0.82	0.65	0.342	7.21
	M. cibodasensis Y34	22.41	0.29	0.76	0.53	0.673	6.25
Ethanol titer	*S. cerevisiaea* K7	33.6	1.24	0.78	0.59	0.371	7.28
	M. cibodasensis Y34	0.41	41.12	0.69	0.42	0.114	6.47

Figure 2B, an increasing response was observed for 62.5 mL hydrolyzate, followed by a slight decrease. Temperature had no effect on response with hydrolyzate. A sharp increase was observed with increase in incubation temperature and time (Figure 2C).

Productivity of ethanol contents in watermelon waste

Figure 3 shows the ethanol yield and titer of both isolates with enzymatic hydrlozate (75%) of WW at 40°C for 8 days. Both strains that showed an increase for both responses interpreted the tolerance of yeast to ethanol.

Discussion

With increased environmental destruction linked to combustion of fossil fuels, alternate biofuels have drawn global attention. Specifically, bioethanol is regarded eco-friendly, ensuring an ecologically sound future. Evaluating in terms of its cost, at present, bioethanol costs approximately US$0.5/L while utilizing first-generation (1G) feedstock (e.g., sugar/starch-based crops). According to the studies conducted in the United States and Brazil, its cost increased by 10x with second-generation (2G) substrates (e.g., lignocellulosic waste biomass). Compared to 2G substrates, cost of bioethanol utilizing 1G ethanologenesis from sugar/starch feedstocks appears remunerative; however, 1G feedstock competes with food items that may lead to hunger, augmenting other problems. Therefore, production of bioethanol using 2G cost-effective and environment-friendly processes are regarded as more positive potentially (Obiora, 2022).

Xylan is discovered in nature as a heterogeneous compound. Complex enzyme systems are required for its breakdown. Microbe-derived enzymes convert xylan into its monomers in an organized manner. A variety of enzymes for breakdown of hemicellulose are present in the environment. Xylan backbones are cleaved at their reducing ends by exo-xylanases to form xylose and short xylo-oligomers (Fushinobu *et al.*, 2005; Ganju *et al.*, 1989; Honda and Kitaoka, 2004; Juturu *et al.*, 2014; Kubata *et al.*, 1994; Kubata *et al.*, 1995; Usui *et al.*, 1999; Tenkanen *et al.*, 2013).

The current study dealt with xylanolytic potential of *B. cereus* XG2 utilizing watermelon peels. The hemicellulosic biomass was hydrolyzed and transformed into xylose. *Bacillus* was identified as one of the possible producer of xylanases among bacteria. Several bacilli with effective xylanolytic activity have been reported, including *Bacillus circulans*, *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus halodurans* (Banka *et al.*, 2014; Gupta *et al.*, 2015; Subramaniyan and Prema, 2002; Thomas *et al.*, 2014). *Bacillus* species, *Stenotrophomonas maltophila* and *Rhodothermus marinus*, Thermotoga species, *Clostridium thermocellum*, and Streptomyces species harbor thermo stable xylanases that are active at temperatures as high as 60–70°C (Kumar and Satyanarayana, 2014; Raj *et al.*, 2013; Thomas *et al.*, 2014). Bacteria is not able to ferment xylose into xylitol. Bacterial xylose isomerases transform xylose into xylulose. Both Embden–Meyerhof–Parnas (EMP) pathway and pentose phosphate pathway convert xylulose to ethanol (Gupta *et al.*, 2019).

In the current study, *B. cereus* XG2 worked efficiently at pH 6. Bacterial xylanases are synthesized at alkaline pH, while fungal xylanases work effectively in acidic conditions. The results of the current study differed from this findings and presented novel characteristics. The biochemical composition of dried WW was analyzed as follows: moisture (0.74±0.05%), hemicellulose (17.20±2.30%), lignin (15.51±0.22%), and cellulose (52.26±0.33). The reducing and total sugars were 16.7±0.05 g/L and 28.7±0.04 g/L, respectively. *B. cereus* XG2 xylanolytic potential was evaluated with WW rinds using PB design. Chaudhary *et al.* (2023) had reported 0.226±0.011 µmol/min/mL xylanolytic potential of *B. cereus* XG2 to convert xylan into xylose.

