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STRATEGIC OPTIMIZATION OF ETHANOL PRODUCTION FROM WATERMELON-RIND WASTE: INTEGRATING ADVANCED MANAGEMENT PRACTICES WITH SEPARATE HYDROLYSIS AND FERMENTATION

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ABSTRACT

Prospectively, this study on converting watermelon-rind waste (WW) into bioethanol is extremely relevant as it accounts the critical issues of waste management especially in agricultural countries like Pakistan and their subsequent utilization for renewable energy production, thus mitigating pollution and favoring energy security. The study using the WW, if implemented at large, can support to manage massive waste–to-valued product. In WW, the reducing sugar contents of 15.9±0.05%, total carbohydrates 28.8±0.05%, total lipid 3.3±0.03% and total protein 3.5±0.03% were calculated. Separate hydrolysis and fermentation (SHF) method was selected here for ethanologenesis. For WW hydrolysis, three saccharification techniques were employed i.e., diluted sulfuric acid, enzymatic using *Enterococcus faecium* XA2 xylanases, and a combination of acidic and enzymatic hydrolysis. Here enzymatic hydrolysis along with dilute sulfuric acid were selected for their effectiveness in complex polysugars into fermentable monosugars while minimizing toxic metabolites release for subsequent fermentation processes. Based on significance elucidated by Placket Burmen (PB) design, combined was selected for subsequent experiment which released 30.43±0.51 g/L reducing sugar and 46.95±0.10 g/L total sugar were recorded. Central composite design (CCD) based optimization for ethanol yield and titer on saccharified WW hydrolyzates were performed. The maximum ethanol yield 0.38±0.1g/g with *Metchnikowia cibodasensis* Y34 was obtained at 32.5ºC with 50% combined treated hydrolyzate of WW upto incubation of eight days. It has been found that SHF can be a valuable strategy to increase the possibility of conversion of hemicellulose to fermentable sugars and in turn into bioethanol production at large. The study stands-out by integrating a novel combination of xylanolytic and ethanologenic microbes using WW leveraging cutting-edge microbial efficiencies that are significantly potent in bioconversions compared to conventional methodologies.

KEYWORD: Bioethanol production, *Enterococcus faeceum*, Saccharification strategies, *Saccharomyces cerevisiae*, Separate hydrolysis and fermentation, Xylanasess

INTRODUCTION

Worldwide, the food-waste crisis is significant, approximating 1.3 billion tons food resources are wasted per annum that accounts one-third of food produced. Contextually, bioethanol production from these food wastes e.g. WW can greatly decrease the waste but also provide an eco-friendly, low-cost value-addition in the form of green energy resource. According to an estimate, production of bioethanol using agri-waste residues like WW can form upto 200 million gallons of this liquid biofuel per year that can reduce GHG emission by approximately 1.5 million tons of carbon dioxide equivalent.[1] However, majority of extraction procedures, when involve chemical techniques, mostly result in more waste generation. Therefore, optimizing the extraction procedures is crucial in maximizing the fermentable sugars and subsequent bioethanol yield. This can minimize the waste generation and ensure the process development to be economically feasible, viable and eco-friendly.

Lignocellulosic biomass (LCB) wastes, principally obtained from agricultural residues, forestry by-products and industrial processes, embody significant global concern. It contains waste such as sugarcane bagasse, wheat straw, corn-stover, wood-chips, and other LCB that is rich in C5 and C6 sugar polymers. The volume of LCB generated waste is enormous, with agricultural residues alone accounts for approximately 140 billion metric tons per annum.

Despite the potential consumption of this waste into value-added products (e.g. bioenergy), much of this LCB waste remains under-utilized that alleviate environmental and social concerns associated with its improper disposal.[2] LCB comprising of sugar polymers i.e. cellulose, hemicelluloses that are fenced by lignin, poses itself as cost-effective, sustainable and renewable feedstock for energy production. Among these, production of second-generation (2G) bio-based valued chemicals (e.g. biofuels) is at its beginning, thought more promising are considered the first generation (1G) feedstock which when used to produce energy, consequence to concede worldwide food security concerns.[3] The consistent use of lignocellulosic biomass (LCB) for bio-based fuels is primarily hindered by its recalcitrance, which makes processing difficult and prevents efficient breakdown into fermentable C5 and C6 sugars.[4] Addressing this resistance requires a combination of chemical, thermal, enzymatic, and microbiological pretreatments, leading to substantial economic inputs.[5] In this regard, pretreatment is a vital step that disrupts the resistant structure of LCB by breaking lignin bonds and lowering the polymerization of cellulose and hemicellulose.[6,7]

In LCB, hemicellulose is amorphous component with little physical strength. Also, in comparison of degree of polymerization of cellulose, hemicelluloses pose less trouble, as it ranges from 100 to 200 units. Xylan, the main hemicellulose found in LCB, is a sugar polymer with repeating β1, 4 xylose monomers as its backbone. Its reducing end sequence contains xylose, rhamnose, and galacturonic acid, which differ depending on the acetyl/methyl glucuronic acid side groups found in various LCB sources.[8] Pakistan cultivates abundant quantities of watermelon with rind that accounts approximately 30-40% of fruit's weight. Annually its production exceeds 850,000 metric tons, resulting about 255,000-340,000 metric tons WW offering for bioethanol production.[9] Several protocols have been explored for the optimum extraction/hydrolysis of xylan into monosugars especially xylose. Acid hydrolysis, alkaline/peroxide extraction, microwave treatment, vapor treatment, high temperature extraction and ionic liquid extraction are examples of established techniques. Generally, mild acid hydrolysis using 0.5%–1% sulfuric acid/hydrochloric acid has been discovered to be a potentially useful method of making hemicellulose susceptible to future hydrolyzing enzyme exposure. Therefore, a synergistic effect of acid and enzymes is required for the best hemicellulose breakdown.[10] In this regard, by breaking down the xylosidic bonds in complex xylan-rich LCB, xylanases presented themselves to be a potentially useful class of enzymes. Microorganisms are often used to produce xylanases for a variety of commercial and industrial uses. Recently, xylanases have drawn a lot of industrial attention for a variety of applications, including the production of food and beverages, animal nutrition, wood pulp bioleaching, bioenergy, chemicals, and pharmaceuticals.[11]

Methodologies e.g. microbial cellulases and xylanases exploitation for LCB degradation have been explored increasingly due to their potential efficiency in converting complex polysaccharides into simple fermentable monosugars. They allow not only improved sugar release from cellulose/xylan feedstocks, in comparison with harmful chemical methods, but also cause better ethanolgenic yield due to less toxic metabolites release.[1] It is crucial to integrate biofuels like bioethanol into our energy system in light of the current global energy crisis. In this regard, producing ethanol through microbial fermentation using fruit wastes as LCB, can be viewed as very beneficial and cost effective in agricultural states such as Pakistan. One of the most effective liquid biofuels available today, ethanol has the potential to replace conventional fuels that are running low. When combined with gasoline, ethanol improves fuel economy and reduces greenhouse gas (GHG) emissions by roughly 40–50%.[12] Xylanases that can convert hemicelluloses into xylose have been found in a variety of bacterial species. *Bacillus amyloliquefaciens, B. halodurans*, *Thermomonospora fusca* and *B. subtilis* are a few examples.[13] High-value products are produced using the xylanases that are harvested from these bacteria in a variety of bioprocesses. However, using commercially available enzymes for these production procedures results in enormous input costs that significantly raise production costs.[11]

