Zoo Botanica
ISSN: 3007-2050 (E), 3007-2042 (P)

Research Article

Cane molasses-based ethanol for optimization by sucrolytic modeling

Samavia Younas[1], Asma Chaudhary[1], Esha Rehman[1], Bushra Bilal[1], Ayesha Aihetasham[2], Syeda Anjum Tahira[3]

[1] *Department of Zoology, Division of Science and Technology, University of Education, Lahore 54770, Pakistan.*
[2] *Institute of Zoology, University of the Punjab, Lahore 54590, Pakistan.*
[3] *Department of Botany, University of Okara 56300, Okara, Pakistan.*

ABSTRACT

To manage waste and address energy crises, the current study focuses on the concept of "Energy from Waste". Molasses, a byproduct in sugar synthesis process, is one of the most prevalent types of organic waste. Due to excessive production and use in distilleries in Pakistan, the non-fermentable portion is drained in spent wash and it is creating serious environmental problems viz soil acidification, manganese deficiency and inhibit seed germination. Effective conversion of non-fermentable sucrose portion of black strap molasses to bioethanol is the main purpose of this study. *Bacillus cereus* FA3s sucrolytic potential for bio-converting sucrose from molasses into reducing sugars for ethanologenesis was assessed. The Plackett-Burman model for hydrolytic screening and Central Composite Design for ethanologenic optimization parameters was employed. With corresponding F (9.53) and p values (0.0451), the model was significant for reducing sugars (75.52 ±0. 019 g/L) obtained via 12 IU of *B. cereus* FA3 crude enzyme dosage at 30°C in 5 days. The ethanolic yield (g ethanol/g of consumed sugars) of standard *Saccharomyces cerevisiae* K7 (0.35±0.05) and experimental *Metschnikowia cibodasensis* Y34 yeasts 0.36 ± 0.09 were also examined from 75 mL molasses hydrolyzate at 32.5°C in 8 days. Significantly better response and positive waste management development were the outcome of this study which will help improve ethanol production in the future.

Keywords: Invertases; sucrose hydrolyzing bacteria; RSM (response surface methodology); molasses as by-product; *Bacillus cereus*.

Correspondence
Asma Chaudhary
asma.ch@ue.edu.pk

Article History
Received: December 01, 2024
Accepted: December 20, 2024
Published: December 31, 2024

INTRODUCTION

In terms of quantity and value, the most valuable product is ethanol, produced through fermentation and comes from the biotechnological industries. Microbial conversion, a critical step in the production of bioethanol, is the fermentation process that yields ethanol from sugar-based feed stocks (Konur, 2023). Several potential crops produce juices with free sugar and are used in the industrial or lab-scale production of bioethanol. Some fruits, sugarcane, sweet sorghum, and sugar beet are good sources of saccharine juices, which are used as a raw material to make ethanol (Ajayo et al., 2022). Due to its significant economic benefits, sugarcane is a commercial crop grown all over the world and is of utmost importance (Dotaniya et al., 2016). Molasses, a byproduct of sugar mills, or juice can be used to produce fuel ethanol. In tropical and subtropical countries, it is the most prestigious feedstock. Variety, growth, and time of harvest affect the amount of sugar in juice (Heinrichs et al., 2017). The sugar processing industry typically produces three byproducts (%): molasses (3–5) after centrifugation, bagasse (25–30) after crushing, and press mud (3–5) after clarification (Tuck et al., 2012). A by-product of the production of sugar is molasses.

The final low-grade massecuite is extracted to produce a heavy, highly viscous liquid from which conventional techniques are unable to extract any more sugar (Solomon, 2011). About 2.5 to 4 % of molasses were produced from one tone of sugarcane. It contains the following: 35% sucrose, 20% water, 9% fructose, 7% glucose, 3% reducing sugars, 4% carbohydrate, 5% nitrogenous and non-nitrogenous compounds each, 5% ash, and 5% others (Teclu et al., 2009; Singh, 2020). Sugarcane molasses, a by-product of sugar processing that is created in large quantities as waste, is the absolute substrate for the ethanol production process in Pakistan. Molasses production has ranged from 2 to 3 million tons over the past ten years (Arshad et al., 2019).

The expansion of the sugar industry and the production of ethanol from molasses can both boost the economic condition of the farmers and ultimately contribute to the Grass Domestic Production (GDP). In the world, Pakistan ranks fifth, seventh, and eighth in terms of sugarcane production, sugar production, and sugar consumption, respectively. Five percent of Pakistan's total cropped area is used for sugarcane cultivation. In Pakistan, a sizable amount of cane molasses has been turned into alcohol during the past ten years. Ethanol is currently used as anhydrous ethanol as fuel or blended with gasoline due to its sufficiently high price on the global market (Bromberg and Cheng, 2010; Arshad and Amjad, 2012; Arshad et al., 2017). Initially, there were thirteen distilleries in Pakistan. Of these 13, 10 are connected to sugar mills. Whereby two are non-operational and eight are operational (Rashid and Altaf, 2008). It is anticipated that there will be 21 distilleries with a combined capacity of 504 million liters (Selladurai et al., 2010; Arshad et al., 2017). Currently, the existing 22 distilleries are functional for converting molasses (2.0 million tons) to alcohol annually (Parkash, 2015; Arshad et al., 2019).