Watermelon waste was saccharified chemically by dilute sulfuric acid for different parameters employing PB design. The highest predicted and experimental values were 29.95, 30.46±0.004 g/L (reducing sugars) and 39.37, 39.82±0.75 g/L (total sugars). The optimized parameters were 6% sulfuric acid, 100°C temperature, and 30 min optimum time. Arumugam and Manikandan (2011) had reported varied values of reducing sugars: 36.67% (banana) and 21.68% (mango). Acidic saccharification

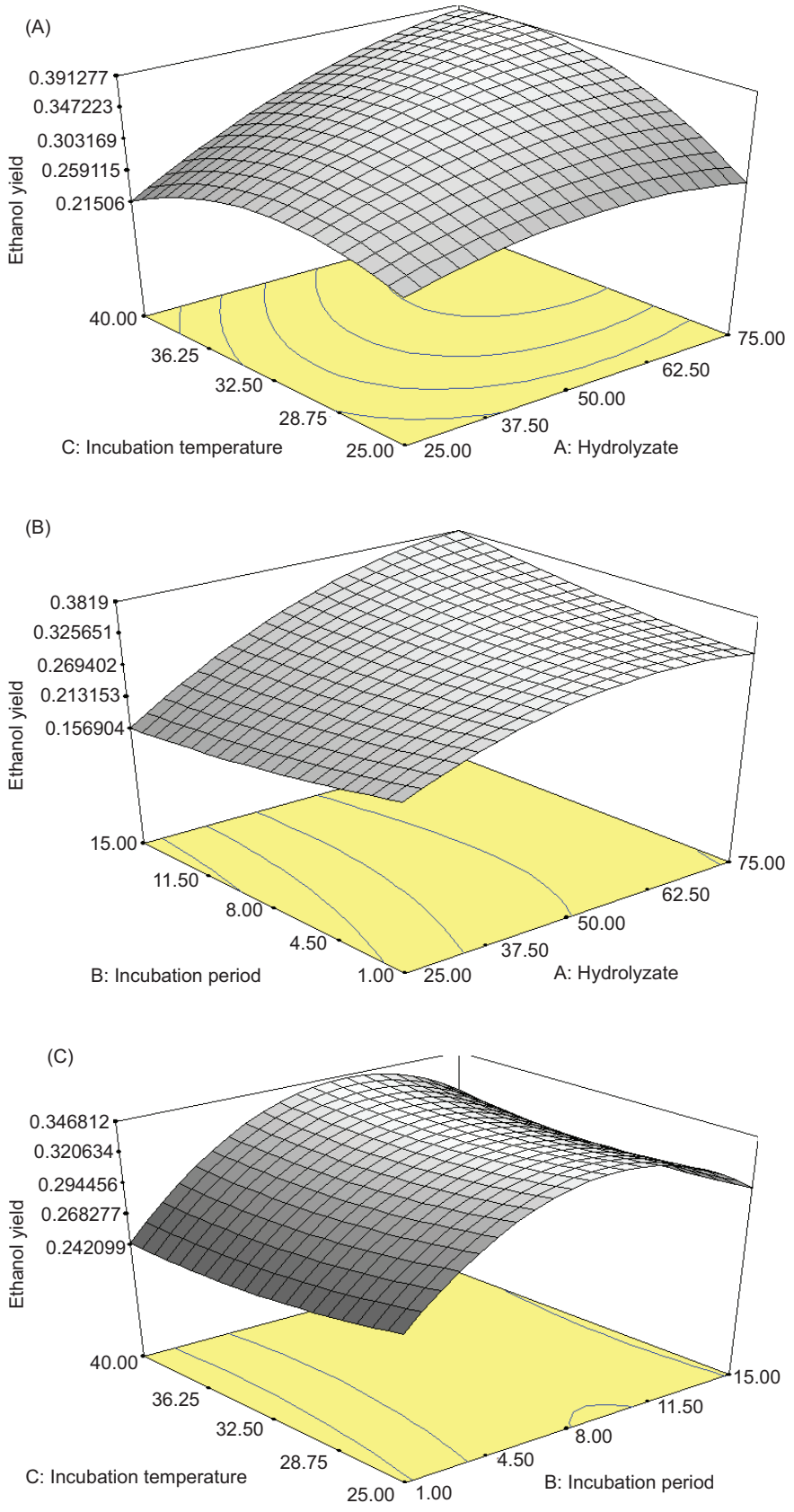

Figure 1. Presentation of 3D surface graphs for ethanol yield in central composite design (CCD) for *S. cerevisiae* K7 yeast isolate indicated by the interactions of hydrolyzate, incubation temperature, and incubation time (A–C).