As the 18th largest global producer of watermelon, Pakistan cultivates 2.41 million tons annually, resulting in approximately 540,000 metric tons of waste that requires proper management. Converting this waste into bioethanol offers a dual benefit: reducing environmental pollution from waste dumping and generating economic profit.[1] Improving cellulose-to-ethanol conversion techniques is vital for advancing the bio economy. To apply this technology industrially, hemicellulose must be processed alongside cellulose. Therefore, screening microbial strains (bacteria and yeast) capable of fermenting both pentose and hexose sugars is required for an economical consolidated bioprocess. Although sugar conversion follows biomass hydrolysis (releasing monosugars like xylose), and many yeasts show strong ethanol-producing capabilities, natural yeast strains rarely exhibit both ethanologenic and xylanolytic traits.[1]

In the bioethanol sector, an efficient fermentation process on hemicellulosic substrate is critical which is still an intractable issue and seeking new solutions. This study aims to investigate locally isolated xylanolytic bacterial strains for the valorization of second-generation (2G) watermelon rind waste (WW), a readily available agro-industrial byproduct. The authors have developed statistical models and comparative methods to identify the most effective hydrolysis procedures to maximize the amount of sugar produced from WW. For this purpose, two bacteria *Bacillus cereus* XG2 and *Enterococcus faecium* XA2 with accession numbers OM970803, OM971654 respectively with best xyanolytic potential were exploited for the saccharification of WW employing statistical

models.[11] Previous research showed that bacteria belonging to the genera Bacillus and Enterobacter are employed extensively because they can produce enzymes and metabolize a variety of substrates.[14] Therefore, WW was saccharified using *Bacillus cereus* XG2 where high temperature, pH and saccharification time (65 ˚C, 9 pH, 5 days for combined treatment) were required to obtain the maximum release of sugars.[15] *Enterococcus faecium* XA2 was selected for enzymatic/combined (acidic and enzymatic) hydrolysis because of its well-established capacity to degrade xylan and to get best set of saccharification parameters by our research group. For ethanol fermentation experiments, two yeast isolates-*Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34-were selected based on their demonstrated high ethanol productivity in a previous study.[16] To enhance the sustainable and economic valorization of watermelon peel waste into ethanol, this study investigated the targeted selection of xylanolytic and ethanologenic microbial strains, employing separate hydrolysis and fermentation (SHF) procedures.

METHODS

Water melon, the most delicious and cheapest fruit being consumed in summer in Pakistan served as raw substrate for the study. The WW were collected from local market, washed properly to process for drying at 60 ˚C. Substrate was pretreated using milling technique. Fine particle size (1 mm) was attained using grinding technique followed by sieving. Storage of dried substrate was done in air tight jars. The processed WW was proceeded for constitutional characterization by following different referenced standard protocols. Ethanolic as well as water extraction of different contents from WW was done. Extract of WW was made by distilled water (10%) for protein and sugar contents while ethanolic extraction (10%) was made for lipids. Phenol Sulfuric method of evaluating carbohydrate content.[17] was employed, for lipid estimation, Zolllner and Kirsch[18] method were used. Evaluation of total protein in the samples was made by Lowry method[19] and DNS method was subjected for measuring reducing sugar (R.S.) and total sugar (T.S.) values.[20] AOAC[21] protocols were adopted for the moisture content measurements. For determining LCB constituents i.e. cellulose, hemicellulose, extractives, lignin, Lin et al.[22] protocols were utilized followed by slight modifications.

Enterococcus faecium XA2 (Accession No. OM971654), with a xylanolytic potential of 0.817 ± 0.036 IU, was used for enzymatic Saccharification.[11] Fermentation studies employed two yeast strains, *Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34, sourced from the Microbiology Laboratory, Dept. of Zoology, University of Education, Lahore, Pakistan.[16]

Water melon waste has hemicellulosic as well as cellulosic contents from 19.38% to 27.74% and 23%.[22] Current study deals with the hydrolysis of hemicellulosic contents of water melon peels. The hydrolysis of cellulosic contents by cellulolytic bacterial isolates is part of the project. WW were hydrolyzed employing three strategies viz dilute sulphuric acid, enzymatic and combined (acidic followed by enzymatic) treatments.

Separate hydrolysis and fermentation strategy was adopted because the current study dealt with the selection of optimization of parameters for three hydrolysis strategies. The hydrolysis was performed at different temperature ranges such as 50-100 for acidic and 55-65 ˚C for enzymatic and combined treatment. Moreover, the both yeast strain (*Saccharomyces cereviceae* K7 and *Metschnikowia cibodasensis* Y34) were mesophilic and showed optimum growth at 30-32 ˚C. By SHF, better adjustment of the specified temperatures for microbes was easily attained. The selection of parameters for three hydrolysis techniques was based on prior research that highlighted the significant influence of certain critical factors on the hydrolysis of biomass.[1,24]

For the screening of hydrolysis parameters, a Placket-Burman (PB) experimental design was employed. This statistical approach is particularly suited for preliminary screening, as it efficiently identifies influential factors while main effects are partially confounded with two-factor interactions. The PB design facilitated the elimination of non-significant variables early in the experimental process, thereby reducing the volume of data collection and computational burden. The PB design comprised 12 experimental runs evaluating multiple parameters across three hydrolysis strategies. The acidic pretreatment parameters were sulfuric acid concentration, 2–6% (v/v), hydrolysis time, 30–60 min, hydrolysis temperature: 50–100°C, substrate loading (peels), 5–10% (w/v). Crude xylanase from *Enterococcus faecium* XA2 was produced in a modified basal medium containing (g/L): yeast extract 0.1, $MgSO_4$ 0.01, $Na_3C_6H_5O_7$ 0.05, and KH_2PO_4 0.2, adjusted to pH 7.0 and incubated at 37°C for 72 hours.[25] Acetate buffer (0.2 M) was used to prepare the watermelon waste (WW) substrate.[26] Initial xylanase activity, determined using xylan as substrate, was 8.17 ± 0.036 IU. For the PB model, the minimum enzyme dosage corresponded to this initial activity, while the maximum level was set at twice this value. Screening for enzymatic parameters included temperature 55–65°C, hydrolysis time 1–5 days, enzyme loading 8.17–16.34 IU, acetate buffer volume 80–90 mL, buffer pH 6–9, WW loading 5–10% (w/v). For combined pretreatment, sulfuric acid hydrolyzate was prepared under conditions yielding maximum responses (Table 1). Screening parameters for the combined approach included temperature 55–65°C, hydrolysis time 1–5 days, enzyme dose 8.17–16.34 IU, acetate buffer volume 25–50 mL, acid hydrolyzate volume 50–75 mL, buffer pH 6–9. For all three PB designs, experimental responses—reducing sugars and total sugars (g/L)—were quantified and analyzed statistically. Maximum theoretical reducing sugar yields were computed using PB modeling tools. The parameter

combinations predicted to optimize reducing sugar production were subsequently validated through saccharification experiments.