The distillery used *Saccharomyces cerevisiae* to anaerobically ferment molasses at pH 4–5 pH and 30–32°C (Roberto Ometto et al., 2009). The microbes produce ethanol by fermenting glucose and reducing sugars, but they do not ferment 35% of the disaccharide sucrose. The primary challenge is the conversion of these non-fermentable sugars into ethanol, which creates a new avenue for environmentally friendly pretreatment methods. The quantity of reducing sugars is increased using the appropriate pretreatment technique. Many microorganism-derived enzymes are crucial in managing the sucrose portion of molasses as waste. Microbes can produce intracellular/extracellular invertase enzymes that hydrolyze the disaccharide sucrose, resulting in fermentable sugars (fructose and glucose) (Sanchez and Cardona, 2008; Chahed Ep Limayem, 2012). The properties of waste can also be altered by these bacterial enzymes, making it more treatable or helping to turn waste into value-added products. Overall, it seems that enzymes have a lot of promising ability for use in many waste treatment applications. The primary tools used to create invert sugars are the enzyme invertase and acid. When the sucrose structure is hydrolyzed by acid, 50% sucrose is inverted. There are no byproducts when sucrose is fully reduced to invert sugar using the invertase enzyme. There are many different enzymes. The biosphere contains a large number of these enzymes, particularly in microbes and plants. One important metabolic enzyme that comes in various isoforms is invertase. The organism's ability to survive is further enhanced by these isoforms (Andjelkovic et al., 2010). According to Kulshrestha and Tyagi (2013), these isoforms seem to control the entry of sucrose into various utilization pathways. According to Gupta and Verma (2015), the microbes chosen for fermentation and invertase production must be productive and adaptable.

The use of bacterial invertases to break down the non-fermentable portion of molasses has been crucial in managing a variety of molasses waste. Additionally, mathematical models for the optimization of fermentation parameters and the enzymatic hydrolysis of disaccharide sugars will be included. Through the integration of bacterial invertases for enzymatic hydrolysis, the study covers the state-of-the-art by utilizing mathematical modeling to convert the maximum amount of reducing sugars into ethanol.

MATERIALS AND METHODS

Selection, Collection and Preservation of Substrate

Sugarcane molasses, a by-product of the sugar processing industry, is produced in bulk as waste. Molasses possesses fermentable as well as non-fermentable sugars (NFS). The NFS consists of sucrose (non-invert sugar), dextran, amylose and amylopectin. The Pakistani distilleries exploit the fermentable and inverted sugar (80-90%) for the production of ethanol. In the present study, the remaining 10% non-inverted sucrose served as a substrate for hydrolysis by bacterial invertases and exploited for ethanol production by yeast isolates. Substrate (Cane molasses, brix 85) was collected from a local sugar factory in the district of Layyah, Punjab. The collected substrate was stored in screwed air-tight jars and was kept at 4°C for 6-8 months. Various contents of molasses were analyzed by using biochemical testing. Total (carbohydrates) and reducing sugars were calculated using the phenol−sulfuric and the 3, 5-dinitrosalicylic acid (DNS) protocols (Dubois et al., 1956; Miller, 1959). The AOAC protocols were employed for moisture contents assessment. The study was planned on the basis of sucrose (38.10±2.4%) from cane molasses as analyzed by Khairul et al. (2022).

Selected Microorganisms for The Study

Sucrose degrading bacterium (*Bacillus cereus* FA3) and ethanologenic yeast isolates (*Metchnikowia cibodasensis* Y34 and *Saccharomyces cerevisiae* K7*)* were collected from already isolated and characterized cultures from Microbiology laboratory, University of Education, Lahore, Pakistan. The bacterial strain was isolated and characterized in 2024 whereas yeast isolates in 2017 and all strains were not genetically modified. Sucrolytic bacterium *Bacillus cereus* FA3 has accession No. OQ450350 and is used for the Saccharification model. *B. cereus FA3* is the selected invertase/sucrose-producing bacterium with the potential of 0.629 IU (Chaudhary et al., 2024). The yeast isolate *Saccharomyces cerevisiae* K7 *and Metchnikowia cibodasensis* Y34 having accession numbers AB693153 and AB693154 were used for fermentation models (Chaudhary and Karita, 2017). Production of ethanol is the study's primary focus. Hence *Saccaromyces cerevisiae* is employed as a control since it is utilized in Pakistani distilleries.

PlacketT Burman Model for Sucrose Hydrolysis of Molasses

Placket Burman Design (PBD) is a well-known type of statistical design (Brereton, 2003). PBD is used for the screening of parameters for hydrolysis of the disaccharide part of molasses caused by *B. cereus* FA3. For the selection of these factors, Design Expert Software (version 6.0.8) was used for the analysis. In this study, six assigned variables viz substrate (molasses brix 85) quantity, buffer volume, *B. cereus* FA3 crude invertase dosage, time period, temperature, and pH were screened in 12 experimental designs. The selection of hydrolysis parameters of the study was based on other research conducted by different authors from the literature (Manoochehri et al., 2020; Chaudhary et al., 2024). In a conical flask, the design-specified molasses and acetate buffer (set as specified pH) concentrations were dispensed and sterilized. Following the addition of the enzyme dose, the flask was incubated at a specific temperature and time-period as specified by the design.

For crude *B. cereus* FA3 invertase, a basal medium with 7 pH and percent composition were prepared by mixing 0.01 grams $MgSO_4$, 0.1 grams of yeast extract, 0.7 grams Na_2HPO_4, 0.05 grams of sodium citrate, and 0.2 grams of potassium dihydrogen phosphate. The bacterial isolate was inoculated in basal medium, incubated at 37°C for three days followed by centrifugation. Filtrate serve as crude enzyme (Bai et al., 2012; Abu Gharbiya et al., 2018). Acetate buffer (g/L) composed of 5.772 g sodium acetate, 1.778 g acetic acid with pH 5 adjusted by HCl to make final volume of liter. Acetate buffer is the best choice for invertase assay due to adjustment of lower pH (>5) (Yucekan and Onal, 2011).

After molasses hydrolysis, samples from each experimental run were drawn out for analysis. Contents of reducing and total sugars were measured using DNS (Miler, 1959) and phenol sulphuric acid methods (Dubois et al., 1956). Statistical tools viz ANOVA and regression were used to assess the significance of the model. The set of parameters was predicted by software that was validated by performing experiments based on predicted parameters.