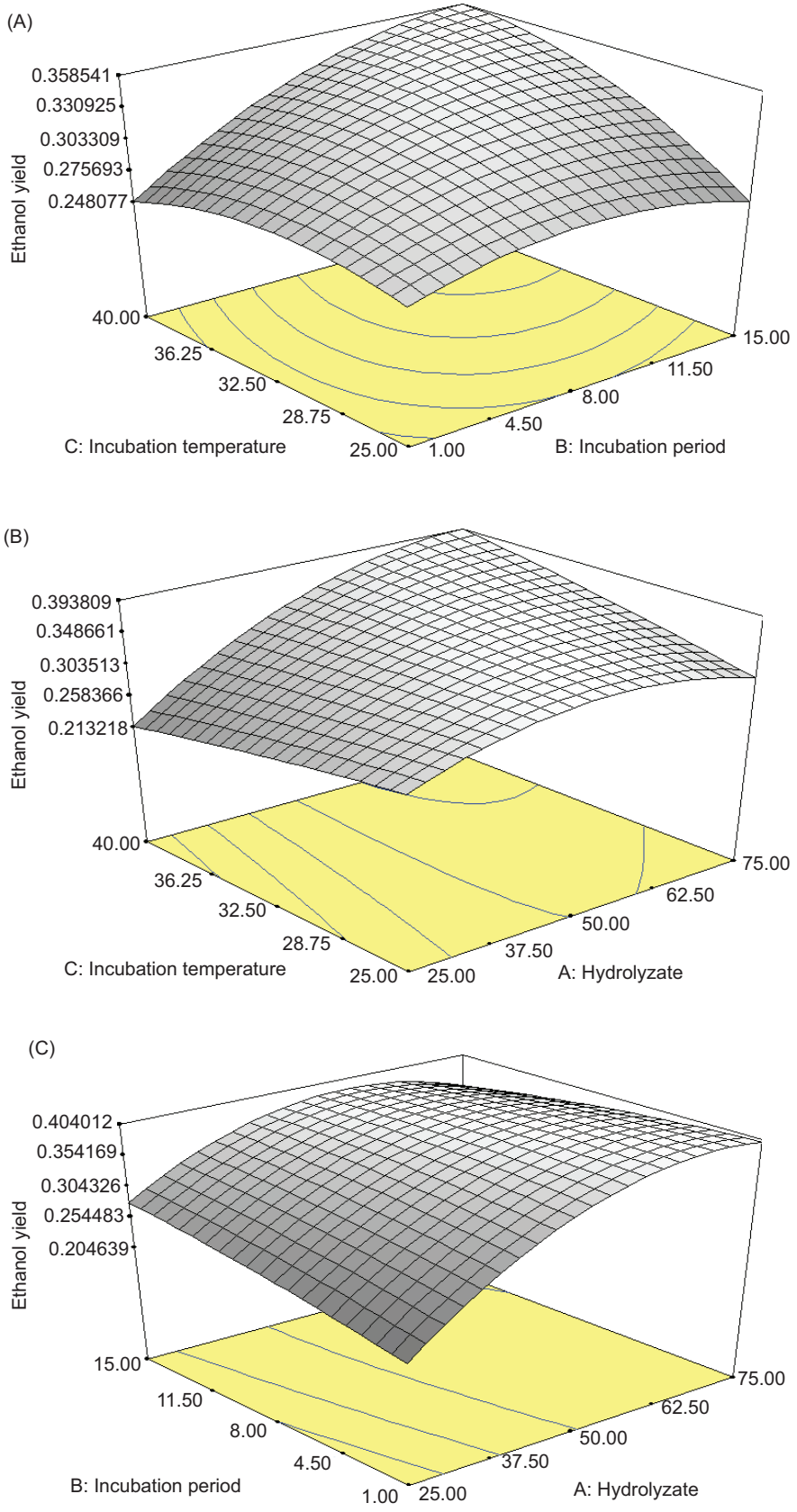

Figure 2. Presentation of 3D surface graphs for ethanol yield in central composite design (CCD) for *Metchnikowia cibodasensis* Y34 yeast isolate indicated by the interactions of hydrolyzate, incubation temperature, and incubation time (A–C).