Only combined hydrolyzate of WW under screening conditions was subjected to ethanologenesis out of the three treatments. Optimization of fermentation parameters was carried out using Central Composite Design (CCD) under Response Surface Methodology (RSM). Design-Expert® software (Version 9.0.3, Stat-Ease, Inc.) was utilized to generate both Placket-Burman screening and CCD optimization models. The CCD included the 20 runs experiment to expedite three parameters**.** The fermentative parameters were WW hydrolyzate 25-75%(v/v): minimal medium 75-25%(v/v), 25-40 ºC incubated for 1-15 days. Three reactions to the experiment were examined: ethanol assay, ethanol yield and yeast growth. MYG (Malt Extract Yeast Extract Glucose) medium was used to create the yeast inoculum, while a minimal medium consisting of (in g) 0.65 yeast extract, 0.26 $(NH_4)_2SO_4$, 0.272 KH_2PO_4, 0.08 $MgSO_4.7H_2O$, 0.03 $CaCl_2$, 0.000042 of $ZnCl_2$, 0.15 $C_6H_8O_7$, and 0.6 $Na_3C_6H_5O_7$.[11] DNS (3, 5-Dinitrosalicylic acid) and potassium dichromate procedures were used to determine the ethanol and reducing sugar concentrations.[20,27] Ethanolic yield (g/g of substrate) was computed by dividing the ethanolic titer (g/L) by sugar utilized (g/L). Ethanolic titer and consumed sugars were computed by following expressions [1, 2] as;

$$\text{Ethanol Estimated (g/L)} = \frac{\text{O.D} \times \text{SF} \times \text{vm}}{100} \quad [1]$$

Where;
O.D = Optical Density of fermentation medium
SF = Standard factor (20.04/mL)
vm = Total volume of fermented WWH medium

$$\text{Reducing sugar consumed} = \text{Initial reducing sugar in fermentation medium} - \text{reducing sugars after fermentation} \quad [2]$$

Theoretical ethanol yield with optimized parameters was calculated using the tools of CCD statistics. A fermentation experiment was conducted to confirm the selected optimal conditions, and the actual yield was computed. To comprehend how factors interact in model, 3D graphs were plotted.

RESULTS

Table 1 showed the quantitatively analyzed contents of WW. The analyzed WW contents (without pretreatment) were reducing sugars as 15.9±0.05, total carbohydrates 28.8±0.05, total lipid 3.3±0.03 whereas total protein 3.5±0.03. By doing the compositional analysis of the sample, contents like hemicellulose, cellulose and lignin contents were also determined (in %) viz 16.20±2.30%, 53.26±0.33% and14.51±0.22 %congruently.

Table 1Quantitative analysis of watermelon waste without pretreatment

Parameter	**Content (%)**
Lignin	14.51 ± 0.22
Cellulose	53.26 ± 0.33
Hemicellulose	16.20 ± 0.30
Moisture	0.76 ± 0.05
Weight loss	16.03 ± 0.5
Carbohydrates (total)	28.8 ± 0.05
Proteins (total)	3.5 ± 0.03
Lipids (total)	3.3 ± 0.03
Reducing sugars	15.9 ± 0.05

All values were presented in form of average of three replicates and S.E.M

The saccharification data presented twelve runs per treatment for two responses i.e., reducing sugar and total sugar estimation (Table 2-4). For each treatment, the parameters responsible for maximum reducing sugars were highlighted in Tables 2-4 whereas by statistical analysis, point prediction of parameters responsible for optimum reducing sugars was done. Its hypothesized that the sugar polymers were converted into monomers by acid, enzymatic and combined treatment (as mentioned in section “validation of predicted contents). Table 2 revealed the data related to responses of acidic hydrolysis. The acidic saccharification parameters were selected based on earlier studies that highlighted the significant influence of a few important variables on biomass hydrolysis.[12] Maximum amount of calculated R.S. and T.S. were 24.35±0.003g/L and 34.83±0.003g/L correspondingly for acid pretreatment (i.e. 6% diluted H_2SO_4) at temperature 100°C for 60 min using 5% (w/v) WW. From the design runs, increase in reducing sugar was observed. The increasing the temperature (50 to 100 °C) and dilute acid concentration (2-6% v/v), improvement in sugar release was noted where as high substrate ratio as well as incubation time has negative impact with low acid concentration.

For enzymatic hydrolysis of WW, the R.S. and T.S. were found to be at higher level after one-day post-inoculationas 20.07±0.03g/L and 35.8±0.04 g/L, respectively with 16.34µmol/ml/min crude enzyme (xylanase), 90ml enzyme buffer (6 pH) at 55°C temperature as presented in Table 3. It is hypothesized that hemicelluloses are converted to soluble sugars by xylanases released from *E. faecium* XA2. The xylanase may perform efficiently at a low pH (6), low temperature (55 °C), less incubation days and at higher enzyme doses. Substrate is also an influential parameter such as a higher enzyme dose (16.34 IU) might be needed to convert more substrate (10% w/v) efficiently whereas less substrate ratio with same enzyme dosage has negative effect.

The optimized parameters for the combined model were hydrolysis time 5 days, xylanase dosage16.34 IU, buffer volume 25mL with pH 9, acid hydrolyzate 75mL at 55°C that released optimum values of reducing and total sugars, 30.43±0.51g/L and 46.95±0.10 g/L respectively (Table 4). In the experimental runs, temperature, pH and acid hydrolyzate concentration were influential factors whereas low enzyme dose (8.17 IU) and substrate ratio (5% w/v) was required for efficient conversion of sugars into monomeric units. It was recorded that high pH (9) with high temperature (65°C) influences the conversion positively while low pH (6) at same temperature affected negatively. Similarly, more acid hydrolyzate concentration with high temperature and pH produced positive effect whereas lower hydrolyzate with same temperature and pH has opposite impact.

Table 2 PB model for acidic hydrolysis of watermelon waste representing various parameters and responses

Run #	Temperature (˚C)	Incubation period (min)	Acid concentration (%)	Peels (% w/v)	Reducing sugars (g/L)	Total sugars (g/L)
1	50	60	6	5	18.21 ± 0.01	18.82 ± 0.01
2	50	30	6	10	0.963 ± 0.004	9.63 ± 0.004
3	100	60	6	5	17.54 ± 0.003	13.17 ± 0.003
4	50	30	2	5	16.83 ± 0.01	26.01 ± 0.01
5	50	60	2	10	12.45 ± 0.007	32.54 ± 0.007
6	100	30	2	10	0.39 ± 0.004	4.37 ± 0.004
7	100	60	6	10	0.44 ± 0.01	2.92 ± 0.01
8	50	30	6	10	14.74 ± 0.002	26.16 ± 0.002
9	100	60	6	5	24.35 ± 0.003	34.83 ± 0.003
10	100	30	2	10	21.098 ± 0.11	33.029 ± 0.11
11	100	60	2	5	6.99 ± 0.04	14.35 ± 0.04
12	50	30	2	5	11.637 ± 7.2	22.97 ± 17.2

All values were presented in form of average of three replicates and S.E.M

Table 3 PB model for enzymatic hydrolysis of WW representing different parameters and responses

Run #	Temperature (ºC)	Incubation period (days)	Enzyme dose (IU)	Buffer concentration (mL)	Peels (%w/v)	pH	Reducing sugar (g/L)	Total sugar (g/L)
1	55	1	16.34	90	10	6	20.07 ± 0.03	35.8 ± 0.04
2	55	5	16.34	90	5	9	9.25 ± 0.01	17.08 ± 0.02
3	65	1	16.34	80	5	6	13.03 ± 0.01	29.73 ± 0.05
4	55	5	16.34	80	10	6	7.88 ± 0.04	13.79 ± 0.08
5	55	1	8.17	80	5	6	2.29 ± 0.02	12.18 ± 0.05
6	65	5	8.17	90	5	6	0.39 ± 0.57	9.01 ± 0.03
7	65	1	8.17	80	10	9	4.38 ± 0.01	20.85 ± 0.02
8	65	5	8.17	90	10	6	11.92 ± 0.02	27.92 ± 0.01
9	65	5	16.34	80	10	9	13.96 ± 0.03	16.1.59 ± 0.03