Central Composite Design (CCD) Based Optimization of Ethanolic Fermentation Factors

The Central Composite Design **(CCD)** was used for the optimization of ethanolic fermentation factors. It is the most common fractional factorial design used in response surface model. The biggest advantage of CCD model is the accuracy of optimization parameters (Bhattacharya, 2021). The three factor CCD model comprised a 20-run experiment was conducted each yeast separately. The factors used for CCD models were enzymatic hydrolyzate of molasses, incubation time, and reaction temperature. In the conical flask, enzymatic hydrolyzate (following predicted set of hydrolysis parameter by PBD). Yeast inoculum was used at 5%. To complete the volume up to 100mL, a sterilized synthetic medium was used. The flask was incubated at temperature and time duration specified by CCD runs. Yeast inoculum was prepared in MYG (malt, yeast extract-glucose) medium at 37°C overnight. The synthetic media (g/L) contained 6.5 grams of yeast extract, 2.6 grams of $(NH_4)_2SO_4$, 2.72 grams of KH_2PO_4, 0.8 grams of magnesium sulphate heptahydrate, 0.3 grams of calcium chloride, 0.00042 grams of zinc chloride, 1.5 grams of citric acid, and 6 grams of sodium citrate (Camelia et al., 2010). The appropriateness of the model was assessed statistically by ANOVA and regression computed by software. The responses were ethanol titer and ethanol yield. Ethanol titer and reducing sugars were assessed following the protocol with acid dichromate and DNS (Miller, 1959; Bennett, 1971).

Yield was measured as:

$$\text{Ethanol yield (g/g)} = \text{Ethanol titer (g/L)} / \text{RS consumed (g/L)} \times 100$$

Validating Optimizing CCD Model

Optimum predicted values based on CCD were assessed utilizing factors from Design Expert Software. Each response was tested in triplicate to ensure the accuracy and reliability of the predicted values.

RESULTS AND DISCUSSION

Compositional Analysis of Cane Molasses

The composition of molasses was measured biochemically. The moisture contents (%) were 19.67. The reducing (fermentable) and total (non-fermentable) sugars were assessed as 17.68, and 52g/L correspondingly (Table 1).

Plackett Burman Design for Enzymatic Molasses Hydrolysis

Spectrophotometric analysis of the reducing sugars and total sugars were performed (Table 2). A high level of reducing sugar contents in enzymatic hydrolysis of molasses with *B. cereus* FA3 reached up to 81.23± 0.26 g/L after 5 days when 12 IU crude enzyme and 50/55 mL molasses/buffer with 5 pH was loaded at 30 ˚C temperature. Total sugars contents were identified up to 204.65±2.99 g/L with the same parameters as was mentioned for reducing sugar response. From responses, it may be deduced that the *B. cereus* FA3 invertase converted the disaccharide part of molasses into monomers efficiently and it was observed by the increase in sugars concentration after hydrolysis.

The data for ANOVA to elucidate the suitability of the model for enzymatic molasses hydrolysis (Table 3**)**. For reducing sugars, the Model F-value of 9.52 implies the model is significant. There is only a 4.51% chance that a Model F-value could occur due to noise. On the other hand, for total sugars, the model was found non-significant with an F-value of 2.91 with a 20.51 % chance of occurrence due to noise. In general terms, the values of ($p > 0.05$) indicated the appropriateness of model.

Computed regression coefficients' statistical data was recorded (Table 4**)**. In enzymatic hydrolysis of molasses, the R-Squared for reducing sugars was 0.9621 which inferred the significance of model along with adj R-Squared of 0.8612. The value of 7.397 explained a sufficient signal for design space navigation in term of adequate precision. In the same way, the value of 5.183 adequate precision and r- squared 0.8859 explained the appropriate signal for total sugars. For model significance, the value of adequate precision must be more than 4. The high values for Coefficient of variation (C.V) 13.81 and press 3073.00 for reducing sugar model interpreted the significance.

Table 1: Compositional analysis of cane molasses.

Parameters	Contents
Moisture (%)	19.67 ± 0.13
Fermentable (reducing) sugars (g/L)	17.68± 0.21
Total sugars (g/L)	52± 1.73

Table 2. PBD for screening molasses hydrolysis factors and responses.

Parameter	Runs											
	1	2	3	4	5	6	7	8	9	10	11	12
Buffer (A) mL	40	55	55	40	55	55	55	40	40	55	40	40
Molasses (B) mL	25	25	50	50	25	25	50	25	25	50	50	50
Crude enzyme (C) IU	6	12	6	12	12	6	12	6	12	6	12	6
Temp (D) ˚C	30	30	37	30	37	30	30	37	37	37	37	30
Time (E) Day	1	1	1	5	1	5	5	5	5	5	1	1
pH (F)	4	4	4	4	5	5	5	5	4	4	5	5
RS (Y1)* g/L	76.28 ± 0.023	51.46 ± 0.036	40.17 ± 0.49	46.11 ± 0.020	25.06 ± 0.031	73.67 ± 0.039	81.23 ± 0.260	73.10 ± 0.031	32.75 ± 0.027	75.91 ± 0.077	71.21 ± 0.029	73.31 ± 0.131’
TS (Y2)* g/L	190.46 ± 1.45	146.46 ± 0.76	116.90 ± 0.23	119.94 ± 1.66	75.96 ± 1.022	192.23 ± 0.922	204.65 ± 2.99	180.04 ± 0.52	96.15 ± 0.31	157.14 ± 2.47	172.01 ± 0.22	188.42 ± 1.076

*Y1, Y2: Responses after enzymatic Saccharification, RS: Reducing sugars, TS: Total sugars

Table 3. Analysis of variance for PBD model of molasses hydrolysis for various responses

