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Original article

Pomegranate peels waste hydrolyzate optimization by Response Surface Methodology for Bioethanol production

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ABSTRACT

Unwanted agricultural waste is largely comprised of lignocellulosic substrate which could be transformed into sugars. The production of bioethanol from garbage manifested an agreeable proposal towards waste management as well as energy causation. The goal of this work is to optimize parameters for generation of bioethanol through fermentation by different yeast strains while *Saccharomyces cerevisiae* used as standard strain. The low cost fermentable sugars from pomegranate peels waste (PPW) were obtained by hydrolysis with HNO_3 (1 to 5%). The optimum levels of hydrolysis time and temperature were elucidated via RSM (CCD) ranging from 30 to 60 min and 50 to 100 °C respectively. The result shows that optimum values (g/L) for reducing sugars was 61.45 ± 0.01 while for total carbohydrates was 236 ± 0.01. These values were found when PPW was hydrolyzed with 3% HNO_3, at 75 °C for one hour. The hydrolyzates obtained from the dilute HNO_3 pretreated PPW yielded a maximum of 0.43 ± 0.04, 0.41 ± 0.03 g ethanol per g of reducing sugars by both *Metchnikowia* sp. Y31 and *M. cibodasensis* Y34 at day 7 of ethanologenic experiment. The current study exhibited that by fermentation of dilute HNO_3 hydrolyzates of PPW could develop copious amount of ethanol by optimized conditions.

1. Introduction

Pakistan is an agricultural country with considerable and excellent products in this field. Consequently, the huge amount of agricultural waste accumulated that lead to serious environmental issues. The large quantity of this waste converted into specific products such as animal fodder as well as fertilizer to overcome the amount of agricultural waste. The agricultural wastes are composed of lignocellulosic components (Adeeyo et al., 2015). Lignocellulose a highly abundant biomass scrutinized to generate

Abbreviations: PPW, Pomegranate peels waste; PPWH, Pomegranate peels waste hydrolysate; RSM, Response Surface Methodology; CCD, Central Composite design; MYG, Malt yeast glucose medium; DNS, Dinitosalicyclic acid.

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fermentable sugars as to get bioethanol. This biomass constitutes agricultural feedstocks, plant derivatives and residues from municipal waste (Aguilar et al., 2002). To manage agro industrial waste to overcome energy crisis, 3 R strategies viz reuse, reduce and recycle are considered as promising technique. Land filling and incineration were the oldest techniques to be used to minimize the massive waste and to protect from hazardous impacts (Forastiere et al., 2009; Mahmoudkhani et al., 2014).

Another biodegradeable waste in the category of lignocellulosic waste is fruit peels that generate on both domestic and commercial level. The consumption of fruit by industries to manufacture various products places a great amount of fruit wastes in the environment (Anderson and Akin, 2008). The discarded peels by household and industries consumption if dumped in environment could lead to serious unhygienic climate. Therefore the mechanizing of bioethanol for the waste management not only reduces these waste quantities but also obliging for energy crisis (Galbe and Zacchi, 2012).

Pomegranate also named as *Punica granatum*; a famous fruit in Pakistan that also highly consumed throughout the world. The fruit contained peels/exocarp 50%, seed pod/aril 40% and seeds 10% (Orzua et al., 2009; Paul et al., 2019). The seeds of pomegranate are consumed as food but the peels as well as arils (90%) are discarded as waste into environment. The Government of Pakistan (GOP, 2019) reported the average annual production and consumption of pomegranate was 40,125 and 4805 tons in Pakistan from 2018 to 2019 which means that Pakistan is producing a large amount of this waste. Pakistani used fresh as well as processed pomegranate fruit in different forms such as fresh/preserved juice, sauces, jams and marmalades (Ay et al., 2012; Bhatnagar et al., 2015). Pomegranate juice extraction is done on domestic and industrial level. The industrial processing of fruit into juice and other products leads to the production of huge perishable as well as degradable waste (peels/seeds) to pose grave hazardous impacts in case of improper disposing off (Kanatt et al., 2010; Mohamad and Khalil, 2014). PPW is categorizes as lignocellulosic waste materials so it can be used to produce bioethanol by dilute acid hydrolysis. This non edible and biodegradable waste could sustain energy crisis by its bioconversion in ethanol and reduce the municipal waste (Talebnia et al., 2008; Khan et al., 2015).

