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Research Article

Comparison of ethanologenic potential of *Saccharomyces cerevisiae* and *Metschnikowia cibodasensis* employing watermelon pulp hydrolysate

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ABSTRACT

The agricultural sector in Pakistan, which is a major contributor to its economy, produces massive biomass waste such as watermelon leftovers. This paper discusses how watermelon pulp can be used as feedstock in bioethanol production and how this is a viable solution to the energy requirements of Pakistan as well as solving the problem of waste management. Fermentable sugars are found in abandoned watermelons, which can be used as a source of bioethanol. In this study, the objective was to hydrolyze the watermelon pulp *Bacillus cereus* FA3 using optimum conditions and ferment the resulting saccharified monomers into ethanol, utilizing yeasts; *Metschnikowia cibodasensis* Y34 strain and *Saccharomyces cerevisiae* K7 strain. To optimize the experimental conditions for hydrolysis and fermentation, Plackett-Burman and central composite designs (CCD) were developed. Under the optimized conditions of the Plackett-Burman model, i.e., 37°C, pH 4, 50:55mL buffer to pulp ratio, 0.63 IU enzyme dosage, 5 days hydrolysis period, maximum reducing sugars of 45.13±0.01 and total sugars of 82.64±0.06 g/L was achieved. Under the optimized conditions viz. 75:25% hydrolysate: synthetic media, 5% (v/v) of yeast inocula, 25 °C, 15 days, the maximum ethanol yield and content with yeast *M. cibodasensis* Y34 strain was recorded as 0.43 ± 0.007g/g and 13.6 ± 0.008g/L whereas *Saccharomyces cerevisiae* yielded 0.41 ± 0.004g/g and 12.2 ± 0.001g/L ethanol respectively. These outcomes underscore the effectiveness of the optimized fermentation process and the promising potential of watermelon pulp valorization for sustainable ethanol production.

Keywords: Watermelon pulp waste; bioethanol production; agro-waste valorization; enzymatic hydrolysis; sustainable energy.

INTRODUCTION

The increasing world requirement of renewable and green energy has fuelled the urge to produce bioethanol using non-food low-cost feedstock. The wastes generated during the fruit-processing and lignocellulosic are one of the most promising sources of second-generation biofuels, especially in developing countries where agricultural wastes are underutilized (Singh et al., 2022; Samantaray et al., 2024). Agriculture in Pakistan is a significant economic activity. The fruits, vegetables and other crops production in Pakistan are played to have large export earnings and employments. Mahmood and Munir (2018) state that 45 percent of the overall labor force and 70 percent of rural population are employed in the agriculture sector of Pakistan. It is a contributor to 80 percent of export of the country. It also contributes more than 20 percent of the GDP of Pakistan and provides raw material to the agro-based industries (Mahmood and Munir, 2018). Having a large agricultural base,

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Pakistan produces millions of tons of crop residues and fruit waste on an annual basis, much of which is disposed of or allowed to rot, with the result of environmental pollution and greenhouse gas emissions (Nawaz et al., 2024). Watermelon waste, particularly rotting or discarded fruit remains is one of these wastes and presents a significant amount of bioethanol precursor, as it contains large amounts of fermentable sugars and organic nutrients, which have been shown to be an ideal source of bioethanol. Watermelon (*Citrullus lanatus*) is the one of the members of cucurbit family (Cucurbitaceae). It belongs to the kingdom of Plantae, order of Cucurbitales as well as to the genus *Citrullus*. It is a juicy fruit that has a long and rich history dating back 5,000 years since its inception in ancient Africa (Nadeem et al., 2022) as it was first planted in the Nile Valley. It was disseminated into Asia and Europe via trade routes through the Mediterranean. Watermelon is currently cultivated in more than 100 countries, and its major manufacturers include China, Turkey, India, Pakistan and United States (Dube et al., 2021). The world output of watermelons reaches 29.6 million tons, which demonstrates the economic influence that this product has on the world (Nadeem et al., 2020; Gebeyhu and Markos, 2023). In Pakistan, it is grown in the anterior areas of Province Punjab and Sindh. Pakistan was among the top watermelon-producing countries in 2022 and 2023 (production ≈ 1.4–1.6 million tons), according to FAO-based summaries and global rankings (FAO/FAOSTAT, 2023) As watermelons are cultivated all over the country, right after their cultivation, biomass or waste watermelons can be sourced from several points in the supply chain. These include unsold, damaged, and rotten fruits from farms and markets, fruits that are discard out due to cosmetic flaws or over-ripeness (Shrefler et al., 2015). Likewise, the processing units and juice industries also generate considerable watermelon waste in the form of pulp, peels, and juice residues (Tarazona-Diaz et al., 2011). This waste not only results in the loss of valuable nutritional resources but also harms the environment. As the waste decomposes, it can produce methane, a potent greenhouse gas, and contaminate soil and ground water (Amicarelli et al., 2021). Moreover, when huge quantities of watermelon waste are disposed, this may also serve as a breeding habitat of diseases carrying insects which can be harmful to human health. Rather than being discarded and left to form a portion of land pollution, this organic waste could be picked and used as a high feedstock to produce bioethanol, which is environmentally friendly, and can be used to achieve renewable energy ambitions. A typical watermelon consists of approximately 68 percent of watermelon pulp or flesh, 30 percent of watermelon rind, and 2 percent of watermelon seeds (Nadeem et al., 2022). Proximate analysis of watermelon indicated that whole watermelon has various nutrients such as, moisture (5.43-6.82%), ash (2.78-3.72%), crude fiber (1.66-3.94%), and true proteins (10.8-13). It has also starch (143.7-172.7mg/g), total sugar (53.7-96.5 mg/g), and reducing sugar (5.6-9.5 mg/g). Key minerals including iron (191-211 mg/kg), copper (20.12-35.03 mg/kg), zinc (68.97-92.57 mg/kg), calcium (98.79-233 mg/kg), and magnesium (79.75-123.9 mg/kg) are also present (Falade et al., 2022). The proportions of sucrose and glucose constitute about 20-40 percent of the total sugars along with fructose which is about 30-50 percent in a ripe watermelon (Bianchi et al., 2018). Yeasts continue to play a central role in fermentation of ethanol in industries, because the most commonly used organism is *Saccharomyces cerevisiae*, which is robust, ethanol tolerant and well-studied (Figueroa et al., 2024). Non-conventional yeasts, however, are also receiving the recent focus of their distinct metabolic abilities, including improved sugar exploitation, resistance to fermentation suppressing factors and possible increases in conversion efficiencies to ethanol (Moon et al., 2025). Hydrolysis and fermentation conditions should be optimized to maximize the yield of ethanol, and in particular with complex fruit-based feed stocks. Placket Burman and central composite design (CCD) are examples of designs that offer statistically powerful frameworks to establish significant parameters and optimal conditions of a process (Chaudhary et al., 2024; Kalangi et al., 2025). The effective extraction of fermentable sugars and high yield of ethanol can be achieved by applying these tools to watermelon pulp hydrolysates. Furthermore, there is lack of data about integrated optimization models (Plackett–Burman and CCD) to simultaneously optimized hydrolysis efficiency and ethanol yield from watermelon pulp hydrolysates.