Responses	Source	Sum of squares	DF	Mean square	F value	P-value	Model suitability
Reducing sugars	Model	4881.62	8	610.20	9.53	0.0451	S*
	A	133.75	1	133.75	2.09	0.2441	
	B	105.07	1	105.07	1.64	0.2902	
	C	678.11	1	678.11	10.59	0.0473	
	D	271.19	1	271.19	4.24	0.1317	
	E	36.10	1	36.10	0.56	0.5072	
	F	332.27	1	332.27	5.19	0.1071	
	Residual	192.06	3	64.02			
	Cor Total	5073.68	11				
Total sugars	Model	156.30	8	19.54	2.91	0.2051	NS*
	A	14.03	1	14.03	2.09	0.2440	
	B	0.19	1	0.19	0.029	0.8755	
	C	12.72	1	12.72	1.89	0.2624	
	D	0.88	1	0.88	0.13	0.7419	
	E	30.16	1	30.16	4.49	0.1242	
	F	25.54	1	25.54	3.80	0.1462	
	Residual	20.14	3	6.71			
	Cor Total	176.44	11				

*S-significant, NS-Non significant

Table 4: Regression analysis for different responses for PBD model of molasses hydrolysis.

Treatments	Responses	C.V*	Press	R Sqr*	Adj R sqr*	Pred R sqr*	Ad pr
Hydrolysis of Molasses by *B. cereus* FA3	Reducing sugars	13.81	3073.00	0.9621	0.8612	0.7166	7.397
	Total Sugars	2.55	322.17	0.8859	0.5815	-0.8260	5.183

*C.V-coefficient variation, R Sqr-R square, Adj R sqr-Adjusted R square, Pred R sqr-Predicted R square, Ad pr-adequate precision.

Validation of Predicted Hydrolytic Parameters and Responses by PB Model via Experimentation

Predicted data from software and experimental values were presented in Table (5). The predicted values for reducing/total sugars in enzymatic hydrolysis of molasses were computed as 72.81 and 167.06 g/L respectively at 30 ˚C with 50/50 mL of buffer/molasses concentration at 5.0 pH and 12 IU enzyme dosage within 5-day hydrolytic time. When molasses underwent hydrolysis under anticipated conditions, the experimental values showed enhancement.

Table 5. Predicted parameters validation for molasses hydrolysis employing PB design.

Technique	Responses	Experimental value (g/L)	Predicted value (g/L)	*Residual	*Error (%)
B. cereus FA3 Hydrolysis	Reducing sugars	75.52±0.019	72.81	2.71	3.72
	Total Sugars	169.45±0.038	167.06	2.39	1.43

*Residual = Exp. value – Pred. value
*Error = Residual / Pred value *100

Optimization for Fermentation Conditions Based on CCD

The values presenting ethanol titer and yield according to different conditions planed by CCD was illustrated in Table (6). Both yeasts generate maximum ethanolic titer at 32.5 ˚C, with 50/50 mL hydrolyzate/synthetic medium when

https://doi.org/10.55627/zoobotanica.002.03.1007

incubated for 8 days. The ethanol yield recorded were 0.32±0.01 and 0.33±0.002 g/g of sugars by both standard, experimental yeast isolates on the same conditions as prescribed for ethanolic titer.

The CCD model's appropriateness for optimized conditions for ethaolic yield response of standard yeast was evident by 57.31, <0.0001F and p values. There is only a 0.01% chance of noise due to "Model F-Value". The 3.84 F with 0.0832 probability value inferred the non-significance of "Lack of Fit" for the response by standard yeast. Non-significant lack of fit narrated the well fitness of the model. In the same way, experimental yeast presented the appropriateness for the ethanolic yield model (14.51 F, 0.0001 p valus) and non-significant lack of fit (0.61 F, 0.7014 p values). There is a 70.14 % chance of occurring of noise due to "Lack of Fit F-value". The 3.27 F values inferred the standard yeast model significance for the second response *i.e.,* ethanolic titer while the experimental yeast implied the non-significance by 2.26 F value (Table 7). The regression analyzed data for various coefficients, adequate precision, and C.V were narrated in Table 8. The validity of the standard yeast yield model was verified by 0.9379 (R^2) and 0.8821 (Adj R-sqrd). The factors of the model corresponded with 15.33 adeq. Prec. and 9.81 C.V. Similarly, R-sqrd (0.9539), Adj R-sqrd (0.9124), Adeq. Prec (17.29), and CV (7.95) presented the yield model suitability for experimental yeast. For the ethanolic titer via standard yeast, the values of R-sqrd, Adj R-sqrd, and Adeq. Prec were 0.7466, 0.5186, and 6.491. On the other hand, R-squared, Adj R-square, and adeq. Prec values for the experimental yeast were 0.6702, 0.3733, and 4.935.

Table 6. Responses to different parameters in CCD model for molasses hydrolyzate fermentation.