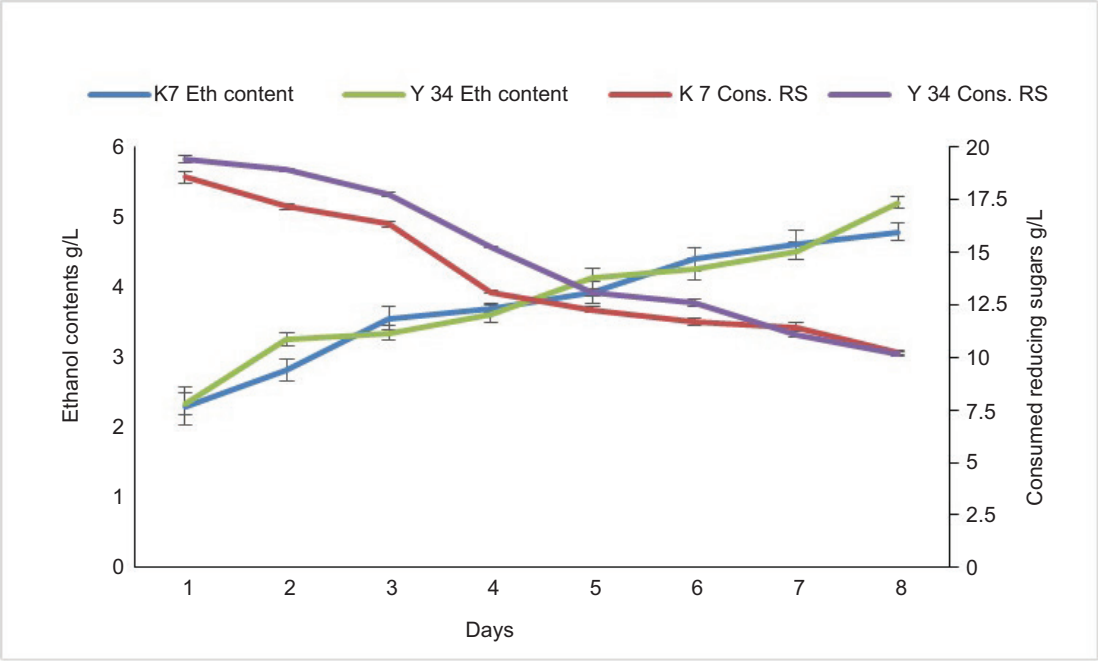

Figure 3. Correlation of ethanol titer (g/L) and consumed reducing sugars (g/L) under optimized conditions elucidated by central composite design (CCD) from *S. cerevisiae* K7 and *M. cibodasensis* Y34 up to 8 days.

not only changes hemicellulose to monomers but also affects the structure of lignocellulose to make it readily accessible to enzymes (Loow *et al.*, 2016; Toquero and Bolado, 2014).

In case of enzymatic hydrolysis of WW, maximum predicted and experimental values for reducing sugars were 26.96, 28.62±0.0007 g/L, and for total sugars, 41.3, 42.3±0.55 g/L. Optimum values were attained in 5 days, at a temperature of 55°C with 9.17-µmol enzyme dose. High enzyme load (9.17 µmol) was reported in the present study for WW hydrolysis. These findings were in contrary to the values reported by Chaudhary *et al.* (2023), that is, 0.917±0.059 µmol/min/mL and 0.817±0.036 µmol/min/mL for bacterial isolates *Bacillus cereus* XG2 and *Enterococcus faecium* XA2, respectively. The xylanolytic potential of termite gut-associated *Candida pseudorhagii* was reported as 1.73 U/mL and 0.98 U/mL (Ali *et al.*, 2017).

Watermelon waste was exposed to combined acidic and enzymatic treatment. The conditions for optimum response were as follows: pH 6, temperature 65°C, enzyme dose, 9.17 µmol/mL/min, and sulfuric acid hydrolyzate. Optimal predicted and experimental reducing sugars were 25.38 g/L and 26.3±0.020 g/L, and the corresponding values for total sugars were 35.4 g/L and 37.2±0.15 g/L. In this treatment, acid hydrolyzate was meant for further hydrolysis by bacterial xylanases. Less sugars were released, compared to enzymatic treatment. Seneesrisakul *et al.* (2017) determined that glucose in the culture medium lowered endoglucanase activity.

The fermentation parameters were optimized by CCD model. The optimized parameters were as follows: enzymatic hydrolysate, 50 mL; synthetic media, 45 mL; temperature, 32.5°C; and incubation time, 8 days. *S. cerevisiae* K7 was used as standard yeast with an optimal yield of 0.37±0.026 g/g. *M. cibodasensis* Y34 was the experimental yeast with an optimal yield of 0.4±0.039 g/g. The present values corroborated the findings of Chaudhary *et al.* (2022), that is, ethanol yield of 0.36±0.02 g/g with *S. cerevisiae* K7 and 0.40±0.01 g/g by *M. cibodasensis* Y34 using WW.