The present study evaluates the comparative ethanol yields of *Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34 using optimized watermelon pulp hydrolysates with *Bacillus cereus* FA3. By integrating process optimization with comparative fermentation analysis, this research contributes to renewable-energy solutions and offers waste-to-biofuel valorization strategy for Pakistan, supporting both environmental sustainability and waste management.

MATERIALS AND METHODS

Substrate collection, preservation and proximate composition

Watermelon (*Citrullus lanatus*), one of the most widely consumed fruit worldwide, was selected as the substrate for this study. Samples were procured from various local markets across Lahore. The fruit was initially washed thoroughly

with distilled water to remove any surface contaminants, after which it was peeled and the pulp was processed using a sterile blunder to extract juice and pulp. The juice was collected in sterile glass bottles and stored at 4 °C until further use for experimental procedures. Compositional assessment of different contents of water melon pulp was done biochemically. Total and reducing sugars were quantified using phenol-sulfuric acid method and 3,5-Dinitrosalicyclic acid (DNS) method, as described by Dubois et al. (1956) and Miller (1959), respectively.

Selected micro-organisms for the study

The micro-organisms used in this study included a sucrose- degrading bacterium (*Bacillus cereus* FA3 strain) and two ethanologenic yeast isolates (*Saccharomyces cerevisiae* K7 strain and *Metschnikowia cibodasensis* Y34 strain). These strains were previously isolated and characterized at Microbiology Laboratory, University of Education, Lahore, Pakistan. The bacterial strain *B. cereus* FA3 (accession number OQ450350) was isolated in 2024 and possesses a high sucrolytic potential with an invertase activity of 0.629 IU, making it suitable for Saccharification processes (Chaudhary et al., 2024). The bacterial isolate was cultured to get purity on fructose supplemented basal medium-g/L (Fructose-20 g, peptone-1.5 g, NH_4SO_4-0.1 g, KH2PO_4- 0.1 g, $MgSO_4$-0.05 g, yeast extract-1 g, agar-2 g, $CaCl_2$- 0.1 (Zhang et al., 2011) The selected two yeast isolates were characterized as *Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34 (AB693154) (Chaudhary and Karita, 2017). All the strains utilized in this study were not genetically modified. *S. cerevisiae* was also employed as a control group because the strain is widely used in commercial distilleries throughout Pakistan to produce ethanol. Yeast isolates were purified on MYG (malt extract-yeast extract-glucose) medium

Sucrose hydrolysis of watermelon pulp employing Plackett-Burman model

A Plackett-Burman Design (PBD) was used to identify the relevant factors that have a significant influence on the hydrolysis of disaccharide fraction of watermelon pulp by *B. cereus* FA3. PBD is an already established statistical screening approach that helps in determining the most significant variables in the experimental procedures (Brereton, 2003). Six independent variables designated for screening were; the volume of watermelon pulp, volume of buffer, dosage of crude enzyme, time of incubation, temperature and pH. To create a 12-run PB experimental matrix, Design Expert Software (version 6.0.8) was used. Each experimental experiment involved the use of sterilized pulp and acetate buffer at a certain pH, addition of particular dosage of crude enzyme and incubation at the use of specific time and temperature conditions.

B. cereus FA3 was cultured in a basal medium. The culture was centrifuged to collect the enzyme-rich supernatant (Bai et al., 2012; Abu-Gharbia et al., 2018). The acetate buffer was composed of 5.722g Sodium acetate and 1.778g glacial acetic acid per liter, with pH adjusted to 4 using HCl. This buffer was selected due to its compatibility with invertase activity at low pH values (Yucekan and Onal, 2011). After enzymatic hydrolysis, samples from each run were collected and analyzed for reducing sugars using DNS method (Miller, 1959) and total sugars via the Phenol- sulfuric acid method (Dubios et al., 1956). The results were statistically analyzed using ANOVA by software.

Validation of predicted PB parameters

To validate the predicted screened conditions obtained through the PB model, experiments were performed in triplicates. The experimental values with less error, confirming the accuracy of the PB based screening of water melon pulp hydrolytic parameters.

Central composite design based optimization of ethanolic fermentation factors

To optimize the ethanolic fermentation process, a central composite design (CCD) was employed. CCD is a widely used response surface methodology that allows accurate estimation of optimum operational parameters (Bhattacharya, 2021). For each yeast isolate, a set of 20 experimental runs was conducted. The three independent variables; enzymatic hydrolysate, incubation time, and fermentation temperature. Each experimental flask was prepared by adding enzymatic hydrolysate (prepared according to PBD- optimized parameters) and 5% yeast inoculum. Sterilized synthetic medium was used to make up the final volume to 100mL.

The yeast inoculum was prepared by cultivating the strains overnight in MYGP (Malt, Yeast extract, Glucose, and Peptone) medium at 30 °C. The synthetic fermentation media consisted of (g/L): 6.5g Yeast extract, 2.6g ammonium sulphate-$(NH_4)_2SO_4$, 2.72g KH_2PO_4, 0.8g magnesium sulphate heptahydrate, 0.3g calcium chloride,0.00042g zinc chloride, 1.5g citric acid, and 6g sodium acetate (Chaudhary et al., 2020). Flasks that were inoculated were then incubated on specified time and temperature as designated in CCD runs. Each run was tested on the basis of the ethanol contents (g/L) as measured by the acid Dichromate method (Bennett, 1971) and the quantity of reducing sugars consumed as measured by the DNS method (Miller, 1959).