Runs	Variables			Standard yeast		Experimental yeast	
	M.H/S.M* mL (X)	Incubation Days (Y)	Temp °C (Z)	Ethanolic Yield	Ethanolic Titer	Ethanolic Yield	Ethanolic Titer
1	50/45	8	45.11	0.29± 0.01	13.01±0.11	0.21±0.01	14.29±0.05
2	25/70	1	40	0.20±0.10	12.6±0.01	0.23±0.02	13.2±0.01
3	75/20	1	40	0.26±0.01	12.4±0.02	0.22±0.01	13.36±0.01
4	25/70	15	40	0.21±0.02	14.13±0.04	0.28±0.10	15.21±0.01
5	75/20	15	25	0.25±0.10	13.21±0.03	0.18±0.05	14.35±0.10
6	7.96/87.04	8	32.5	0.17±0.01	11.79±0.08	0.13±0.15	12.17±0.01
7	75/20	1	25	0.12±0.01	12.09±0.07	0.15±0.09	13.32±0.02
8	50/45	8	32.5	0.28±0.10	14.29±0.08	0.25±0.10	15.28±0.10
9	25/70	15	25	0.23±0.10	14.79±0.01	0.24±0.07	15.23±0.01
10	50/45	8	19.8	0.18±0.01	13.09±0.10	0.14±0.01	14.28±0.10
11	50/45	19.7	32.5	0.26±0.10	12.85±0.11	0.21±0.10	13.26±0.02
12	50/45	8	32.5	0.27±0.02	14.91±0.02	0.28±0.11	15.97±0.01
13	50/45	-3.77	32.5	0.16±0.02	10.27±0.07	0.13±0.08	11.26±0.02
14	50/45	8	32.5	0.32±0.01	15.32±0.02	0.33±0.02	16.78±0.10
15	50/45	8	32.5	0.27±0.01	14.28±0.03	0.30±0.02	5.27±0.01
16	92.0/3.0	8	32.5	0.19±0.02	10.33±0.06	0.13±0.06	10.39±0.01
17	50/45	8	32.5	0.28±0.00	14.31±0.09	0.31±0.02	15.28±0.00
18	25/70	1	25	0.17±0.02	12.32±0.11	0.19±0.06	13.17±0.00
19	50/45	8	32.5	0.29±0.01	15.02±0.70	0.28±0.01	16.29±0.02
20	75/20	15	40	0.17±0.01	13.36±0.21	0.25±0.11	14.37±0.01

*Ratio of molasses hydrolyzate (M.H) and synthetic medium (S.M). Yeast inoculum 5% was added in each run to make the fermentation medium volume up to 100 mL.

Validation of Optimized Parameters of Fermentation by CCD Model

Predicted optimum factors for ethanolic yield (g/g) and titer (g/L) for both yeasts were 0.31, 15.64 (K7) and 0.34, 16.55 Experimental findings for ethanolic yield and titer was enhanced when predicted conditions were applied for an experiment (Table 9).

Interrelationship of Different Variables for Ethanolic Yield Model

The interrelationship of factors for both yeasts were presented by the following equations:

Ethanolic Yield response for K7 yeast = $+0.30+0.088X-0.0034Y+0.0058Z-0.024X^2+0.00407Y^2+0.005384Z^2+0.005XY-0.015XZ-0.0075YZ+2.833$

Ethanolic Yield response for Y34 yeast = $+0.31+0.084X+0.013Y-0.004Z-0.025X^2-0.00538Y^2-0.00584Z^2-0.00875XY+0.011XZ-0.00375YZ+3.083$

Positive and negative signs in the equations represent the antagonistic and synergistic effects on the response.

Table 7: Analysis of variance for responses in CCD model for molasses hydrolyzate fermentation

Responses	Yeasts	Source*	Sum of squares	D.F	Mean of square	F value	P value
Ethanolic yield	Standard yeast	Mod	0.071	9	7.854	57.31	<0.0001 S*
		Res	1.370	10	1.370		
		LOF	1.087	5	2.174	3.84	0.0832 NS*
		PE	2.833	5	5.667		
		CT	0.072	19			
	Experimental yeast	Mod	0.065	9	7.189	14.51	0.0001 S
		Res	4.955	10	4.955		
		LOF	1.872	5	3.743	0.61	0.7014 NS
		PE	3.083	5	6.167		
		CT	0.70	19			
Ethanolic Titer	Standard yeast	Mod	13.99	9	1.55	3.27	0.0393 S
		Res	4.75	10	0.47		
		LOF	4.73	5	0.95	227.33	<0.0001 S
		PE	0.021	5	4.160		
		CT	18.74	19			
	Experimental yeast	Mod	16.69	9	1.85	2.26	0.1103 NS
		Res	8.22	10	0.82		
		LOF	8.20	5	1.64	391.51	<0.0001 S
		PE	0.021	5	4.187		
		CT	24.91	19			

*Mod-Model, Res-Residual, LOF-Lack of fit, PE-Pure error, CT-Cor total, *S-significant, NS-Non significant

Table 8. Analysis of regression for responses in CCD model for molasses hydrolyzate fermentation.

Responses	Yeasts	*C.V*	Press	*R Sqr	*Adj R sqr	*Pred R sqr	*Ad pr
Ethanolic yield	Standard yeast	9.81	0.043	0.9379	0.8821	0.6584	15.33
	Experimental yeast	7.95	0.019	0.9539	0.9124	0.8315	17.290
Ethanolic Titer	Standard yeast	24.21	35.74	0.7466	0.5186	-0.9267	6.491
	Experimental yeast	31.76	61.99	0.6702	0.3733	-1.4884	4.935

*C.V-coefficient variation, R Sqr-R square, Adj R sqr-Adjusted R square, Pred R sqrd-Predicted R square, Ad pr-adequate precision. (Y34) under hydrolyzate/synthetic medium-75/20 mL, 32.5°C for 8 days.

Table 9: Optimum molasses fermentation responses to validate the predicted values of CCD model.

Responses and yeasts	Experimental value	Predicted value	*Residual	*% Error
Ethanolic yield K7	0.35±0.05	0.31	0.04	11.4
Ethanolic yield Y34	0.36±0.09	0.34	0.02	5.55
Ethanolic titer K7	15.82±0.13	15.64	0.18	3.19
Ethanolic titer Y34	16.89±0.29	16.55	0.34	2.05

*Residual = Exp. value – Pred. value, *Error = Residual / Pred. value *100

DISCUSSION

Being an agrarian country, Pakistan generates enormous amounts of crop waste throughout the year. Certain crop juices that contain free sugar can be used directly to produce bioethanol. Sugarcane, sugar beet, and sweet sorghum are certain sugar-based feedstocks whose sugars especially glucose/other monomers are fermented microbially to produce ethanol. Among these crops, molasses is generated as a byproduct of sugar synthesis process and is considered as a promising sugary feedstock for ethanologenesis. A considerable amount of fermentable sugars, mostly fructose and glucose, are present in molasses. Either bacteria or yeast can ferment these sugars to produce ethanol. Sugarcane molasses, a waste product of sugar processing, is the only substrate used in Pakistan to produce ethanol (Driouch et al., 2010; Solomon, 2011). Molasses is widely used for the industrial production of bioethanol in Brazil. One limitation is that the higher molasses concentration results in less ethanol being produced because of osmotic pressure (Barbosa et al., 2016).