A well-known statistical tool RSM used to accomplish the purpose of optimization by determining optimum values of factors (Dubois and Lasida, 2010). The work envisaged the PPW biotransformation to fermentable reducing sugars by nitric acid hydrolysis with ultimate ethanol genesis via yeast strains.

2. Materials and methods

2.1. Substrate collection

PPW were collected from different sites of Lahore and rinsed to get rid of dirt particles. Afterwards washed out using distilled water then dehydrated in the hot air oven at 60 °C. These dried peels were then powdered to get a size of (~1mm) using a mixing grinder.

2.2. Proximate composition

Proximate composition includes protein, reducing sugar as well as total carbohydrate contents from biomass. After a day incubation (30 °C, 150 rpm), the aqueous PPW filtrate (1:100) was processed by DNS reagent (Hu et al., 2008), phenol–sulphuric acid method (Nielsen, 2010) and Folin-Ciocalteu method (Blainski et al., 2013) correspondingly. Whereas the ethanolic filtrate of PPW (1:10) obtained after keeping at room temperature for a day was estimated for total lipids by sulfo phosphor vanillin reagent (Zöllner and Kirsch, 1962). The moisture and ash contents were determined by AOAC protocols (AOAC, 2012). The amount of extractives, lignin, hemicellulose and the cellulose were calculated by gravimetric protocol (Ayeni et al., 2013) with a little modification.

2.3. Hydrolysis and optimization of PPW by CCD

The PPW was hydrolyzed with nitric acid for the breakdown of cellulosic contents into fermentable sugars. PPW were hydrolysed by nitric acid having ratio of 1:10 in conical flask (250 mL) by covering with aluminium foil. The experiment was planned to assess two responses with three factorial level viz nitric acid concentration, saccharification time and temperature. The optimization of factors for Saccharification was conducted using CCD (design expert software). Experiment was designed with 20 runs executing in triplicates with the help of RSM. To calculate the optimum values of responses, an optimization design was chosen to analyse the characteristic factors. The saccharified mixture at specified experimental conditions was proceeded in shaking incubator revolving at 100 rpm. The experimental mixture was subjected to filtration and the neutralized using NaOH pellets. Charcoal with 2.5% concentration was used to detoxication followed by filteration again of PPWH.

The mathematical optimization design with range of parameters was executed employing CCD approach with RSM up to three factor levels (Table 1). The CCD designed a matrix for three parameters (independent variables) of experiment (Table 2). The CCD model represented low, middle and high values for hydrolysis parameters. The base for current model was provided by computed data from various research studies whose prominence was biomass saccharification influenced by some parameters (Akram, 2015; Pervaiz, 2016; Nasim, 2016; Siddique, 2016). The present study highlighted the optimized findings of certain parameters for nitric acid hydrolysis.

The experiment was carried out with dilute HNO_3 hydrolysis with various concentrations to envisage variability in responses. The experimental data obtained by CCD matrix with predicted temperature as well as time was analyzed by the subsequent regression equation (Y) on various responses i.e reducing sugars (Yr) and Total sugars (Yts) as;

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + e \quad (1)$$

where Y stated predicted response, X_1, X_2 and X_3 mentioned input variables while β_0, β_1, β_2, β_3, β_{11}, β_{22}, β_{33}, β_{12}, β_{13} and β_{23} mentioned coefficients of regression. ANOVA was used to analyze interaction of all factor. While R^2 plus Adjusted R^2, the determination coefficient exhibit the accuracy with fitting quality of the polynomial design. The Response surface graphs constructed to check the effect by factors upon response both individual and interactive. The optimum area was identified through main parameters in graphs (Bashir et al., 2010).