Ethanol Yield was calculated using formula:

Ethanol yield (g/g) = Ethanol contents (g/L) / reducing sugar consumed (g/L) × 100
The suitability of the CCD model was statistically validated using ANOVA.

Validating of optimized conditions selected from CCD model

To validate the predicted optimal conditions obtained through the CCD model, confirmatory experiments were conducted for 15 days. Each experiment was replicated three times to ensure statistical reliability and reproducibility of the results. The correlation of ethanol yield with yeast growth was plotted.

RESULTS AND DISCUSSION

Compositional analysis of watermelon pulp

The composition of watermelon pulp was measured biochemically. The moisture and ash contents (%) were 88±0.16, 0.28±0.01 respectively. The total and reducing sugars were assessed as 75.11±0.02, 37.45±0.14 (g/L) correspondingly as shown in table (1).

Table 1. Proximate analysis of watermelon pulp.

Parameters	Contents*
Total sugars(g/L)	75.11±0.02
Reducing sugars (g/L)	37.45±0.14
Ash (%)	0.28±0.01
Moisture (%)	88.0±0.16

*Data for contents are expressed as mean ± S.E.M., based on three replicates.

Plackett-Burman design for enzymatic hydrolysis

Spectrophotometric analysis data for reducing and total sugars of all experimental run in PB model were presented in Table 2. The enzymatic hydrolysis of watermelon pulp using *B. cereus* FA3 resulted in a high concentration of reducing sugars reaching 45.13±0.01 after 5 days under conditions of 0.65IU enzyme dose, water melon pulp: buffer ratio 55:50 mL, pH 4, and at 37 °C. Under the same conditions, total sugar content reached 82.64±0.06 g/L. These results suggested that the *B. cereus* FA3 based sucrase enzyme effectively hydrolyzed sucrose into glucose and fructose, as indicated by the increased total sugar contents following hydrolysis. The ANOVA based data for reducing sugar (table 3) indicated that the PB model is statistically significant, with F-value of 16.16 and a p-value of 0.0024, suggesting that the model accounts for a significant portion of the variability in the response. Specifically, Factor F1-buffer (F=14.48, p =0.009, F3-enzyme dose (F=22.15, p =0.003, and F6-pH (F=9.69, p=0.0208) had statistically significant effects, indicating a meaningful influence on reducing sugar levels. The residual mean square was 1.29, and the model explained approximately 92.3% of the total variance (104.06 out of 112.78), indicating a strong model of fit and high predictive accuracy. In ANOVA, residual of the model led to unexplained variation. In this model, the relatively low residual mean square as compared to factor mean squares supports the adequacy of the model for reducing sugars. Similarly, the ANOVA results for total sugars in saccharified water melon pulp (table 3) also showed a statistically significant model (F=9.31, p=0.0066), confirming that the predictors collectively explain substantial variation in the response. Among the individual factors, Factor F1-buffer (F=11.24, p =0.0154) and Factor F4-Temperature (F=28.33, p=0.0018) significantly influenced total sugar content. Factor F6-pH (F=3.84, p =0.0978) showed a moderate but non-significant effect. The model accounts for 88.6% of the total variation (709.11 out of 800.49), indicating a good fit and reliable explanatory power. The df-6 and mean Square-15.2298 of the residual accounts for experimental error and unexplained variability of the model. The residual mean relative to model mean square indicated the model fitness.

Validation of predicted hydrolytic parameters and responses using PB model through experimental evaluation

The comparison between experimental and predicted values (table 4) indicated a good model of accuracy under optimized conditions; 37 °C, pH 4, 50:55mL buffer to pulp ratio, 0.65 IU invertase dosage, and a 5 days hydrolysis period. For reducing sugars, the experimental value was 45.13 while predicted value was 40.00g/L, resulting in a residual of 5.13 and a percentage error of 12.8%. The error 12.8% seems high but the deviation is acceptable and adds to the reliability of PBD. The deviation may be due to variable enzyme dosage and biomass association along with certain environmental factors viz temperature, pH etc. For total sugars, the experimental value was 82.64g/L compared to predict as 79.00g/L, yielding a residual of 3.64 and a 4.6% error. These low percentage errors confirm the reliability and predictive accuracy of the regression model.

CCD based optimization of fermentation conditions for ethanologenesis

Table (5) recorded the contents of ethanol and yield values of different conditions described in the CCD model. The

Table 2. PBD for screening of watermelon pulp hydrolysis factors and effect on responses.

Run	F1-Buffer (mL)	F2-Pulp (mL)	F3-Enzyme dose (IU)	F4-Temp (Celsius)	F5-Time (Days)	F6-p H	RS (g/L)	TS (g/L)
1	40	50	0.65	30	1	5	35.14±0.06	60.29±0.06
2	40	25	0.65	30	1	4	34.69±0.07	59.82±0.07
3	40	50	1.3	37	5	4	40.94±0.01	68.94±0.05
4	55	50	0.65	30	5	4	43.86±0.05	63.98±0. 09
5	40	25	0.65	37	5	5	45.13±0.01	82.64±0.06
6	55	25	1.3	37	1	5	36.12±0.03	61.39±0.06
7	40	25	1.3	30	5	5	35.83±0.06	62.73±0.04
8	55	50	0.65	37	1	5	36.74±0.04	60.97±0.09
9	40	50	1.3	37	1	4	37.65±0.01	61.42±0.05
10	55	25	1.3	30	1	4	34.87±0.05	59.98±0.06
11	55	50	1.3	30	5	5	41.78±0.03	77.24±0.05
12	55	25	0.65	37	5	4	40.97±0.04	80.29±0.07

*Data for responses are expressed as mean ± S.E.M., based on three replicates.

Table 3. Analysis of Variance of PBD model of watermelon pulp hydrolysis for various responses.