The present study focuses on the conversion of non-fermentable sugar-sucrose in molasses employing *B. cereus* FA3 invertase potential for enzymatic hydrolysis. The invertase/sucrase potential of selected bacterium was determined to be 0.629±0.015 IU. Invertase or beta-fructo furanosidase is involved in catalysis of sucrose hydrolysis to D-fructose and D-glucose (equimolar) and forming inverted sugar as an outcome. In the current study, the pH and temperature with efficient activity were noted as 5 and 30 ˚C. From the literature, it was found that bacterial invertases exhibited activity in all pH i.e., acidic, neutral, and alkaline with an optimum of 4.5 pH (Toledo et al., 2019). Bacteria are considered as the main source for the production of intracellular and extracellular invertases though other sources are animals, plants, yeasts, and fungi. Among bacteria, invertase Actinomycetales (Anthrobacter, Streptomyces, Brevibacterium), and some Bacillus strains are the best examples of invertase producers. *Bacillus cereus* is capable of producing invertase due to better safety, efficiency, and scalability (Yoon et al., 2007).

In this study, the Placket-Burman design (PBD) was followed by CCD of response surface methodology (RSM) to optimize conditions for ethanol production. Placket-Burman and central composite models are widely used by researchers for screening and optimization of experimental factors (Yan et al., 2011; Awad et al., 2013). Maximum sugars viz 81.23± 0.26 g/L (reducing sugars), 204.65 ± 2.99 g/L (total sugars) were observed at 5 pH, 30 ˚C, 12 IU enzyme dosage, 50% molasses substrate when hydrolyzed for 5 days. Yun et al. (2002) and Khandekar et al. (2014) described the higher hydrolytic activity of invertase enzyme at lower concentrations of substrate. The pH 5 is considered as the best pH for the efficient activity of invertase in *Aspergillus niger* (Manoochehri et al., 2020). Similar results were found in the present hydrolysis experiment. The values of maximum reducing sugars in the study varied from the findings of Yan et al. (2011) where 164.8 g/L reducing sugars at 4.82 pH, 55 ˚C and 142.2 IU glucoamylase load. The values of total sugars were in agreement with the finding of 22.17 ±0.66 % after pretreatment with sulphuric acid in cane molasses (Raharja et al., 2019). Pretreatments are considered as the best techniques to increase the sugar contents by converting polymeric unit into monomers (Jayanti et al., 2019).

From CCD models in current study, maximum ethanolic yield and titer was noted as 0.35±0.05, 5.82±0.09 (K7) and 0.36±0.09, 6.89±0.29 (Y34) under optimized conditions viz 75/20 mL hydrolyzate and synthetic medium, 32.5 ˚C and 8 days. Shankar et al. (2015) employed RSM, which is based on the central composite design, to maximize the ethanol production process of *S. cerevisiae* MTCC 170 at 35 ˚C, and 4% glucose with 10% ethanol yield. The ethanol yields were consistant with the findings of Hawaz et al. (2024) that were 0.33 g/g of ethanol yield with *S. cerevisiae* isolate TA2 and 0.30 g/g with *Wickerhamomyces. anomalus* isolate HCJ2F under laboratory conditions. The findings of Yadav et al. (2011) and Naito et al., 2019 were corroborated our finding of ethanol yield who observed7.5 g/L ethanol titer, a yield of 0.3 g/g and 7.6 g/L by S. cerevisiae OVB11 and S. cerevisiae H28 respectively. The selection of a better substrate for fermentation is worth considering for better end-product production. The yeast isolates converted the sugar monomers into ethanol and carbon dioxide in a fermentation medium (Vasconcelos, 2015; Raharja et al., 2019). In fermentation experiments, the substrate concentration is one of the main factors that can affect cell growth, end-product formation, and yeast cell metabolism (Grahovac et al., 2016). A high concentration of substrate will inhibit cell growth. The present finding showed the maximum values at a 3:1 ratio of substrate with synthetic medium and yeast inoculum. Ergun and Mutlu (2000) observed cell inhibition at 40% substrate concentration in beet molasses fermentation. High sugars repress the enzymes in the fermentation pathways of yeast and slow down the conversion of sugars into ethanol as low ethanol titer was seen in the experiment with high hydrolyzate concentration (Zohri and Mostafa (2000); Reddy and Reddy (2006). The high sugar contents in the fermentation medium inhibited the yeast cells' ability to use sugar. The ethanolic yield in the current study by *M. cibodasensis* Y34 and *S. cerevisiae* K7 corroborated the finding of Chaudhary et al. (2024) with the mango juice by following the CCD model. Predicted values from the software were validated experimentally. Santiago Urbina et al. (2011) successfully enhanced ethanol production up to 8% by adhering to the model’s predicted parameters. Statistical models such as Plackett Burman and Central composite designs are considered as efficient screening and optimization tools by saving time and reducing the number of experiments.

CONCLUSION

It was concluded that the highest reducing sugars by *Bacillus cereus* FA3 hydrolyzed molasses were 75.52±0.019 g/L. After eight days of fermentation, the yeast *Metchnikowia cibodasenis* Y34 produced a maximum ethanol yield of 0.36±0.09 g/g sugar consumed. Both *Bacillus cereus* FA3 and *Metchnikowia cibodasensis* Y34 are anticipated to be ethanol tolerant and possess a positive waste-to-bioethanol conversion potential. By utilization of non-fermentable sugars, there is a fair margin to increase ethanol yield per ton of molasses.