10	65	1	16.34	90	5	9	13.74 ± 0.02	22.18 ± 0.02
11	55	1	8.17	90	10	9	8.06 ± 0.04	15.72 ± 0.001
12	55	5	8.17	80	5	9	2.26 ± 1.07	21.43 ± 3.44

All values were presented in form of average of three replicates and S.E.M

Table 4 PB model for combined hydrolysis of WW representing various parameters and responses

Run #	1	2	3	4	5	6	7	8	9	10	11	12
Temperature (˚C)	55	65	65	65	55	65	55	55	55	65	55	65
Peels (%w/v)	5	10	5	10	5	5	10	5	10	5	10	10
Xyanase dose (IU)	8.17	16.34	16.34	8.17	16.34	8.17	8.17	16.34	8.17	8.17	16.34	16.34
Buffer concentration (mL)	25	50	25	25	25	50	50	50	25	50	50	25
Acid Hydrolyzate (mL)	50	50	75	75	75	75	75	50	50	50	75	50
pH	9	9	9	9	6	6	9	9	6	6	6	6
Reducing sugars (g/L)	4.03 ± 0.09	5.47 ± 0.61	9.31 ± 0.84	25.47 ± 0.71	30.43 ± 0.51	17.73 ± 0.80	19.2 ± 0.89	20.09 ± 0.57	13.84 ± 0.55	10.68 ± 0.08	15.53 ± 0.37	5.46 ± 0.17
Total sugars (g/L)	14.63 ± 0.83	22.26 ± 0.72	24.96 ± 0.7	35.88 ± 0.8	46.95 ± 0.10	30.04 ± 0.15	30.01 ± 0.16	32.27 ± 0.64	29.65 ± 0.67	29.92 ± 0.12	28.57 ± 0.80	27.51 ± 0.05

All values were presented in form of average of three replicates and S.E.M

The statistically analyzed data for three treatments were recorded in Table 5. The acidic PB model exhibited an F-value of 94.90 for reducing sugars, indicating statistical significance. However, the associated probability value suggested approximately 7.97% likelihood that this result occurred due to random noise. For total sugars, the F-value of 3.23 indicated non-significance of model relative to experimental noise. Notably, model terms with "Prob > F" < 0.05 were identified as statistically significant contributors. For enzymatic hydrolysis treatment, the Placket-Burman model (reducing sugars) demonstrated non-significance with an F-value of 2.32, though approximately 14% of the observed effect may be attributed to experimental noise. Similarly, for total sugars, the model was not significant with F-value of 0.43 and an 84.13% probability that observed variations resulted from noise. As combined hydrolysis treatment is concerned, the PB model (reducing sugars) was statistically significant (F-value = 21.74), with only a ~5% probability that the observed F-value arose from random variation. Model significance was not established for total sugars, with a 47.47% probability that the observed effects were attributable to noise. Table 6 presented the regression coefficients analyzed data for three treatments. According to acidic PB model for reducing sugars, the close agreement between Pred R-Sq (0.85) and Adj R-Sq (0.99) confirmed strong model reliability and predictive capability. In the same way for T.S., the Adeq Precision value of 6.48 suggested an adequate signal-to-noise ratio, supporting model reliability despite limited predictive R-squared. For enzymatically hydrolyzed reducing sugar model, the high R-Sq (0.9919) and Adj R-Sq (0.956), along with an Adeq Precision of 16.146, confirmed excellent model fit and sufficient signal for navigating the design space. For T.S., the negative Pred R-Sq and low Adeq Precision (1.856) indicated that the model lacks predictive power and adequate discrimination for this response. For reducing sugars of combined treatment, the substantial discrepancy between R-Sq (0.8230) and Adj R-Sq (0.3508), along with a negative Pred R-Sq, suggested limited predictive reliability. The Adeq Precision of 4.468 marginally meets the adequacy threshold. For T.S., the low Adj R-Sq (0.0850) relative to R-Sq (0.6673) indicated poor model robustness, with only ~8.5% of variability.

Table 5 Statistically analyzed data for various responses with different PB designed watermelon waste treatments

Treatment	**Response**	**Source**	**Sum of squares**	**DF**	**Mean square**	**F Value**	**p Value**
	RS	M	4608.74	10	460.87	24.89	0.007 (S)

Acidic WW hydrolysis		R	4.86	1	4.86		
		C	4613.60	11			
	TS	M	17035.45	10	1703.54	3.23	0.40 (NS)
		R	526.46	1	526.46		
		C	17561.91	11			
Enzymatic WW hydrolysis	RS	M	87.87	9	9.76	2.32	0.14 (NS)
		R	0.71	2	0.36		
		C	88.59	11			
	TS	M	35919.28	10	3591.93	0.43	0.84 (NS)
		R	8332.40	1	8332.40		
		C	44251.68	11			
Combined WW treatment	RS	M	11015.71	8	1376.94	21.74	0.005 (S)
		R	2369.80	3	789.93		
		C	1.50	19			
	TS	M	76745.9	7	10963.7	1.14	0.47 (NS)
		R	38271.13	4	9567.78		
		C	1.150+005	11			

Abbreviations: RS-Reducing sugars; TS-Total sugars; M-Model; R-Residual; C-Core total; S-Significant; NS-Not significant

Table 6 Regression coefficients analyzed data for various responses with PB designed WW hydrolysis strategies

Treatment	Response	C.V	PRESS	R squared	R squared (adjusted)	R squared (predicted)	Adequate precision
Acidic WW hydrolysis	RS	7.67	699.35	0.998	0.98	0.8484	31.79
	TS	22.46	75809.7	0.97	0.67	0.7167	6.48
Enzymatic WW hydrolysis	RS	10.02	25.72	0.9919	0.9556	0.7096	16.146
	TS	92.99	1.2 + 006	0.8117	-1.0713	-26.1146	1.856
Combined WW hydrolysis	RS	78.02	37916.74	0.8230	0.3508	0.8327	4.468
	TS	96.96	3.44 + 005	0.6673	0.0850	0.9947	3.642

Abbreviations: RS-Reducing sugars; TS-Total sugars

Table 7 presented both predicted and experimentally validated values for all three Placket-Burman (PB) experimental designs. For acidic hydrolysis using 6% sulfuric acid, the model predicted total sugar (T.S.) and reducing sugar (R.S.) yields of 36.44 g/L and 25.13 g/L, respectively, under optimized conditions of 5% WW substrate (w/v), 30 minutes reaction time, and 50°C. In the case of enzymatic hydrolysis, predicted yields for total sugars and reducing sugars were 27.75 g/L and 40.35 g/L, respectively, following a 24-hour incubation period at 55°C, with an enzyme loading of 16.34 IU, 90 mL acetate buffer (pH 6), and 10% WW substrate (w/v).

For the combined acidic-enzymatic treatment, predicted reducing sugar and total sugar yields were 30.11 g/L and 47.65 g/L, respectively, achieved after five days of incubation at 55°C, using an enzyme load of 16.34 IU, 25 mL acetate buffer (pH 6), and 75 mL acid hydrolyzate derived from WW substrate. Notably, experimental sugar yields consistently exceeded the model-predicted values across all hydrolysis treatments when WW substrate was processed under the optimized parameters identified by the Placket-Burman screening design, thereby validating the practical effectiveness and predictive utility of the statistical approach.