	Source	Sum of Squares	DF	Mean Square	F Value	Prob>F	
Reducing Sugars in saccharified pulp	Model	104.0553	5	20.8111	16.1594	0.002	Significant
	F1	18.6501	1	18.6501	14.4820	0.009	
	F2	35.07	1	35.07	10.64	0.290	
	F3	28.5208	1	28.5208	22.1467	0.003	
	F4	27.90	1	27.90	4.24	0.132	
	F5	0.1587	1	0.1587	0.1232	0.738	
	F6	45.2408	1	45.2408	9.6945	0.021	
	Residual	5.8669	6	1.2878			
	Cor Total	112.7822	11				
Total Sugar in saccharified pulp	Model	709.1076	5	141.8215	9.3123	0.007	Significant
	F1	171.2341	1	171.2341	11.2434	0.015	
	F2	0.91	1	0.91	0.149	0.971	
	F3	2.7170	1	2.7170	0.1784	0.688	
	F4	431.4002	1	431.4002	28.3260	0.002	
	F5	36.06	1	36.06	6.49	0.134	
	F6	58.4767	1	58.4767	3.8396	0.098	
	Residual	91.3789	6	15.2298			
	Cor Total	800.4865	11				

Table 4. Predicted parameter validation of PBD for watermelon pulp hydrolysis.

Contents	Experimental value	Predicted value	Residual	% Error
Reducing Sugar (g/L)	45.13	40.00	5.13	12.8
Total sugar (g/L)	82.64	79.00	3.64	4.6

two yeast strains had the highest ethanol concentration at 25 °C with the ratio of 75:25 mL of hydrolysate to synthetic media after 15 days incubation. In this case, the yield of ethanol by *M. cibodasensis* Y34 and *S. cerevisiae* K7were 0.40 ± 0.03 and 0.39 ± 0.02g/g, correspondingly.

The ANOVA (Table 6) ethanol yield data with the use of *S. cerevisiae* K7 revealed that the model was statistically significant ($F=5.48$, $p<0.005$), which proved that the model was useful to explain the variation in the production of ethanol. The significance of Lack of Fit test ($F=5851.25$, $p<0.0001$) indicated that there was some deviation between the model and the observed data. Nonetheless, low values of pure error and residual mean square indicated that the

precision of model was good. The reliable predictive capability of model was accounted for a total sum of squares of 0.085 and 19 degrees of freedom. The effectiveness of the strain under optimal conditions was observed by 0.39 ±0.03 g/g yield. In table 6, the ANOVA *of M. cibodasensis* Y34 fermentation showed that the volume of hydrolysate, incubation time, and temperature had a significant impact on the yield of ethanol (0.40 ± 0.03g/g) and ethanol content (13.5 ± 0.07 g/L), although the Lack of Fit ($p < 0.001$) indicated considerable unexplained variability. There was also a significant effect on yeast growth ($p = 0.044$), with the adequate model fit ($p = 0.343$). These results were confirmed by the validation experiments, whereby the same results were identical in terms of ethanol yield, content, and biomass production after 15 days and thus validated the strength of the optimized fermentation conditions in the production of bioethanol.

Table 5. CCD based matrix of yeast response on hydrolyzed watermelon pulp**.**

Exp.	Variables			*Metschnikowia cibodasensis* (Y34)			*Saccharomyces cerevisiae* (K7)		
	Hydrolysate (mL)-X	Days-Y	Temp °C-Z	Ethanol Yield	Ethanol Production	Yeast Growth	Ethanol Yield	Ethanol Production	Yeast Growth
1	50	8	45.11	0.30± 0.02	7.09±0.09	1.56±0.03	0.26±0.02	6.00±0.09	1.31±0.01
2	25	1	40	0.23±0.02	4.04±0.08	0.60±0.07	0.21±0.03	3.08±0.09	0.60±0.02
3	50	8	19.8	0.34±0.03	11.1±0.01	2.05±0.01	0.34±0.02	10.09±0.01	1.14±0.04
4	75	1	40	0.24±0.01	2.40±0.01	0.53±0.02	0.25±0.02	2.11± 0.01	1.77± 0.04
5	50	19.7	32.5	0.37±0.04	13.0±0.09	2.65±0.01	0.35±0.05	11.51±0.09	1.36±0.04
6	25	15	40	0.37±0.01	11.3±0.03	1.83±0.01	0.38±0.04	9.97± 0.01	1.47± 0.03
7	50	8	32.5	0.37±0.01	12.1±0.08	1.13±0.02	0.33±0.09	10.53± 0.02	1.39±0.04
8	75	15	25	0.40±0.03	13.5±0.07	2.14±0.02	0.39±0.02	11.75±0.05	2.60±0.02
9	50	-3.77	32.5	0.09±0.01	0.56±0.02	0.92±0.02	0.11±0.01	0.56± 0.03	1.29 ±0.05
10	7.96	8	32.5	0.38±0.002	13.0±0.08	2.45±0.0006	0.36±0.07	11.55±0.02	1.90 ±0.07
11	50	8	32.5	0.37±0.03	12.1±0.08	2.37±0.02	0.33±0.04	10.51± 0.02	1.11±0.05
12	75	1	25	0.28±0.01	4.05±0.09	0.45±0.01	0.26±0.05	3.37±0.09	0.75±0.01
13	50	8	32.5	0.37±0.05	12.1±0.08	2.38±0.005	0.34±0.04	10.58±0.08	1.18± 0.01
14	25	15	25	0.36±0.06	11.6±0.01	2.20±0.004	0.34±0.05	10.06±0.08	0.61±0.02
15	50	8	32.5	0.38±0.01	12.1±0.009	2.34±0.01	0.33±0.004	10.55±0.05	1.24±0.07
16	92	8	32.5	0.34±0.01	8.26±0.01	1.62±0.03	0.32±0.01	7.58±0.05	2.12±0.04
17	50	8	32.5	0.37±0.06	12.0±0.001	2.34±0.01	0.33±0.01	10.54±0.01	1.19±0.07
18	25	1	25	0.25±0.07	3.01±0.008	0.76±0.03	0.26±0.07	2.96± 0.09	0.43±0.03
19	50	8	32.5	0.37±0.02	12.0±0.001	2.33±0.01	0.26±0.01	10.55± 0.01	1.44±0.02
20	75	15	40	0.34±0.03	11.0±0.01	1.93±0.09	0.35±0.01	10.86±0.03	1.90±0.02