REFERENCES

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

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.

Andjelkovic, U., Picuric, S., & Vujčić, Z. (2010). Purification and characterization of *Saccharomyces cerevisiae* external invertase isoforms. *Food Chemistry*, *120*(3), 799-804.

AOAC (2016). *Official methods of analysis* (G. W. Latimer Jr. Eds., 20th ed.). Association of Official Analytical Chemists International, Washington.

Arshad, M., & Amjad, M. (2012). Medicinal use of sunflower oil and present status of sunflower in Pakistan: A review study. *Science, Technology and Development*, *31*(2), 99-106.

Arshad, M., Abbas, M., & Iqbal, M. (2019). Ethanol production from molasses: Environmental and socioeconomic prospects in Pakistan: Feasibility and economic analysis. *Environmental Technology & Innovation*, *14*, 100317.

Arshad, M., Hussain, T., Iqbal, M., & Abbas, M. (2017). Enhanced ethanol production at commercial scale from molasses using high gravity technology by mutant *S. cerevisiae*. *Brazilian Journal of Microbiology*, *48*, 403-409.

Awad, G. E., Amer, H., El-Gammal, E. W., Helmy, W. A., Esawy, M. A., & Elnashar, M. M. (2013). Production optimization of invertase by *Lactobacillus brevis* Mm-6 and its immobilization on alginate beads. *Carbohydrate Polymers*, *93*(2), 740-746.

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.

Barbosa, H. S., Silveira, E. D. A., Miranda Jr, M., & Ernandes, J. R. (2016). Efficient very-high-gravity fermentation of sugarcane molasses by industrial yeast strains. *Journal of the Institute of Brewing*, *122*(2), 329-333.

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* (pp 1-21). IntechOpen.

Brereton, R. G. (2007). *Applied chemometrics for scientists* (pp 9-62). John Wiley & Sons.

Bromberg, L., & Cheng, W. K. (2010). *Methanol as an alternative transportation fuel in the US: Options for sustainable and/or energy-secure transportation* (Final report, pp 1-78). Sloan Automotive Laboratory, Massachusetts Institute of Technology. Cambridge.

Camelia, B., Cristiana, T., & Gabriela, B. (2010). Yeast isolation and selection for bioethanol production from inulin hydrolysates. *Innovative Romanian Food Biotechnology*, (6), 29-34.

Chahed Ep Limayem, A. (2012). Biological strategies and mathematical approaches for limiting bacterial contaminants and chemical pollutants in bioethanol fermentations (pp 1-24). ProQuest LLC.

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.

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

Dotaniya, M. L., Datta, S. C., Biswas, D. R., Dotaniya, C. K., Meena, B. L., Rajendiran, S., Regar, K.L. & Lata, M. (2016). Use of sugarcane industrial by-products for improving sugarcane productivity and soil health. *International Journal of Recycling of Organic Waste in Agriculture*, 5, 185-194.

Driouch, H., Roth, A., Dersch, P., & Wittmann, C. (2010). Optimized bioprocess for production of fructofuranosidase by recombinant *Aspergillus niger*. *Applied Microbiology and Biotechnology*, 87, 2011-2024.

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

Ergun, M., & Mutlu, S. F. (2000). Application of a statistical technique to the production of ethanol from sugar beet molasses by *Saccharomyces cerevisiae*. *Bioresource Technology*, *73*(3), 251-255.

Grahovac, J., Jokić, A., Dodić, J., Vučurović, D., & Dodić, S. (2016). Modelling and prediction of bioethanol production from intermediates and byproduct of sugar beet processing using neural networks. *Renewable Energy*, *85*, 953-958.

Gupta, A., & Verma, J. P. (2015). Sustainable bio-ethanol production from agro-residues: a review. *Renewable and Sustainable Energy Reviews, 41*, 550-567.

Hawaz, E., Tafesse, M., Tesfaye, A., Kiros, S., Beyene, D., Kebede, G., Boekhout, T., Groenwald, M., Theelen, B., Degefe, A., Degu, S., Admasu, A., Hunde, B. & Muleta, D. (2024). Bioethanol production from sugarcane molasses by co-fermentation of *Saccharomyces cerevisiae* isolate TA2 and *Wickerhamomyces anomalus* isolate HCJ2F-19. *Annals of Microbiology, 74*(1), 13.

Heinrichs, R., Otto, R., Magalhães, A., & Meirelles, G. C. (2017). Importance of sugarcane in Brazilian and world bioeconomy. In Knowledge-driven developments in the bioeconomy: technological and economic perspectives (pp 205-217). Springer Nature link.

Jayanti, A. N., Sutrisno, A., Wardani, A. K., & Murdiyatmo, U. (2019). Bioethanol production from sugarcane molasses by instant dry yeast (effect of pretreatment and fermentation temperature). *In IOP conference series: Earth and Environmental science* (vol. 230, No. 1, pp. 012102). IOP Publishing.

Khairul, S. A. M., Mahyudin, N. A., Abas, F., Jamaludin, N. S. & Ab Rashid, N. K. M. (2022). The proximate composition and metabolite profiling of sugarcane (*Saccharum officinarum*) molasses. *Malaysian Applied Biology*, *51*(2), 63-68.

Khandekar, D. C., Palai, T., Agarwal, A., & Bhattacharya, P. K. (2014). Kinetics of sucrose conversion to fructo-oligosaccharides using enzyme (invertase) under free condition. *Bioprocess and Biosystems Engineering*, *37*, 2529-2537.

Konur, O. (2023). Metabolic engineering for the bioethanol production: Scientometric study. *In Bioethanol Fuel Production Processes. II* (pp. 302-326). CRC Press.