Table 7 PB model validation for predicted parameters for different watermelon waste hydrolytic treatments

Treatments	Responses	Predicted amount (g/L)	Experimental amount (g/L)	Residual	Percent error
Acidic WW hydrolysis	RS	25.13	25.67 ± 0.003	0.54	2.148
	TS	36.44	37.16 ± 0.16	0.72	1.97
Enzymatic WW hydrolysis	RS	27.75	28.07 ± 0.003	0.32	1.15
	TS	40.35	41.34 ± 0.004	0.99	2.45
	RS	30.11	31.48 ± 0.014	1.37	4.5

Combined WW hydrolysis	TS	47.65	47.83 ± 0.005	0.18	0.37

Abbreviations: RS,-Reducing sugars; TS-Total sugars
Residual = Experimental value – Predicted value
Error = Residual / Predicted value × 100

Table 8 presented the ethanol titer and yield values under various fermentation conditions optimized using Central Composite Design (CCD). Both *Saccharomyces cerevisiae* K7 and *Metchnikowia cibodasensis* Y34 achieved maximum ethanol yields at 32.5°C when incubated with 50 mL of hydrolyzate for eight days. Specifically, *S. cerevisiae* K7 produced an ethanol yield of 0.35 ± 0.01 g/g and ethanol contents of 5.14 ± 0.01 g/L, whereas *M. cibodasensis* Y34 yielded 0.38 ± 0.01 g/g ethanol and 6.78 ± 0.03 g/L ethanol content under same conditions.

The ANOVA results for the CCD models were summarized in Table 9. The model predicting ethanol yield was statistically significant for both yeast strains, with F-values of 3.64 (p = 0.0285) for *S. cerevisiae* K7 and 3.26 (p = 0.039) for *M. cibodasensis* Y34. In contrast, the models for ethanol contents were not statistically significant, exhibiting F-values of 1.40 and 1.29 for *S. cerevisiae* K7 and *M. cibodasensis* Y34, respectively. These non-significant results indicated approximately 30.32% and 34.49% probabilities that the observed effects could be attributed to random experimental noise.

Table 10 presented the regression coefficients, coefficient of variation (CV), and adequate precision values for all models. R-sq and Adj R-sq values of model for ethanolic yield employing ethanologenic yeast *S. cereviseae* K7, were found to be 0.766 and 0.556, corroboratively, indicated the model reliability of around 76.6% associated to the variables. Value of "Adeq Precision" i.e. 5.375 with 30.3 CV pointed towards model reliability. In the same way, R-sq and Adj R-sq values for ethanolic yield employing yeast isolate *M. cibodasenis*Y34 were 0.75 and 0.52 directed towards model reliability with agreement of 8.022, 43.7 Adeq Precision as well as CV correspondingly. R-sq and Adj R-sq values for ethanolic content from yeast *S. cereviseae* K7 were 0.557 and 0.159 with Adeq Precision value of 4.718 whereas the R-sq and Adj R-sq values for ethanolic content employing yeast *M. cibodasenis*Y34 were 0.539 and 0.123 and Adeq Precision value of 4.733 were recorded, The values represented the less reliability of model.

Table 8 Central Composite Design matrix with optimized fermentation parameters for ethanol titer and ethanol yield responses

Parameters				**K7 yeast**		**Y34 yeast**	
Run #	**WW hydrolyzate (mL)**	**Time (Days)**	**Temperature (ºC)**	**Ethanol content (g/L)**	**Ethanol yield (g/g)**	**Ethanol content (g/L)**	**Ethanol yield (g/g)**
1	50	8	45.1	4.18 ± 0.01	0.28 ± 0.01	2.5 ± 0.002	0.19 ± 0.02
2	25	1	40	0.34 ± 0.03	0.11 ± 0.1	2.32 ± 0.006	0.09 ± 0.01
3	75	1	40	4.36 ± 0.001	0.15 ± 0.1	4.04 ± 0.004	0.30 ± 0.1
4	25	15	40	2.96 ± 0.001	0.23 ± 0.02	3.35 ± 0.03	0.26 ± 0.2
5	75	15	25	4.72 ± 0.005	0.11 ± 0.03	2.16 ± 0.01	0.22 ± 0.3
6	7.95	8	32.5	5.02 ± 0.04	0.1 ± 0.03	4.48 ± 0.01	0.31 ± 0.41
7	75	1	25	4.78 ± 0.07	0.20 ± 0.02	2.54 ± 0.01	0.13 ± 0.1
8	50	8	32.5	5.14 ± 0.01	0.35 ± 0.01	6.78 ± 0.03	0.38± 0.1
9	25	15	25	2.4 ± 0.08	0.16 ± 0.04	2.15 ± 0.01	0.3 ± 0.05
10	50	8	19.8	5.3 ± 0.004	0.35 ± 0.01	1.84 ± 0.01	0.24 ± 0.01
11	50	19.7	32.5	5.72 ± 0.001	0.24 ± 0.03	4.85 ± 0.01	0.32 ± 0.04
12	50	8	32.5	4.66 ± 0.004	0.3 ± 0.04	2.09 ± 0.004	0.24 ± 0.04
13	50	-3.77	32.5	6.5 ± 0.002	0.11 ± 0.05	2.97 ± 0.004	0.28 ± 0.2
14	50	8	32.5	5.52 ± 0.01	0.28 ± 0.01	2.84 ± 0.001	0.2 ± 0.03
15	50	8	32.5	5.28 ± 0.006	0.26 ± 0.11	2.73 ± 0.12	0.23 ± 0.11
16	92.04	8	32.5	4.9 ± 0.01	0.24 ± 0.2	4.35 ± 0.01	0.29 ± 0.01
17	50	8	32.5	4.84 ± 0.02	0.37 ± 0.3	3.01 ± 0.01	0.21 ± 0.03
18	25	1	25	3.46 ± 0.03	0.12 ± 0.02	3.42 ± 0.01	0.25 ± 0.01
19	50	8	32.5	5.08 ± 0.005	0.34 ± 0.01	5.52 ± 0.01	0.35 ± 0.02
20	75	15	40	4.18 ± 0.06	0.28 ± 0.01	0.8 ± 0.12	0.19 ± 0.04

All values were presented in form of average of three replicates and S.E.M

Table 9 Statistically analyzed data for various responses for the fermentation of watermelon waste

Response	Yeast isolate	Source	Squares' sum	Mean of square	D.F	F-value	P-value
Ethanol yield	K7 Yeast	M	0.11	0.012	9	3.64	0.0285 (S)
		R	0.033	3.341	10		
		L	0.019	3.78	5	1.31	0.3886 (NS)
		P	0.014	2.89	5		
		C	0.14		19		
	Y34 Yeast	M	0.058	6.48	9	3.26	0.039 (S)
		R	0.020	1.99	10		
		L	6.922	1.38	5	0.54	0.7454 (NS)
		P	0.013	2.59	5		
		C	0.078		19		
Ethanol titer	K7 Yeast	M	6.91	0.77	9	1.4	0.3 (NS)
		R	5.49	0.55	10		
		L	5.22	1.04	5	19.2	0.03 (S)
		P	0.27	0.05	5		
		C	12.4		19		
	Y34 Yeast	M	3.21	0.36	9	1.29	0.33 (NS)
		R	2.75	0.28	10		
		L	1.93	0.39	5	2.35	0.18 (NS)
		P	0.82	0.16	5		
		C	5.96		19		

Abbreviations: M-Model; R-Residual; L-Lack of fit; P-Pure error; C-Core total; S-Significant; NS-Not significant