*Data for responses are expressed as mean ± S.E.M., based on three replicates. Ethanol yield-g/g, Ethanol production-g/L, Yeast growth-O.D**.**

Inter-relationship of different variables for ethanol yield

The inter-relationship of factors for both yeasts were represented by the following equations:

Ethanolic Yield Response for *S. cerevisiae* K7 =

0.30343 + 0.00728459*X +0.0361761*Y -0.0223059*Z-0.000238*X*Y-1.9459X*Z+1.77349*Y*Z-0.0097X2-0.00223Y2+1.654Z2+0.0025

Ethanolic Yield Response for *M. cibodasensis* Y34 =

+0.21727 +0.021199*X +0.010083*Y +0.0097016*Z-0.002299*X*Y-0.006108*X*Z+0.002023*Y*Z+1.0097X2+0.0823 Y2+0.0654 Z2+0.0039

The signs in the equations denoted the nature of the interaction, with positive values indicating synergistic and negative values indicating antagonistic effects on the response.

Validation of optimized fermentation selected from CCD model

The application of optimized conditions based on the CCD model led to improve the experimental outcomes for both yeasts. Under these conditions, *S. cerevisiae* K7 achieved an ethanol yield of 0.41g/L and an ethanol content of 12.2g/L while *M. cibodasensis* Y34 produced slightly higher yield of 0.43g/L and ethanol content of 13.6g/L (table 7).

Figure (1) indicated a positive correlation between fermentation time, yeast growth, and ethanol production. In the early fermentation days (1-4), ethanol production as well as yeast growth was low for both strains, indicating lag phase. From days 5 to 10, a steady increase in growth and ethanol yield was observed. The maximum ethanol yield was

achieved between days 13 and 15 in log phase with both yeast strains. *M. cibodasensis* Y34 demonstrated greater ethanol yield efficiency, making it a promising candidate for bioethanol production from watermelon pulp hydrolysate.

Table 6. ANOVA analysis of yeasts response on ethanol yield and content.

Responses	Yeasts	Origin	Sum of square	DF	Mean Square	F value	P > value
Ethanol Yield	Y34	Model	0.066	6	0.011	3.57	0.026 Significant
		Residual	0.040	13	0.00306		
		Lack of Fit	0.040	8	0.005	62935.22	<0.0001 Significant
		Pure Error	0.0039	5	0.0079		
		Cor Total	0.11	19			
	K7	Model	0.061	6	0.010	5.48	<0.005 Significant
		Residual	0.024	13	0.001847		
		Lack of Fit	0.024	8	0.00319	5851.25	<0.0001 Significant
		Pure Error	0.0025	5	0.005129		
		Cor Total	0.085	19			
Ethanol Contents	Y34	Model	237.54	6	39.59	5.31	0.006 Significant
		Residual	96.95	13	7.46		
		Lack of Fit	96.95	8	12.12	0.001306	<0.0001 significant
		Pure Error	0.00464	5	0.0009281		
		Cor Total	334.49	19			
	K7	Model	188.96	6	31.49	5.56	<0.005
		Residual	73.68	13	5.67		
		Lack of Fit	73.67	8	9.21	15872.35	<0.0001 Significant
		Pure Error	2.901E-003	5	0.005802		
		Cor Total	262.64	19			

The current study has validated the effective valorization of watermelon (*Citrullus lanatus*) pulp waste to bioethanol by an integrated approach that involves enzymatic hydrolysis and a statistically optimized fermentation. Since Pakistan has a large agricultural production and hence fruit wastes are produced, watermelon pulp transformation to bioethanol will be a sustainable solution towards renewable energy production and waste management of the environment. Recent studies have highlighted the relevance of fruit-based agro-wastes as bioethanol feedstock since they contain high sugar content and low levels of lignin complexity compared to lignocellulosic residues (Shah et al., 2019; Mgeni et al., 2024). Using watermelon pulp for bioethanol has a number of advantages to Pakistan. It offers a two-fold solution to the issue of agricultural waste management and the existence of renewable energy source. The use of it could also lower environmental pollution in the form of the decomposition of the organic waste that may cause the emission of methane and water pollution (Amicarelli et al., 2021). It can also facilitate the implementation of a circular economy in the agricultural sector as this would enable the addition of value to the waste streams, thereby boosting the lives of rural populations and establishing new bioenergy markets (Scapini et al., 2023). The highest reducing sugar and total sugar concentration of 45.13±0.01 g/L and 82.64±0.06 g/L respectively obtained by Plackett Burman model has demonstrated that Bacillus cereus FA3 is effective in enzymatic hydrolysis of watermelon pulp. Efficiencies of bacterial sucrase/invertase systems have been reported in fruit wastes with high content of sucrose, such as mango, pomegranate, and watermelon residue (Saleem et al., 2022; Chaudhary et al., 2024). The results of the ANOVA

Table 7. Optimum watermelon pulp hydrolysate fermentation validation.

	Metschnikowia cibodasensis (Y34)			*Saccharomyces cerevisiae* (K7)		
Days	Ethanol Yield*	Ethanol Content*	Sugar consumption*	Ethanol Yield*	Ethanol Content*	Sugar consumption*
1	0.18±0.004	2.08±0.04	11.1±0.01	0.17±0.005	1.91±0.009	10.7±0.08
2	0.19±0.001	2.20±0.005	11.2±0.08	0.19±0.004	2.07±0.01	10.7±0.09
3	0.21±0.001	2.97±0.01	14.0±0.06	0.19±0.005	2.11±0.014	10.9±0.08
4	0.22±0.001	3.16±0.0009	14.2±0.06	0.22±0.006	2.66±0.014	12.0±0.04
5	0.24±0.001	3.91±0.01	16.0±0.01	0.22±0.005	2.70±0.009	12.1±0.07
6	0.26±0.005	4.50±0.09	17.1±0.02	0.24±0.005	3.31±0.0009	13.6±0.04
7	0.27±0.0005	5.23±0.01	18.8±0.01	0.26±0.01	3.77±0.091	14.1±0.08
8	0.30±0.0003	6.29±0.01	20.9±0.02	0.27±0.005	4.13±0.0009	15.0±0.06
9	0.31±0.0001	7.87±0.01	24.9±0.02	0.29±0.004	4.71±0.008	16.0±0.09
10	0.32±0.0009	8.29±0.01	25.7±0.03	0.31±0.004	5.63±0.01	17.9±0.09
11	0.33±0.0009	9.15±0.03	26.9±0.01	0.33±0.005	6.61±0.01	19.9±0.03
12	0.35±0.001	10.0±0.04	27.8±0.03	0.35±0.003	7.53±0.01	20.9±0.07
13	0.42±0.0009	12.4±0.01	29.1±0.03	0.37±0.004	8.64±0.02	22.9±0.08
14	0.40±0.0007	12.2±0.03	29.8±0.03	0.38±0.005	9.73±0.02	25.0±0.06
15	0.43±0.0007	13.6±0.008	31.2±0.03	0.41±0.004	12.2±0.001	29.7±0.05