2.4. Percent saccharification of PPWH

For computation of percent sacharrification, 5 g PPW were dispemsed in 50 mL of 3% HNO3 at 75 °C for 60 min. The reducing sugars were calculated by DNS method. The saccharification percentage (2) was expressed as (Mithra et al., 2018);

$$\text{Saccharification } (\%) = \frac{\textit{Reducing sugars } \left(\frac{g}{L}\right) \times 0.9}{\textit{Substrate } \left(\frac{g}{L}\right) \times 10} \times 100 \quad (2)$$

Table 1
Coded values for experimental variables employing Central Composite Design for PPW hydrolysis by nitric acid.

Actual values of coded levels				
Variables	Coded symbol	Low level	Centre point	High level
Acid concentration	X_1	1%	3%	5%
Hydrolysis temperature	X_2	50 °C	75 °C	100 °C
Hydrolysis time	X_3	30 min	45 min	60 min

Table 2
Central Composite Design matrix presenting experimental variables for HNO_3 scarification of PPW.

Experimental Runs	HNO_3 conc. X_1 (%)	Temperature X_2 (°C)	Hydrolysis time X_3 (min)
1	3	75	45
2	5	50	30
3	5	100	60
4	5	100	30
5	1	100	30
6	3	75	45
7	5	50	60
8	3	75	45
9	1	100	60
10	1	50	30
11	1	50	60
12	3	75	45
13	6.36	75	45
14	0.36	75	45
15	3	75	70.23
16	3	75	45
17	3	75	19.77
18	3	75	45
19	3	117.04	45
20	3	32.96	45

2.5. PPWH ethanologenesis

The ability to produce Bioethanol via yeast strains *S. cerevisiae* K7, *M. cibodasensis* Y34, *Metschnikowia* sp. Y31 and was chosen for this study. *Saccharomyces cerevisiae* K7 was delivered by Japan Brewing Society as generous gift while author used already isolated *M. cibodasensis* Y34 and *Metschnikowia* sp. Y31 as experimental yeast which had ability to produce ethanol (Chaudhary and Karita, 2017).

2.6. Detoxification and fermentation of PPWH with different yeast strains

After saccharification, PPWH contains not only fermentable sugars but also few inhibitors like phenolics, furfural etc. These compounds in hydrolyzate were subjected to inhibit the fermentation of sugars by microorganisms. To achieve high fermentation ability, detoxification of hydrolyzate is necessary before the fermentation experiment.

For detoxification of PPWH, charcoal treatment was performed using 2.5% activated charcoal in PPWH while kept on shaking for one hour. Subsequently the supernatant was neutralized with pellets of sodium hydroxide followed by filtration (Mussatto and Roberto, 2005; González et al., 2003).

2.7. PPWH ethanologenesis by various yeast isolates

For proceeding of fermentation experiment, neutralized PPWH: synthetic medium:inoculum were mixed in 50:45:5 ratio in conical flasks of 250 mL capacity with aluminium foil cover. The inoculums was prepared with MYG medium by adding 0.3 g yeast and malt extract, 0.5 g peptone along with 1 g of glucose in 100 mL of distilled water. Already revived yeast strains (500 μl) were added in medium then incubated for a day at 30 °C. The synthetic medium's composition was yeast extract (3.575 g), ammonium sulphate (1.43 g), potassium dihydrogen phosphate (1.496 g), magnesium sulphate (0.44 g), calcium chloride (0.165 g), zinc chloride (0.00023 g), citric acid (0.825 g) along with trisodium citrate (3.3 g). The mixture formed in distilled water (550 mL) was autoclaved (15 min, 121 °C).