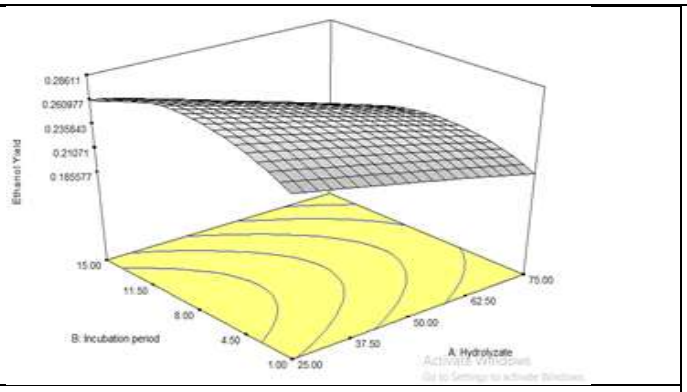
Table 10 Regression coefficients analyzed data for various responses with CC designed fermentation of WW hydrolyzates by yeast isolates

Response	Yeast isolate	CV	Press	R squared	Adjusted R squared	Predicted R squared	Adequate precision
Ethanol yield	K7	30.3	32.8	0.766	0.556	3.344	5.375
	Y34	43.7	58.2	0.75	0.52	26,711	8.022
Ethanol titer	K7	33.2	40.8	0.557	0.159	-2.292	4.718
	Y34	22.3	16.1	0.538	0.123	-1.701	4.733

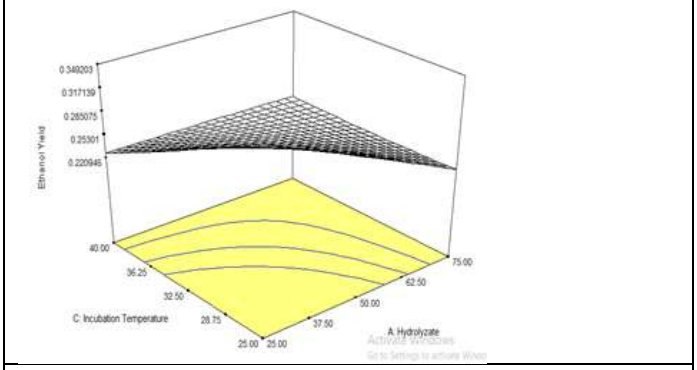
Figures 1 (*S. cereviseae*K7) and 2 (*M. cibodasensis* Y34) presented the interaction of different variables for the response i.e. ethanol yield. Three dimensional presentation was used to interpret the effect of all parameters with both yeast. In Figure 1a, where incubation time proceeded then ethanolic yield was also increased sharply whereas increase in hydrolyzate content caused slight reduction in ethanolic response. Figure 1b showed sharp decrease in ethanolic yieldwith increase in incubation temperature and hydrolyzate. In Figure 1c where incubating temperature was increased, it resulted in great reduction of ethanolic yield and increased incubating time cause slight reduction of ethanol yield (response). Hydrolyzate concentration influenced the ethanolic yield positively as it might provide more nutrients for efficient working of *S. cereviseae*K7 yeast. The incubation time and temperature has negative impact on ethanol yield. As high/low temperature might cause stress on yeast growth and metabolic activities which impair bioethanol production. The yeast may produce end product (ethanol) at 32.5 ^{o}C instead of 25 and 40 ^{o}C. In the same way, incubation time has opposite effect on yield. It is hypothesized that the accumulation of ethanol contents and certain metabolites in fermentation medium might affected the yeast growth adversely as days passed.

Figure 2a presented sharp elevation in yield with increase in incubation period and hydrolyzate. In Figure 2b whereas increase in incubating temperature resulted in slight decrease in ethanolic yield whereas increased hydrolyzates consequenced marked improvement in response and slight reduction was observed as incubating temperature and incubating period were increased (Figure 2c). Similar trend of interaction of factors with ethanolic yield was recorded with *M. cibodasensis* Y34 as was seen with standard yeast. Hydrolyzate showed progressive impact on ethanolic yield of yeast as a source of nutrients. Incubation time and temperature have

adverse effect on ethanol yield as these factors cause a stress on yeast growth/metabolism and ethanol/metabolite toxicity.



(a)



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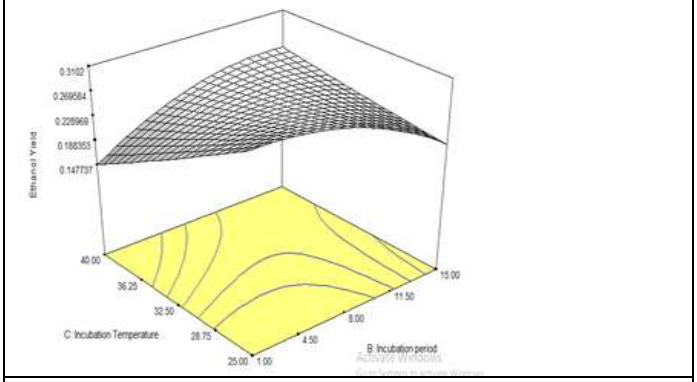
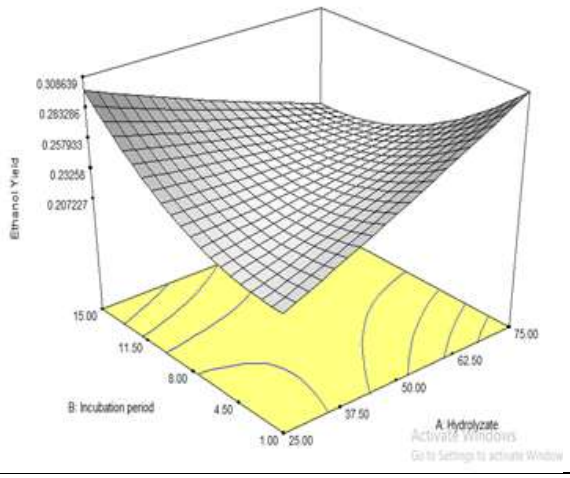
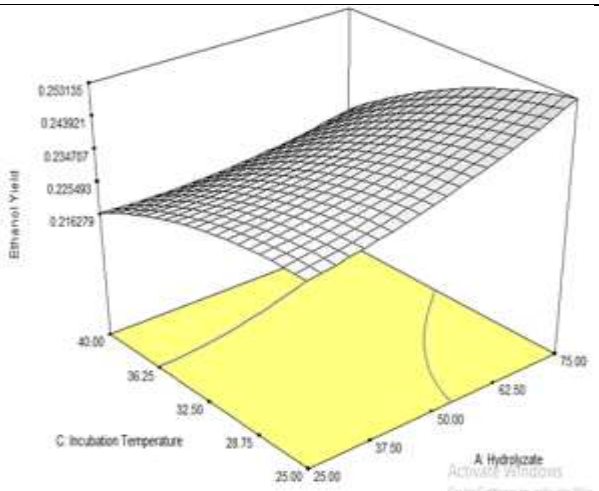


(c)

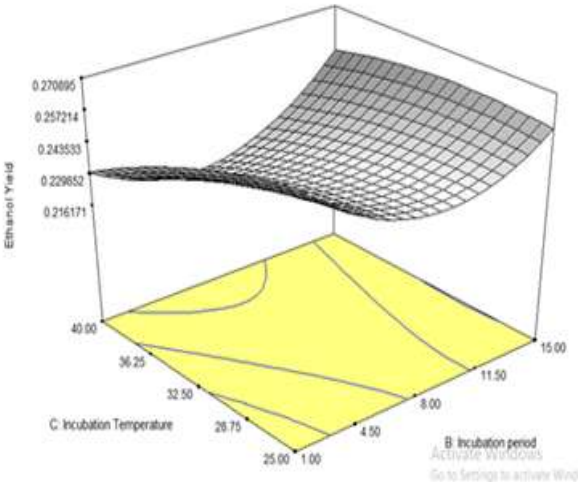
Fig. 1 Influence of Hydrolyzate Concentration, Fermentation Temperature, and Incubation Period on Ethanol Yield by *Saccharomyces cerevisiae* K7