Conclusion

The study discovered that optimum reducing sugars in enzymatic hydrolyzate were 28.62±0.007 g/L determined after 5 days, with 9.17 µmol/min/mL crude enzyme, at pH 6 and temperature 65°C. The maximum ethanol yield of 0.4±0.0035 g/g was estimated with *Metchnikowia cibodasenis* Y34, using 50 mL of enzymatic hydrolyzate at 32.5°C for 8 days. This yeast was assumed to have a promising potential for converting fruit waste into bioethanol.

Recommendations

A comparative study of hydrolysis and fermentation of WW offers an alternative method for waste processing and management. The processed waste serves as raw materials and source/substrate for the production of biofuel. This work could be extended to batch and continuous fermentation. The fermentors could be designed for the production of bioethanol on a commercial scale.

Conflict of Interest

The authors declare no conflict of interest.

Conceptualization, Asma Chaudhary; **Methodology**, Ayesha Aihetasham; **Software**, Smavia Younas; **Validation**, Nimra Basheer; **formal analysis**, Nageen Hussain, **investigation**, Asma Chaudhary; **Resources**, Tariq Aziz; data curation, Sumaira Naz.; writing—original draft preparation, Smavia Younas.; **writing—review and editing**, Thamer H Albekairi; **visualization**, Nimra Basheer; **Supervision**, Asma Chaudhary and Nageen Hussain.; **project administration**, Tariq Aziz

Funding

No external funding was received.

Acknowledgments

The authors express their gratitude to the Research Supporting Program (RSPD2024R568) of King Saud University Riyadh Saudi Arabia.

References

Abu-Gharbia M.A., El-Sawy N.M., Nasr A.M. and Zedan L.A. 2018. Isolation, optimization and characterization of cellulases and hemicellulases from Bacillus cereus LAZ 518 isolated from cow dung using corn cobs as lignocellulosic waste. J Pharm Appl Chem. 4(1): 1–13. https://doi.org/10.18576/jpac/040201

Alex S., Saira A., Nair D.S., Soni K.B., Sreekantan L., Rajmohan K., et al. 2017. Bioethanol production from watermelon rind by fermentation using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Indian J Biotechnol. 16(4): 663–666. https://api.semanticscholar.org/CorpusID:91263801.

Ali S.S., Wu J., Xie R., Zhou F., Sun J. and Huang M. 2017. Screening and characterizing of xylanolytic and xylose-fermenting yeasts isolated from the wood-feeding termite, Reticulitermes chinensis. PLoS One, 12(7): e0181141. https://doi.org/10.1371/journal.pone.0181141

Alvira P., Tomas-Pejo E., Ballesteros M. and Negro M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol. 101(13): 4851–4861. https://doi.org/10.1016/j.biortech.2009.11.093

Annamalai N., Thavasi R., Jayalakshmi S. and Balasubramanian T. 2009. Thermostable and alkaline tolerant xylanase production by Bacillus subtilis isolated form marine environment. Indian J Biotech. 8(3): 291–297. https://api.semanticscholar.org/CorpusID:53466911

Arumugam R. and Manikandan M. 2011. Fermentation of pretreated hydrolyzates of banana and mango fruit wastes for ethanol production. Asian J Exp Biol Sci. 2(2): 246–256.

Association of Official Analytical Chemists (AOAC). 2012. Official Methods of Analysis of AOAC International, 18th Ed. AOAC, Gaithersburg, MD.

Azhar S., Henriksson G., Theliander H. and Lindström M.E. 2015. Extraction of hemicelluloses from fiberized spruce wood. Carbe Polym. 117: 19–24. https://doi.org/10.1016/j.carbpol.2014.09.050

Aziz T, Shah Z, Sarwar A, Ullah N, Khan AA, Sameeh MY, et al. 2023. Production of bioethanol from pretreated rice straw, an integrated and mediated upstream fermentation process. Biomass Conv. Bioref. 1–11. https://doi.org/10.1007/s13399-023-04283-w

Bai Y., Huang H., Meng K., Shi P., Yang P., Luo, H., et al. 2012. Identification of an acidic α-amylase from Alicyclobacillus sp. A4 and assessment of its application in the starch industry. Food Chem. 131(4): 1473–1478. https://doi.org/10.1016/j.foodchem.2011.10.036