These experimental flasks were incubated for 10 days without shaking at 30 °C ± 0.02. The sugars in hydrolyzate were used as nutrients while synthetic medium specifically served as nitrogen, vitamins as well as minerals source. The fermentation experiment was assessed daily up to 10 days. The ethanol and reducing sugars along with growth of yeast was analysed subsequently. The growth of yeast in fermentation was evaluated using spectrophotometer at 600 nm as described by Yang et al. (2018). According to Mithra et al. (2018), fermentation efficiency (3) was calculated as;

$$Fermentation\ \ Efficiency(\%) = \frac{Practical\ \ ethanol\ \ yield}{Theoretical\ \ ethanol\ \ yield} \times 100 \quad (3)$$

2.8. Statistical interpretation

All experimental data was attained in triplicates. Statistical protocols (ANOVA and regression) were applied to evaluate the data using Design Expert (ver. 6.0.9, Stat-Ease, Minneapolis). Data related to fermentation was interpreted by one-way ANOVA employing Dunkun's multiple ranges tests (SPSS ver. 16.0, USA).

3. Results

3.1. Compositional components of PPW

Data presented in Table 3 manifested the compositional analysis of pomegranate peels. Different contents (g/L) were as total carbohydrates 79.6 ± 0.04, total protein 15.6 ± 0.002, total lipids 3.1 ± 0.005 and reducing sugars 25.1 ± 0.02. Hemicellulose (%) was calculated as 28.20 ± 1.06 while crude cellulose + insoluble lignin and soluble lignin (%) were 36.36 ± 0.20 and 15.27 ± 1.25. Moreover, the moisture and ash contents (%) were 7.65 ± 0.07 and 11.4 ± 0.03 correspondingly.

Table 3
Compositional study of pomegranate peels.

Contents	Quantity
Moisture Contents (%)	7.65 ± 0.07
Ash contents (%)	11.4 ± 0.03
Total Proteins (gL^{-1})	15.6 ± 0.002
Total Lipids (gL^{-1})	3.1 ± 0.005
Reducing sugar Contents (gL^{-1})	25.1 ± 0.02
Total Carbohydrate (gL^{-1})	79.6 ± 0.04
Extractives (%)	22.20 ± 0.13
Soluble lignin Contents (%)	15.27 ± 1.25
Hemicellulose Contents (%)	28.20 ± 1.06
Crude Cellulose + insoluble lignin (%)	36.36 ± 0.20

Each value corresponds to means of triplicates ± S.E.M.

3.2. Optimization of hydrolysis factors by CCD

Dilute HNO_3 hydrolysis of PPW releases the fermentable sugars from lignocellulosic biomass and undergo in fermentation for ethanol synthesis. The experimental values for reducing sugars obtained via acid pre-treatment in 20 runs CCD model were tabulated (Table 4).

The optimum reducing sugars experimental value (61.45 ± 0.01) and predicted value (50.2181) were estimated with dilute nitric acid hydrolysis at 3% HNO_3 concentration with 75 °C temperature within 60 min of time. The quadratic regression equation attained by ANOVA was for reducing sugars was:

$$Yr = +42.06 - 7.13X_1 + 1.53X_2 + 4.32X_3 - 1.80X_1^2 - 5.02X_2^2 + 3.83X_3^2 + 1.04X_1X_2 + 0.36X_1X_3 + 1.66X_2X_3 + 91.97 \quad (4)$$

Synergistic and antagonistic interaction was expressed by positive and negative symbols respectively. Significance and validation of the model was analyzed by the F-value (7.23) and P-value (0.0035) as computed in Table 5. The F value greater than four and p values smaller than 0.0500 exhibited the significance of model. The non-significance of Lack of fit of model was evident with 1.23 F and 0.4313 p values. The coefficient R^2 value (0.8786) and R^2_{adj} value (0.7571) were mentioned in Table 6, which deliberated the reliability of model. A ratio of "Adeq Precision" of 9.313 and CV of 12.71% indicated the adequate signal.