(a)

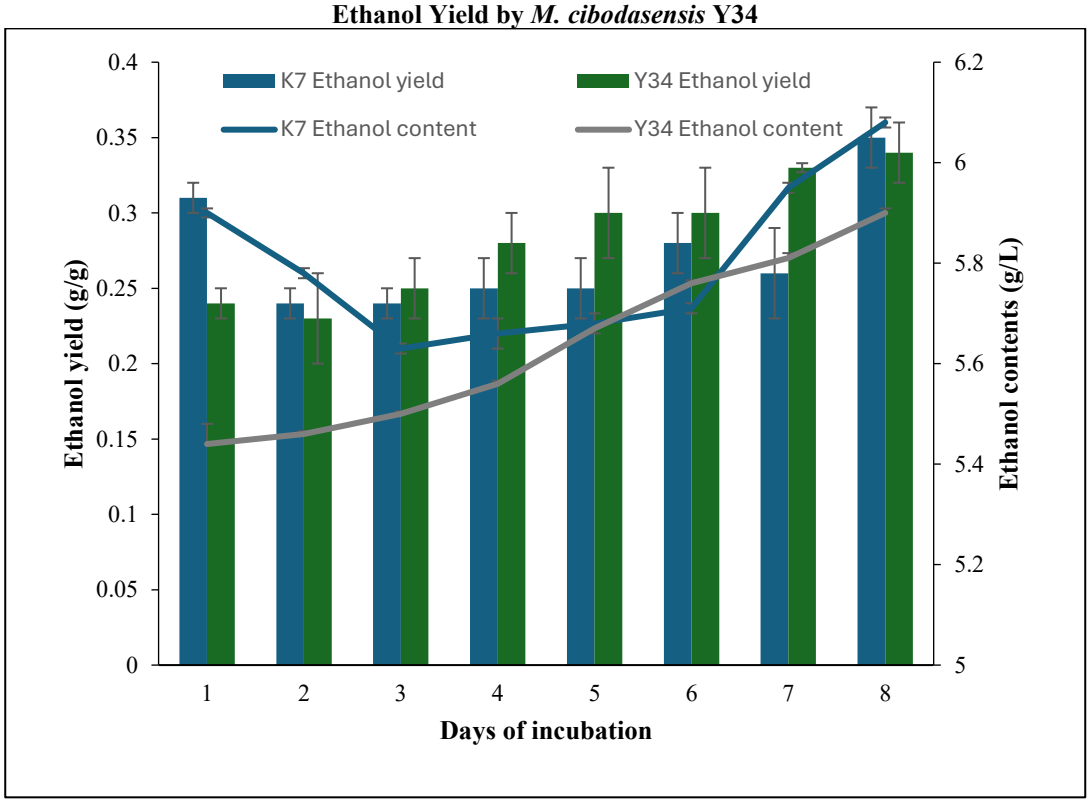


(b)



(c)

Fig. 2 Influence of Hydrolyzate Concentration, Fermentation Temperature, and Incubation Period on Ethanol Yield by *M. cibodasensis* Y34



Ethanol yield and titer for both isolates fermenting combined-treated WW hydrolyzate (50%) at 32.5°C were presented in Figure 3. Ethanol production by both strains increased steadily through day 8, indicating that this duration corresponds to the maximum ethanol tolerance limit of the yeasts under these fermentation conditions

DISCUSSION

For the industrial production of ethanol, xylan has become the most demanding carbon source used for fermentation.[28] Hydrolyzation of xylan into xylose can be conducted both enzymatically and chemically.[29] Enzymatic hydrolysis of xylan is carried out by xylanases and produce xylose.[30] Reportedly the most common inducers of the inducible enzyme i.e., xylanases are xylan. Current study focuses on the comparison of acidic, enzymatic, and combined pretreatments. Separarte hydrolysis and fermentation also known as SHF is a process used for bioethanol production involves successive events of microbial hydrolysis and fermentation using WW as substrate. According to Zhang et al.[31] fruit wastes produced by industries can be very potent raw materials for the production of industrially important secondary metabolites by microbes. Utilization of wastage food as substrate for microbes produces various food items and other useful products i.e., ethanol, bioenergy, acids, biofertilizers, and single cell protein.[32] The microbial hydrolysis of lignocellulosic biomasson is a cost-effective, energy-efficient, and eco-friendly process.[33]

Enterococcus faecium XA2 xylanolytic potential was evaluated on watermelon rinds waste using Placket Burman Design. Previously, Chaudhary et al.[11] reported 0.818±0.036 IU xylanolytic potential of *Enterococcus faecium* XA2 to convert xylan into xylose.

The lignocellulosic material's recalcitrant structure needs to be broken up by mechanical or physiochemical pretreatment techniques (such as acid/enzymatic, combined acid + enzymatic treatments) in order to make these sugar linkages accessible. The application of these pretreatment procedures is reliant on the feedstock since which determines the cellulose, hemi-cellulose, and lignin composition.[34] In the study, acidic hydrolysis was carried out by dilute sulphuric acid. It was hypothesized that the lignocellulose was converted into monomers i.e., pentose and hexose by acid hydrolysis and eventually into bioethanol through fermentation. Results have shown that the maximum reducing sugars were 24.35±0.003 g/L and total sugars were 34.830.16g/L at 100°C for 60 min with 5% of substrate (WW) and 6% diluted H_2SO_4 which is in accordance with previous finding.[12] In another study, similar findings were observed with 6% diluted sulfuric acid at 100°C for 30 min that corroborated the values of current experiment.[35] Asli et al. [36] reported the optimum total sugar content (1.12 ± 0.01 mg m/L) with the predicted value (1.21) while total sugar contents were near to the experimental value. These findings were contrary to the values of current study. Acidic pretreatment increased the reducing sugars because this pretreatment of

watermelon peels waste (WPW) effectively forms soluble sugars from hemicellulose. Acidic pretreatment alters the lignocellulosic structure to facilitate its availability for enzymes in addition to the conversion of hemicellulose to monomers.[37] Furthermore, a number of investigations revealed that the process of dilute acid hydrolysis led to biomass swelling, which in turn resulted in a loosening of the structural connections between lignin and xylan.[38] Chen et al.[39] also discovered that all hemicelluloses were totally destroyed at a temperature of 190 °C, leaving a large amount of debris on the biomass surface. Regarding acid concentration, Hanim et al.[40] recognized that acid activity was correlated with H^+ concentration. Higher hydrogen ion concentration led to more glucosidic bond cleavage and, in turn, faster hydrolysis of hemicelluloses to sugars.[41] It is important to note that the impact of acid strength on biomass pretreatment appeared to be more pronounced at lower concentrations. In the design, maximum sugars were released at 6% (v/v) acid concentrations. Same findings were observed by Gil et al. [42] by increasing acid concentration from 2.75 to 5.0 %.