Kulshrestha, S., Tyagi, P., Sindhi, V., & Yadavilli, K. S. (2013). Invertase and its applications–a brief review. *Journal of Pharmacy Research*, *7*(9), 792-797.

Manoochehri, H., Hosseini, N. F., Saidijam, M., Taheri, M., Rezaee, H., & Nouri, F. (2020). A review on invertase: Its potentials and applications. *Biocatalysis and Agricultural Biotechnology, 25*, 101599.

Miller, G. L. (1959). Modified DNS method for reducing sugars. *Analytical Chemistry, 31*(3), 426-428.

Naito, Y., Okai, M., Ishida, M., Takashio, M., & Urano, N. (2019). Bioethanol production from molasses by yeasts with stress-tolerance isolated from aquatic environments in Japan. *Advances in Microbiology*, *9*(12), 1000-1011.

Parkash, A. (2015). Modeling of ethanol production from molasses: A review. *Industrial Chemistry, 3*, 108.

Raharja, R., Murdiyatmo, U., Sutrisno, A., & Wardani, A. K. (2019). Bioethanol production from sugarcane molasses by instant dry yeast. In *IOP conference series: Earth and Environmental Science* (vol. 230, pp. 012076). IOP Publishing.

Rashid, T., & Altaf, Z. (2008). Potential and environmental concerns of ethanol production from sugarcane molasses in Pakistan. *Nature Precedings*, 1-1.

Reddy, L. V. A., & Reddy, O. V. S. (2006). Rapid and enhanced production of ethanol in very high gravity (VHG) sugar fermentation by *Saccharomyces cerevisiae*: Role of finger millet (*Eleusine coracana* L.) flour. *Process Biochemistry, 41*(3), 726-729.

Roberto Ometto, A., Zwicky Hauschild, M., & Nelson Lopes Roma, W. (2009). Lifecycle assessment of fuel ethanol from sugarcane in Brazil. *The International Journal of Life Cycle Assessment, 14*, 236-247.

Sanchez, O. J., & Cardona, C. A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology, 99* (13), 5270-5295.

Santiago-Urbina, J. A., Ventura-Canseco, L. M. C., Ayora-Talavera, T. D. R., Ovando-Chacon, S. L., & Luc Dendooven, G. M. F. (2011). Optimization of ethanol production from mango pulp using yeast strains isolated from" taberna": A Mexican fermented beverage. *African Journal of Microbiology Research, 5*(5), 501-508.

Selladurai, G., Anbusaravanan, N., Shyam, K. P., Kandhasamy, P., & Balamuthu, K. (2010). Recycling of distillery sludge from sugarcane industry using bioresource technology. *Journal of Applied Scientific Research, 6*, 218-223.

Shankar, T., Sathees, R., & Anandapandian, K. T. K. (2015). Statistical optimization for ethanol production by *Saccharomyces cerevisiae* (MTCC 170) using response surface methodology. *Journal of Advancement in Medical and Life Sciences, 2*(3), 6-10.

Singh, P. (2020). Sugar industry: a hub of useful bio-based chemicals. *Sugar and sugar derivatives: changing Consumer Preferences*, 171-194.

Solomon, S. (2011). Sugarcane by-products based industries in India. *Sugar Tech*, *13*, 408-416.

Teclu, D., Tivchev, G., Laing, M., & Wallis, M. (2009). Determination of the elemental composition of molasses and its suitability as carbon source for growth of sulphate-reducing bacteria. *Journal of Hazardous Materials, 161*(2-3), 1157-1165.

Toledo, L. E. T., García, D. M., Cruz, E. P., Intriago, L. M. R., Pérez, J. N., & Chanfrau, J. M. P. (2019). Fructosyltransferases and invertases: useful enzymes in the food and feed industries. *Enzymes in Food Biotechnology*, 451-469.

Tuck, C. O., Pérez, E., Horváth, I. T., Sheldon, R. A., & Poliakoff, M. (2012). Valorization of biomass: deriving more value from waste. *Science, 337*(6095), 695-699.

Vasconcelos, J. N. D. (2015). Chapter 15: *Ethanol Fermentation in Sugarcane agricultural production, bioenergy and ethanol*. Academic Press, USA.

Yan, S., Li, J., Chen, X., Wu, J., Wang, P., Ye, J., & Yao, J. (2011). Enzymatically hydrolysis of food waste and ethanol production from the hydrolysate. *Renewable Energy*, *36*(4), 1259-1265.

Yoon, M. H., Choi, W. Y., Kwon, S. J., Yi, S. H., Lee, D. H., & Lee, J. S. (2007). Purification and properties of intracellular invertase from alkalophilic and thermophilic *Bacillus cereus* TA-11. *Journal of Applied Biological Chemistry, 50*(4), 196-201.

Yadav, K. S., Naseeruddin, S., Prashanthi, G. S., Sateesh, L., & Rao, L. V. (2011). Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of Saccharomyces cerevisiae and *Pichia stipitis*. *Bioresource Technology*, 102(11), 6473-6478.

Yucekan, I., & Onal, S. (2011). Partitioning of invertase from tomato in poly (ethylene glycol)/sodium sulfate aqueous two-phase systems. *Process Biochemistry*, 46(1), 226-232.

Yun, H. S., Yoon, I. S., & Kang, B. G. (2002). Rapid repression of vacuolar invertase in mungbean hypocotyl segments and regulation by sucrose, auxin and light. *Plant Growth Regulation*, *38*, 181-189.

Zohri, A. A. & Mostafa, E. (2000) Ethanol production from dates in Saudi Arabia on industrial scale. *Microbiology, 28*(2), 76-81.

Winne, P. H. (2001). Self-regulated learning viewed from models of information processing. In B.J. Zimmerman & D.H. Schunk (Eds.), *Self-regulated learning and academic achievement* (2nd ed., pp. 160-192). Lawrence Erlbaum Associates.