Three D response surface charts indicated interactive effect of parameters for response i.e., release of reducing sugars. Fig. 1A showed that reducing sugars had direct increasing effect for temperature but no change was recorded for HNO_3 concentration. Same increasing trend with hydrolysis time was exhibited in Fig. 1B while acid concentration have no interactive effect. Fig. 1C described that sharp increase in reducing sugars with effect of time was observed and temperature upto 75 °C caused increase in reducing sugars followed by decrease. The percent saccharification yield in PPWH using HNO_3 was found to be 1.525. The cellulosic contents in PPWH were transformed into fermentable sugars applying dilute nitric acid.

Total Carbohydrates contents were also observed in PPWH by nitric acid. The optimum carbohydrate contents were recorded with 3% of HNO_3 concentration with 75 °C temperature for an hour while the experimental and predicted values (g/L) were 236 ± 0.01 and 228.041 correspondingly. The interactive effect of variables was evident by quadratic equation.

$$Yts = +221.48 + 22.73X_1 - 17.30X_2 + 2.52X_3 - 44.94X_1^2 - 20.37X_2^2 + 4.04X_3^2 - 12.94X_1X_2 + 10.69X_1X_3 + 1.54X_2X_3 + 5168.03 \quad (5)$$

The positive and negative symbols interpreted synergistic and antagonistic interrelationship. The statistical tool ANOVA confirmed the model's significance representing the 4.11, 0.0235 as F and p-values. The non-significance of Model's lack of fit was shown by 1.00-F and 0.5156-p values. The values of regression coefficients were 0.8043- R^2 and 0.6086- R^2adj. The values of CV 20.03% and adequate precision 6.796 envisaged the appropriateness of the model.

In Fig. 2(A), graph showed increase in total sugars slightly with HNO3 concentrations and sharp along with temperature. Fig. 2(B) depicted the increase in response upto 4% nitric acid followed by decrease whereas only increasing trend was recorded with time. In Fig. 2(C) the carbohydrate contents decrease was illustrated with a small increase in the hydrolysis temperature but increase was recorded with time.

3.3. Detoxification of PPWH for fermentation by phenol estimation

The main purpose of nitric acid detoxification was to minimise the quantity of phenolics. A reduction of 54% of phenol contents in PPWH was recorded. The phenolic compounds of PPWH were reduced to 0.66 ± 0.03 from 1.21 ± 0.02 via detoxification.

3.4. Fermentation of detoxified nitric acid PPWH

For ethanologenesis, PPW were treated at maximum saccharification parameters (3% HNO_3, 75 °C, 1 h) to prepare hydrolyzate. Maximum g/g Ethanol yield was 0.41 ± 0.03, 0.43 ± 0.04, with *M. cibodasensis* Y34 and *Metschnikowia* sp. Y31 at day 7 (Fig. 3). The *S. cerevisiae* K7 used as standard yeast synthesized the highest yield as 0.40 ± 0.02 at day 8. *Metschnikowia* sp. Y31, *M. cibodasensis* Y34 and *S. cerevisiae* K7 synthesized the ethanol titer (g/L) 12.99 ± 0.40, 11.56 ± 0.31 and 10.72 ± 0.38 correspondingly. The reducing

Table 4
CCD matrix for experimental variables presenting different responses employing HNO_3 hydrolysis of PPW.