*Ethanol yield-g/g, Ethanol production-g/L, Sugar consumption-g/L, values are mean±S.E.M (standard error mean).

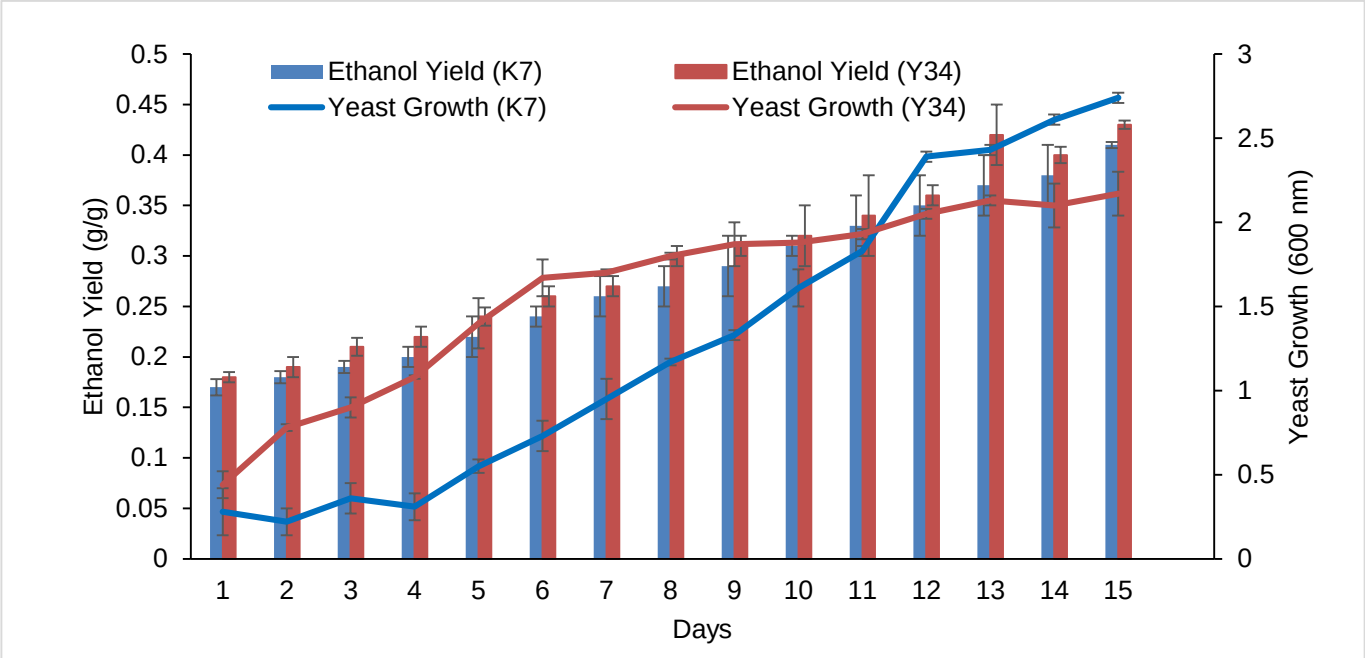

Figure 1. Correlation of Ethanol Yield and yeast growth in fermentation medium containing hydrolyzed water melon pulp.

analysis showed that the model was statistically significant in reducing (F=16.16, p=0.002), total sugars (F=9.31 p=0.006) with model df -5 and residual error df-6 and the models accounted more than 88 percent of the variance. In overall model the selected parameters significantly influenced reducing and total sugar production. The residual mean square (1.2878, 15.2298) for reducing and total sugars represented experimental error and unexplained variation, confirming adequate model fit. The selected parameters were 0.65IU enzyme dose, water melon pulp: buffer ratio 55:50mL, PH 4 and 37 ^{o}C. The fact that the predicted and experimental values are very close and the percentage error is less than 8% justifies the accuracy and reliability of the model. This proves the efficiency of *B. cereus* FA3 sucrase to degrade sucrose into fermentable sugars in water melon pulp. The enzyme was active in acidic pH (pH 4) and moderate temperatures (37 ^{o}C) and these findings were in agreement with previous reports with optimal enzyme performance under slightly acidic conditions (Jatoi et al., 2025; Kalangi et al., 2025).

The fermentative potential of *Saccharomyces cerevisiae* and *Metschnikowia cibodasensis* are of great interest in the bioethanol industry. The *Metschnikowia cibodasensis*Y34 in the current study had maximum ethanol yield and contents. *S. cerevisiae*, which has traditionally been used in baking and brewing, is famously known to produce ethanol commercially. This yeast is genetically stable and can withstand the stress-induced conditions such a low pH and high concentration of ethanol (Lane et al., 2018). Contrary to *Metschnikowia cibodasensis*, a species belonging to the genus *Metschnikowia* demonstrates high-level ethanol tolerance and effective fermentation ability particularly in non-traditional substances such as saccharified watermelon pulp (Mendon procurement et al., 1993; Kurtzman et al., 2011; Quirós et al., 2014). These yeasts have provided a significant possibility for optimized bioethanol production, whereby *S. cerevisiae* served as a strong control strain and *M. cibodasensis* is a possible substitute fermentative organism in advanced biofuel applications.