Bajpai P. 2016. Structure of Lignocellulosic Biomass, In: Bajpai, P. (Ed.), Pretreatment of Lignocellulosic Biomass for Biofuel Production, Springer Briefs in Molecular Science. Springer Singapore, Singapore, pp. 7–12. https://doi.org/10.1007/978-981-10-0687-6_2

Banka A.L., Albayrak Guralp S. and Gulari E. 2014. Secretory expression and characterization of two hemicellulases, xylanase, and β-xylosidase, isolated from *Bacillus subtilis* M015. Appl Biochem Biotechnol. 174: 2702–2710. https://doi.org/10.1007/s12010-014-1219-1

Bennett G.A., Lagoda A.A., Shotwell O.L. and Hesseltine C.W. 1981. Utilization of zearalenone-contaminated corn for ethanol production. J Am Oil Chem Soc. 58(11): 974–976. https://doi.org/10.1007/BF02659774

Bouacem K., Bouanane-Darenfed A., Boucherba N., Joseph M., Gagaoua M., Ben Hania W., et al. 2014. Partial characterization of xylanase produced by *Caldicoprobacter algeriensis*, a new thermophilic anaerobic bacterium isolated from an Algerian hot spring. Appl Biochem Biotechnol. 174: 1969–1981. https://doi.org/10.1007/s12010-014-1153-2

Bradner J.R., Sidhu R.K., Gillings M. and Nevalainen K.M.H. 1999. Hemicellulase activity of antarctic microfungi. J App Microbiol. 87(3): 366–370. https://doi.org/10.1046/j.1365-2672.1999.00827.x

Camelia B., Cristiana T. and Gabriela, B. 2010. Yeast isolation and selection for bioethanol production from inulin hydrolysates.

Innov Rom Food Biotechnol. (6): 29–34. https://www.gup.ugal.ro/ugaljournals/index.php/IFRB/article/view/3350

Chakdar H., Kumar M., Pandiyan K., Singh A., Nanjappan K., Kashyap P.L., et al. 2016. Bacterial xylanases: biology to biotechnology. 3 Biotech. 6: 1–15. https://doi.org/10.1007/s13205-016-0457-z

Chaudhary A. and Karita S. 2017. Screening of yeast isolates from flowers for effective ethanol production. Turk J Biol. 41(6): 890–900. https://doi.org/10.3906/biy-1704-7

Chaudhary A., Hussain I., Ahmad Q.A, Hussain Z., Akram A.M. and Hussain A. 2022. Efficient utilization of melon peels to produce ethanol: a step toward sustainable waste management. Biomass Convers Bioref. 14: 3463–3475. https://doi.org/10.1007/s13399-022-02687-8

Chaudhary A., Hussain A., Shehzadi A., Manzoor M., Shahbaz M. and Deepanraj B. 2023. Production of ethanol from xylan by indigenous xylanolytic and ethanologenic bacteria isolated from fruit wastes. Sustain Energy Technol Assessments. 57: 103216. https://doi.org/10.1016/j.seta.2023.103216

Chen W., Chen Y., Yang H., Xia M., Li K., Chen X., et al. 2017. Co-pyrolysis of lignocellulosic biomass and microalgae: products characteristics and interaction effect. Bioresour Technol. 245: 860–868. https://doi.org/10.1016/j.biortech.2017.09.022

Dubois M., Gilles K.A., Hamilton J.K., Rebers P.T. and Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem. 28(3): 350–356. https://doi.org/10.1021/ac60111a017

Fushinobu S., Hidaka M., Honda Y., Wakagi T., Shoun H. and Kitaoka, M. 2005. Structural basis for the specificity of the reducing end xylose-releasing exo-oligoxylanase from *Bacillus halodurans* C-125. Journal of Biological Chem. 280(17): 17180–17186. https://doi.org/10.1074/jbc.M413693200

Ganju R.K., Vithayathil P.J. and Murthy S.K. 1989. Purification and characterization of two xylanases from *Chaetomium thermophile* var. coprophile. Can J Microbiol. 35(9): 836–842. https://doi.org/10.1139/m89-140

Guerriero G., Hausman J.F., Strauss J., Ertan H. and Siddiqui K.S. 2016. Lignocellulosic biomass: biosynthesis, degradation, and industrial utilization. Eng Life Sci. 16(1): 1–16. https://doi.org/10.1002/elsc.201400196

Gupta A., Ahmad A., Chothwe D., Madhu M.K., Srivastava S. and Sharma V.K. 2019. Genome-scale metabolic reconstruction and metabolic versatility of an obligate methanotroph *Methylococcus capsulatus* str. Bath. Peer J. 7: e6685. https://doi.org/10.7717/peerj.6685