During saccharification of hemicellulose by enzymes, the maximum levels of reducing sugars and total sugars were determined after 24 h of incubation, for 16.34 IU crude enzyme (xylanase), 6 pH at 55°C temperature. The maximum recorded amount of reducing sugars was 20.07 g/L. While the predicted reducing sugar and total sugar values after 24hrs were 27.75g/L and 40.35g/L, respectively for 16.34 µmole/min/mL enzyme, pH 6 and 10g peels at 55ºC. Watermelon waste is selected as the substrate for the current study despite having less sugar than other potential substrates for bioethanol production. This is because of the annual global production of approximately 540,000 metric tons of fruit waste that needs to be properly disposed of and utilized. As Pakistan grows 2.41 million tons of watermelons annually, ranking it 18th in the world. Watermelon is a summertime favorite fruit of people of Pakistan that is inexpensive and readily available. Pollution results from the perishable and sugary waste of watermelon, which is attractive to WW still reduce environmental pollution when managed properly –serving as good technique in sense of waste management.[15] Chaudhary et al. [11] reported that the bacterial isolates *Bacillus cereus* XG2 and *Enterococcus faecium* XA2 pests and provides a good growing medium for microorganisms. Bioethanol production from xlan have proficient potential to hydrolyze i.e., 0.817 ± 0.036 and 0.917 ± 0.059 IU, respectively. The maximum xylanolytic activity was reported by Ali et al. (2017) i.e., 0.98 U/ mL and 1.73 U/mL with D-xylose and xylan by an isolated (termite gut) yeast species *Candida pseudorhagii*. Without pretreatment enzymatic hydrolysis is not very effective (20% yield), while pretreatment has reportedly spiked up the sugar contents up to 90%.[43]

Enzymatic hydrolysis is considered a more appropriate pathway for the hydrolysis of biomass because it uses less energy.[1, 44] In current study, the conversion of hemicellulose to xylose in pretreated biomassis accomplished by xylanase enzymes.[45] Due to this conversion ability, xylanase appeared as an emerging enzyme that enhances the yield of fuel.[46] Xylanolytic bacterial and fungi possess diverse enzymatic machineries for xylan hydrolysis. Endoxylanase initially degrades xylan to unbranched xylooligosaccharides (XOSs) (xylobiose, xylotriose, xylotetraose) as intermediates, which are then quickly hydrolyzed into single xylose by β-xylosidase The pathways that are involved in the breakdown of xylan are the pentose phosphate pathway, glycolysis, and pyruvate metabolism. Xylanases catalyze the anomeric backbone of xylan by double/single displacement mechanism in which the main configuration is retained or inverted.[47]

In combined hydrolysis the reducing sugars and total sugars were highest i.e., 31.48±0.014 g/L and 47.83±0.005g/L after 5 days of incubation, at 55°C for 16.34 IU crude xylanase, 25mL enzymatic+75mL acid hydrolyzate. The expected values of total sugar and reducing sugars were 47.65g/L and 30.11g/L, respectively. The enzymatic hydrolysis showed lesser production of reducing sugars as compared to acidic and combined acid/enzymatic pretreatment. The diluted acid preferentially hydrolyse hemicelluloses followed by increased enzymatic degradability. Xu and Huang[48] used a two-stage acid hydrolysis system. In the first stage, pentoses were recovered by hydrolyzing hemicelluloses hydrolysate while the second stage improves the digestibility of substrate by enzymes under severe conditions. In the same way, Auxenfans et al.[49] (2014) improved substrate hydrolysis by two fold employing combined action of dilute acid and ionic liquid. The combined treatments affected synergistically the enzymatic hydrolysis and removal/degradation of hemicellulose as well as cellulose.[37] Seneesrisakul et al. [50] showed that glucose was present in culture medium which results into decreased endoglucanase production (activity), which means increased amount of glucose in a medium can result decreased activity of bacteria.

*Metchnikowia cibodasensis*Y34 and *Saccharomyces cerevisiae* K7 yeasts showed an estimated maximum ethanol yield i.e., 0.35m 0.38 g/g of reducing sugar, in 8 days at 32.5ºC. Due to its high stress and osmotolerance, *S. cerevisiae* is able to withstand the fair inhibitors concentration found in hydrolysates. It has potential for utilization of diverse sugars. Xylose is imported by *S. cerevisiae* via facilitated diffusion. Compared to xylose, this transport mechanism is more selective for glucose. This yeast lacks a functional metabolic pathway, despite having homologs of the genes that metabolize xylose. Furthermore, there is usually a lag phase following uptake, during which metabolic enzymes are produced to initiate metabolism. The fermentation process was accelerated by *S. cerevisiae* when these native genes were overexpressed. A byproduct that accumulated was xylitol. This byproduct determined the existence of xylose metabolic pathway in the yeast.[51] Ali et al.[52] reported that in xylose

containing medium *Candida stipites* utilized most of the sugar in the 1st day of incubation with ethanol value of 3.57 g/L and 0.088 g yield of ethanol g/g xylose. Although 2.83 g/L ethanol with yield of 0.090 g of ethanol g/L xylose was estimated in medium containing sugar cane bagasse hydrolyzate after 2 days of incubation.[53] These findings were in contrary to the values in current study. The findings of ethanol yield g/g 0.29±0.003 and 0.30 ± 0.003 produced from *M. cibodasensis* Y34 and S. cerevisiae K7, respectively reported by Chaudhary et al. [54] were in accordance to our findings.

The yeasts (*S. cereviseae* K7, *M. cibodasensis* Y34) used for the experimentation were mesophilic. It has long been recognized that temperature, incubation period, substrate and ethanol levels influences metabolism, growth and sensitivity to alcohol/metabolite toxicity of mesophilic yeast during sugar fermentation. The alteration in the structure of the cell membrane causes the decrease in functionality of yeast. *S. cerevisiae* has low tolerance to ethanol.[55] The hydrolyzate contain the nutrients and sugar contents that affected positively the ethanolic yield but with high temperature and incubation period, adverse effect on ethanol production is observed. High temperatures may potentially function as a stressor for yeast by impeding cell growth and reducing metabolic activity, ultimately leading to a decrease in ethanol yield.[56] The causes of reduction in metabolism might be disruptions in the redox balance within cells, lysis of cells, and release of vitamins and amino acid in medium which in turn drop the production of bioethanol.[57] Yeast produced metabolites in the fermentation medium, including lactate, trehalose, glycerol, acetic acid, and succinic acid, which also contribute to the suppression of metabolism. The high sugar concentrations during *S. cerevisiae* fermentations caused the water activity to drop to below-average levels, which in turn inhibited the metabolism.[58] In current investigation, increase in hydrolyzate concentration (substrate) influenced positively whereas increased incubation period and temperature have adverse effect. A decrease in ethanol yield was observed with ripe carabao mango peelings by increasing fermentation time.[59] According to Gawande and Patil[60] this decline can be associated to the accumulation of harmful metabolic byproducts and inhibitors during fermentation, which have an impact on yeast growth and reduce yeast cell biomass. On the contrary, increase in incubation time leading to increased ethanol titer was reported by Osei et al. [61] with maize, old waste papers, corn and sugarcane bagasse substrates.

CONCLUSIONS AND RECOMMENDATIONS

According to the study, combined (acidic + enzymatic) saccharified samples exhibited substantial amounts of reducing sugars during 5 days of incubation. The concentration was 31.48±0.014g/L using 16.34 IU crude enzyme (xylanase) and 75% WW hydrolyzate at pH 6 and 55°C. After 8 days, the 50% hydrolyzate, *Metchnikowia cibodasensis* Y34 generated a maximum ethanol yield of 0.38g/g of fermentable sugars at 32.5°C. This yeast shows promise in using WW biomass to convert waste to bioethanol.

Saccharification and fermentation of watermelon peels are compared to offer an alternate approach to waste management and raw material utilization for the production of biofuels such as bioethanol. The results of this study point to the possibility of producing huge quantities using both batch and continuous fermentation techniques. This might result in a greater production of bioethanol by commercial fermenters.

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