The ANOVA test on the production of ethanol revealed that both *Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34 had significant response to the factors of fermentation including the volume of hydrolysate, incubation period and temperature. In the case of *S. cerevisiae* K7, the model was significant ($F = 5.48$, $p < 0.005$) and much of the variation in the ethanol yield was attributed to it, with the highest yield being 0.41±0.004 g/L with ethanol content 12.2 ± 0.001 under optimal conditions. Nonetheless, the appropriateness in lack of fit ($p < 0.0001$) indicated certain differences between the predicted and experimental values. Likewise, *M. cibodasensis* Y34 was significantly affected by these factors on ethanol yield (0.43 ±0.007 g/L) and ethanol content (13.6 ± 0.008 g/L), and the growth of the yeast ($p = 0.0436$), which up to reached 2.17 ± 0.01 (OD). Though there is a significant lack of fit ($p < 0.0001$). These optimized conditions were verified in successful validation experiments using both yeasts. *M. cibodasensis* Y34 had slightly higher ethanol yield and biomass than *S. cerevisiae* K7 indicating to its possible use as an effective alternative to conventional yeast in bioethanol production. This high performance can be explained by the high adaptability of non-conventional yeasts to fruit-based substrates and their capacity to effectively metabolize mixed sugars (Moon et al., 2025). Scapini et al. (2023) described that water melon pulp has high potential for bioethanol generation up to 90 percent of yield during numerous fermentation cycles. In addition, watermelon juice, with a high content of amino nitrogen (~ 400 mg N/L), favored fast fermentation in pH 5 to reach 0.41-0.46 g/g of ethanol yields at 25% sugar, and 0.36-0.41 g/g at 35% sugar (Fish et al., 2009). Ajavo et al. (2022) achieved similar findings on paper mulberry fruit juice (PMFJ) that produced an ethanol concentration of 73.69g/L, productivity of 4.61 g/L/hr., and yield of 0.48g/g. The positive association was found between the increase in yield of ethanol and yeast growth indicating the simultaneous formation of biomass and ethanol production in the exponential growth cycle of fermentation. The considerable increase in ethanol was observed after the fifth day and reached its peak till day 15 with the highest yeast biomass. Comparable growth- production correlations have been documented with fruit juice and pulp fermentations, in which nutrient rich feedstock enhance persistent metabolic activity (Bashir et al., 2025).

The data obtained by the current study allow adopting agro-waste valorization as part of the system of renewable energy in Pakistan. The use of discarded watermelon pulp to produce bioethanol is twofold, as it reduces pollution caused by organic waste, and allows energy security (Mgeni et al., 2024; Chaudhary et al., 2024). The improved ethanol production capability of *M. cibodasensis* Y34 highlights the potential of unconventional yeasts in advanced bioethanol production practices.

CONCLUSION

Bacillus cereus FA3 efficiently hydrolyzed watermelon pulp, producing maximum reducing (45.13 ± 0.01 g/L) and total sugar (82.64 ± 0.06 g/L). Fermentation with *M. cibodasensis* Y34 and *S. cerevisiae* K7 yielded maximum ethanol concentrations of 0.43 ± 0.007 g/L and 0.41 ± 0.004 g/L, respectively, after 15 days. Non-conventional yeasts such as *M. cibodasensis* Y34 may prove valuable for future bioethanol applications due to their enhanced fermentative potential.

AUTHOR'S CONTRIBUTION

The experimentation was conducted by Afifa Syeda and Sher Ali as part of their study. Asma Chaudhary is responsible for the design and conduction of experiments. Ayesha Aihetasham is acknowledged for facilitation of the substrate collection and analysis. Amina Asghar and Pakeeza Fatima is accredited for biochemical profiling and statistical analysis. The financial support was given by University of Education, Lahore, Pakistan.

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AVAILABILITY OF DATA AND MATERIAL

The collected and analyzed data is presented in the form of tables and figures.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The current study was checked and approved by the relevant team.

CONSENT FOR PUBLICATION

All authors have reviewed the manuscript and approved it for publication.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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REFERENCES

Abu-Gharbia, M. A., El-Sawy, N. M., Nasr, A. M., & 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. Journal of Pharmaceutical and Applied Chemistry, 4(2), 1-13.

Ajayo, P. C., Huang, M., Zhao, L., Tian, D., Jiang, Q., Deng, S., ... & Shen, F. (2022). Paper mulberry fruit juice: a novel biomass resource for bioethanol production. Bioresources and Bioprocessing, 9(1), 3.

Amicarelli, V., Lagioia, G., & Bux, C. (2021). Global warming potential of food waste through the life cycle assessment: An analytical review. Environmental Impact Assessment Review, 91, 106677.

Bai, L., Hu, H., & Xu, J. (2012). Influences of configuration and molecular weight of hemicelluloses on their paper-strengthening effects. Carbohydrate Polymers, 88(4), 1258-1263.

Bashir, T., Nisar, A., Habib, G., & Gul, H. (2025). Efficient production of bioethanol and methanol from unappetizing pumpkin by separate saccharification and fermentation process using *Saccharomyces cerevisiae*. Biofuels, 1-12.

Bennett, C. (1971). Spectrophotometric acid dichromate method for the determination of ethyl alcohol. The American Journal of Medical Technology, 37(6), 217-220.

Bhattacharya, S. (2021). Central composite design for response surface methodology and its application in pharmacy. In Response surface methodology in engineering science. IntechOpen.

Bianchi, G., Rizzolo, A., Grassi, M., Provenzi, L., & Scalzo, R. L. (2018). External maturity indicators, carotenoid and sugar compositions and volatile patterns in 'Cuoredolce®'and 'Rugby'mini-watermelon (Citrullus lanatus (Thunb) Matsumura & Nakai) varieties in relation of ripening degree at harvest. Postharvest Biology and Technology, 136, 1-11.

Chaudhary, A., & Karita, S. (2017). Screening of yeast isolates from flowers for effective ethanol production. Turkish Journal of Biology, 41(6), 890-900.