Gupta V., Garg S., Capalash N., Gupta N. and Sharma P. 2015. Production of thermo-alkali-stable laccase and xylanase by co-culturing of Bacillus sp. and *B. halodurans* for bio bleaching of kraft pulp and deinking of waste paper. Bioproc Biosyst Eng. 38(5): 947–956. https://doi.org/10.1007/s00449-014-1340-0

Honda Y. and Kitaoka M. 2004. A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. J Biol Chem. 279(53): 55097–55103. https://doi.org/10.1074/jbc.M409832200

Huang X., Li Z., Du C., Wang J. and Li S. 2015. Improved expression and characterization of a multi domain xylanase from *Thermoanaerobacterium aotearoense* SCUT27 in *Bacillus subtilis*. J Agric Food Chem. 63(28): 6430–6439. https://doi.org/10.1021/acs.jafc.5b01259

Isikgor F.H. and Becer C.R. 2015. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polymer Chem. 6(25): 4497–4559. https://doi.org/10.1039/C5PY00263J

Juturu V., Teh T.M. and Wu J.C. 2014. Expression of *Aeromonas punctata* ME-1 exo-xylanase X in E. coli for efficient hydrolysis of xylan to xylose. Appl Biochem Biotech. 174: 2653–2662. https://doi.org/10.1007/s12010-014-1216-4

Kamble R.D. and Jadhav A.R. 2012. Isolation, purification, and characterization of xylanase produced by a new species of Bacillus in solid state fermentation. Int J Microbiol. 2012: 683193. https://doi.org/10.1155/2012/683193

Kassim M.A., Hussin A.H., Meng T.K., Kamaludin R., Zaki M.S.I.M. and Zakaria W.Z.E.W. 2022. Valorisation of watermelon (*Citrullus lanatus*) rind waste into bioethanol: An optimization and kinetic studies. Int J Environ Sci Technol. 19: 2545–2558. https://doi.org/10.1007/s13762-021-03310-5

Khan S., Nisar A., Wu B., Zhu Q.L., Wang Y.W., Hu G.Q., et al. 2022. Bioenergy production in Pakistan: potential, progress, and prospect. Sci Total Environ. 814: 152872. https://doi.org/10.1016/j.scitotenv.2021.152872

Kubata B.K., Suzuki T., Horitsu H., Kawai K. and Takamizawa K. 1994. Purification and characterization of Aeromonas caviae ME-1 xylanase V, which produces exclusively xylobiose from xylan. Appl Environ Microbiol. 60(2): 531–535. https://doi.org/10.1128/aem.60.2.531-535.1994

Kubata B.K., Takamizawa K., Kawai K., Suzuki T. and Horitsu, H. 1995. Xylanase IV, an endoxylanase of *Aeromonas caviae* ME- 1 which produces xylotetraose as the only low molecular weight oligosaccharide from xylan. Appl Environ Microbiol. 61: 1666–1668. https://doi.org/10.1128/aem.61.4.1666-1668.1995

Kumar V. and Satyanarayana T. 2014. Production of endoxylanase with enhanced thermostability by a novel polyextremophilic *Bacillus halodurans* TSEV1 and its applicability in waste paper deinking. Proc Biochem. 49(3): 386–394. https://doi.org/10.1016/j.procbio.2013.12.005

Kumar R., Singh S. and Singh O.V. 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol. 35(5): 377–391. https://doi.org/10.1007/s10295-008-0327-8

Li Z, Tian-Tian L, Aziz T, Min Z, Sarwar A, Zhennai Y, et al. 2023. Purification of Galacto-oligosaccharide (GOS) by fermentation with Kluyveromyces lactis and Interaction between GOS and casein under simulated acidic fermentation conditions. World J Microbiol Biotechnol.;39(12):342. https://doi.org/10.1007/s11274-023-03791-1

Lin L., Yan R., Liu Y. and Jiang W. 2010. In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: cellulose, hemicellulose and lignin. Bioresour Technol. 101(21): 8217–8223. https://doi.org/10.1016/j.biortech.2010.05.084

Loow Y.L., Wu T.Y., Md. Jahim J., Mohammad A.W. and Teoh W.H. 2016. Typical conversion of lignocellulosic biomass into reducing sugars using dilute acid hydrolysis and alkaline pretreatment. Cellulose. 23: 1491–1520. https://doi.org/10.1007/s10570-016-0936-8