Chaudhary, A., Akram, A. M., Ahmad, Q.U.A., Minahal, Q., Ara, C., Andleeb, S., Iqtedar, M. and Ali, Q., 2020. Rsm-Based Fermentative Ethanologenesis Employing Acid Hydrolysate Watermelon Peels. Plant Cell Biotechnology and Molecular Biology, 21(63&64):63-77.

Chaudhary, A., Hussain, Z., Ajmal, H., Abdul Rehman, R., Abbas, G., Aihetasham, A., & Tahira, S. A. (2024). Efficient Bioconversion of Mango Waste into Ethanol Employing Plackett–Burman and Central Composite Models. ACS Omega, 9(38), 39652-39662.

Dube, J., Ddamulira, G., & Maphosa, M. (2021). Watermelon production in Africa: challenges and opportunities. International Journal of Vegetable Science, 27(3), 211-219.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28(3), 350-356.

Falade, O. S., Otemuyiwa, I. O., Adekunle, A. S., Adewusi, S. A., & Oluwasefunmi, O. (2020). Nutrient composition of watermelon (Citrullis lanatus (Thunb.) Matsum. &Nakai) and egusi melon (*Citrullus colocynthis* (L.) Schrad.) seeds. Agriculturae Conspectus Scientificus, 85(1), 43-49.

FAO (2023). FAOSTAT. Food and Agriculture Organization of the United Nations, Rome, Italy. http://www.fao.org/faostat/en/#data.

Figueroa, R. H. H., López-Malo, A., & Mani-López, E. (2024). Antimicrobial activity and applications of fermentates from lactic acid bacteria–a review. Sustainable Food Technology, 2(2), 292-306.

Gebeyhu, B., & Markos, G. (2023). Assessment of soil mulching field management, and deficit irrigation effect on productivity of watermelon varieties, and Aqua Crop model validation. Heliyon, 9(11).
Jatoi, A. S., Nguyen, H. M., & Ahmed, J. (2025). Enhanced acido-thermophilic invertase production via a derepressed mutant of Pichia kudriavzevii in batch cultures. Biomass Conversion and Biorefinery, 1, 713–727.
Kalangi, C., Rathod, N. J., Madhuri, K. S., Kumar, S., Pandey, V., Sahni, M., & Leon-Castro, E. (2025). Performance optimization of ethanol blends in diesel model using Taguchi and grey relational approach. Scientific Reports, 15(1), 36048.
Kurtzman, C., Fell, J. W., & Boekhout, T. (Eds.). (2011). The yeasts: a taxonomic study. Elsevier.
Lane, S., Xu, H., Oh, E. J., Kim, H., Lesmana, A., Jeong, D., ... & Kim, S. R. (2018). Glucose repression can be alleviated by reducing glucose phosphorylation rate in Saccharomyces cerevisiae. Scientific Reports, 8(1), 2613.
Mahmood, K., & Munir, S. (2018). Agricultural exports and economic growth in Pakistan: an econometric reassessment. Quality & Quantity, 52(4), 1561-1574.
Mendonça-Hagler, L. C., Hagler, A. N., & Kurtzman, C. P. (1993). Phylogeny of Metschnikowia species estimated from partial rRNA sequences. International Journal of Systematic Bacteriology, 43(2), 368-373.
Mgeni, S. T., Mero, H. R., Mtashobya, L. A., & Emmanuel, J. K. (2024). The prospect of fruit wastes in bioethanol production: A review. Heliyon, 10(19).
Miller, G. L. (1959). Modified DNS method for reducing sugars. Analytical Chemistry, 31(3), 426-428.
Moon, S. Y., An, N. Y., & Lee, J. Y. (2025). Transforming non-conventional yeasts into key players in biotechnology: advances in synthetic biology applications. Frontiers in Microbiology, 16, 1600187.
Nadeem, M., Navida, M., Ameer, K., Siddique, F., Iqbal, A., Malik, F., ... & Javaria, S. (2022). Watermelon nutrition profile, antioxidant activity, and processing. Food Science and Preservation, 29(4), 531-545.
Quirós, M., Rojas, V., Gonzalez, R., & Morales, P. (2014). Selection of non-Saccharomyces yeast strains for reducing alcohol levels in wine by sugar respiration. International Journal of Food Microbiology, 181, 85-91.
Saleem, A., Hussain, A., Chaudhary, A., Ahmad, Q. U. A., Iqtedar, M., Javid, A., & Akram, A. M. (2022). Acid hydrolysis optimization of pomegranate peels waste using response surface methodology for ethanol production. Biomass Conversion and Biorefinery, 12(5), 1513-1524.
Samantaray, B., Mohapatra, S., Mishra, R. R., Behera, B. C., & Thatoi, H. (2024). Bioethanol production from agro-wastes: a comprehensive review with a focus on pretreatment, enzymatic hydrolysis, and fermentation. International Journal of Green Energy, 21(6), 1398-1424.
Scapini, T., Bonatto, C., Dalastra, C., Bazoti, S. F., Camargo, A. F., Júnior, S. L. A., ... & Treichel, H. (2023). Bioethanol and biomethane production from watermelon waste: a circular economy strategy. Biomass and Bioenergy, 170, 106719.
Shah, K. R., Vyas, R., & Patel, G. (2019). Bioethanol production from pulp of fruits. Bioscience Biotechnology Research Commmunications, 12(2), 464-471.
Shrefler, J., Brandenberger, L., Rebek, E., Damicone, J., & Taylor, M. (2015). Watermelon production. Oklahoma Cooperative Extension Service, HLA-6236.
Tarazona-Díaz, M. P., Viegas, J., Moldao-Martins, M., & Aguayo, E. (2011). Bioactive compounds from flesh and by-product of fresh-cut watermelon cultivars. Journal of the Science of Food and Agriculture, 91(5), 805-812.
Yücekan, İ., & Önal, S. (2011). Partitioning of invertase from tomato in poly (ethylene glycol)/sodium sulfate aqueous two-phase systems. Process Biochemistry, 46(1), 226-232.
Zhang, L., Zhao, H., Gan, M., Jin, Y., Gao, X., Chen, Q., ... & Wang, Z. (2011). Application of simultaneous saccharification and fermentation (SSF) from viscosity reducing of raw sweet potato for bioethanol production at laboratory, pilot and industrial scales. Bioresource Technology, 102(6), 4573-4579.