Loow Y.L., Wu T.Y., Tan K.A., Lim Y.S., Siow L.F., Md. Jahim J., et al. 2015. Recent advances in the application of inorganic salt pretreatment for transforming lignocellulosic biomass into reducing sugars. J Agric Food Chem. 63(38): 8349–8363. https://doi.org/10.1021/acs.jafc.5b01813

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193: 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Narjis K, Najeeb U, Abid S, Tariq A, Metab A, Abdulrahman A. 2023. Isolation and Identification of Protease Producing Bacillus Strain from Cold Climate Soil and Optimization of its Production by applying Different Fermentation Conditions. Appl Ecol Environ Res. 21(4):3391-3401. http://dx.doi.org/10.15666/aeer/2104_33913401 .

Miller G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 31(3): 426–428. https://doi.org/10.1021/ac60147a030

Obiora C. 2022. Optimal cost of production of bioethanol: a review. Available at SSRN: https://ssrn.com/abstract=4171036 or http://dx.doi.org/10.2139/ssrn.4171036. https://doi.org/10.2139/ssrn.4171036

Raj A., Kumar S. and Singh S.K. 2013. A highly thermostable xylanase from *Stenotrophomonas maltophilia*: purification and partial characterization. Enzyme Res. 2013: 429305. https://doi.org/10.1155/2013/429305

Rashid R. and Sohail M. 2021. Xylanolytic Bacillus species for xylooligosaccharides production: a critical review. Bioresourc Bioprocess. 8(1): 1–14. https://doi.org/10.1186/s40643-021-00369-3

Saleem A., Hussain A., Chaudhary A., Ahmad Q.U.A., Iqtedar M., Javid A., et al. 2020. Acid hydrolysis optimization of pomegranate peels waste using response surface methodology for ethanol production. Biomass Conv Bioref. 12: 1513–1524. https://doi.org/10.1007/s13399-020-01117-x

Seneesrisakul K., Guralp S.A., Gulari E. and Chavadej S. 2017. *Escherichia coli* expressing endoglucanase gene from Thai higher termite bacteria for enzymatic and microbial hydrolysis of cellulosic materials. Elect J Biotechnol. 27: 70–79. https://doi.org/10.1016/j.ejbt.2017.03.009

Shah TA, Majeed T, Rahman SU, Ihsan T, Aziz T, Alharbi M, et al. 2023. Synergistic treatment of crude enzymes from Bacillus sp. strains to boost anaerobic fermentation of rice straw. Biomass Conv. Bioref. 1-10. https://doi.org/10.1007/s13399-023-05090-z

Subramaniyan S. and Prema P. 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit Rev Biotechnol. 22(1): 33–64. https://doi.org/10.1080/07388550290789450

Tenkanen M., Vršanská M., Siika-aho M., Wong D.W., Puchart V., Penttilä M., et al., 2013. Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity. FEBS J. 280(1): 285–301. https://doi.org/10.1111/febs.12069

Thomas L., Ushasree M.V. and Pandey A. 2014. An alkalithermostable xylanase from *Bacillus pumilus* functionally expressed in *Kluyveromyces lactis* and evaluation of its deinking efficiency. Bioresour Technol. 165: 309–313. https://doi.org/10.1016/j.biortech.2014.03.037

Toquero C. and Bolado S. 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour Technol. 157: 68–76. https://doi.org/10.1016/j.biortech.2014.01.090

Usui K., Ibata K., Suzuki T. and Kawai, K. 1999. XynX, a possible exo-xylanase of Aeromonas caviae ME-1 that produces exclusively xylobiose and xylotetraose from xylan. Biosci biotechnol biochem. 63(8): 1346–1352. https://doi.org/10.1271/bbb.63.1346

Ullah, N., Mujaddad-ur-Rehman, M., Sarwar, A., Nadeem M., Nelofar R., Irfan M., et al. 2023. . Effect of bioprocess parameters on alkaline protease production by locally isolated Bacillus cereus AUST-7 using tannery waste in submerged fermentation. Biomass Conv. Bioref. 1-10. https://doi.org/10.1007/s13399-023-04498-x

Zöllner N. and Kirsch K. 1962. Über die quantitative Bestimmung von Lipoiden (Mikromethode) mittels der vielen natürlichen Lipoiden (allen bekannten Plasmalipoiden) gemeinsamen Sulfophosphovanillin-Reaktion. Z Gesamte Exp Med. 135: 545–561. https://doi.org/10.1007/BF02045455