Runs	HN_{O3} conc. (%)	Temp (°C)	Time (min)	Red sugars ± SEM (g/L)	Total Carbs ± SEM (g/L)	Weight loss ± SEM (%)	Extractive ± SEM (%)	Hemicellulose ± SEM (%)	Soluble Lignin ± SEM (%)	Crude Cellulose + Insoluble lignin ± SEM (%)
1	3	75	45	47.7 ± 0.00	113.5 ± 0.02	39.5 ± 0.09	23.8 ± 0.39	27.7 ± 0.08	13.8 ± 0.24	34.6 ± 0.39
2	5	50	30	28.5 ± 0.02	218.7 ± 0.05	42.1 ± 0.24	28.5 ± 0.12	24.6 ± 0.17	13.8 ± 0.17	32.9 ± 0.45
3	5	100	60	45.8 ± 0.00	140.9 ± 0.10	44.7 ± 0.02	30.2 ± 0.14	27.2 ± 0.25	12.9 ± 0.15	29.5 ± 0.14
4	5	100	30	27.9 ± 0.02	139.8 ± 0.22	41.0 ± 0.07	21.0 ± 0.17	26.0 ± 0.14	13.3 ± 0.14	39.5 ± 0.18
5	1	100	30	49.7 ± 0.00	122.0 ± 0.21	40.7 ± 0.18	26.9 ± 0.15	25.9 ± 0.03	14.0 ± 0.08	33.0 ± 0.18
6	3	75	45	29.0 ± 0.06	215.7 ± 0.26	47.1 ± 0.87	31.3 ± 0.16	27.9 ± 0.04	11.2 ± 0.07	29.5 ± 0.14
7	5	50	60	50.9 ± 0.02	101.5 ± 0.04	43.0 ± 0.12	25.5 ± 0.24	26.1 ± 0.21	14.2 ± 0.01	34.0 ± 0.03
8	3	75	45	28.7 ± 0.04	197.6 ± 0.00	48.0 ± 0.38	25.0 ± 0.13	24.7 ± 0.05	12.1 ± 0.28	38.0 ± 0.19
9	1	100	60	48.8 ± 0.00	230.4 ± 0.02	51.1 ± 0.12	36.9 ± 0.09	26.2 ± 0.17	13.1 ± 0.22	23.6 ± 0.08
10	1	50	30	46.2 ± 0.03	202.2 ± 0.03	50.5 ± 0.09	37.5 ± 0.13	23.2 ± 0.04	11.4 ± 0.12	27.7 ± 0.08
11	1	50	60	42.8 ± 0.03	150.0 ± 0.25	47.5 ± 0.02	37.8 ± 0.14	22.8 ± 0.01	11.5 ± 0.20	27.8 ± 0.32
12	3	75	45	37.6 ± 0.02	255.4 ± 0.02	44.8 ± 0.02	35.1 ± 0.17	25.8 ± 0.12	11.6 ± 0.12	27.3 ± 0.32
13	6.36	75	45	46.7 ± 0.02	165.6 ± 0.10	47.3 ± 0.01	35.5 ± 0.11	26.6 ± 0.03	10.5 ± 0.07	27.2 ± 0.22
14	0.36	75	45	29.4 ± 0.00	106.4 ± 0.10	46.7 ± 0.11	36.2 ± 0.02	25.4 ± 0.10	12.0 ± 0.73	26.2 ± 0.15
15	3	75	70.23	34.9 ± 0.09	179.7 ± 0.21	44.2 ± 0.01	32.9 ± 0.06	26.3 ± 0.13	12.0 ± 0.24	28.6 ± 0.39
16	3	75	45	33.1 ± 0.01	227.2 ± 0.31	44.2 ± 0.02	31.0 ± 0.02	27.1 ± 0.10	13.9 ± 0.14	27.8 ± 0.30
17	3	75	19.77	41.3 ± 0.01	236.3 ± 0.01	44.7 ± 0.02	37.9 ± 0.10	20.0 ± 0.34	11.0 ± 0.15	30.9 ± 0.24
18	3	75	45	65.4 ± 0.04	273.7 ± 0.08	46.7 ± 0.18	38.6 ± 0.13	27.8 ± 0.16	11.8 ± 0.33	21.7 ± 0.30
19	3	117.04	45	43.5 ± 0.04	178.8 ± 0.07	47.0 ± 0.18	37.5 ± 0.22	24.9 ± 0.18	12.5 ± 0.22	24.9 ± 0.21
20	3	32.96	45	37.3 ± 1.01	191.9 ± 0.00	46.3 ± 0.21	32.2 ± 0.13	26.8 ± 0.08	11.7 ± 0.24	29.1 ± 0.43

Each value constitute mean of triplicates ± S.E.M.

Table 5
ANOVA for hydrolysis quadratic model computing two responses in nitric acid treated PPWH.

Contents	Source	Sum of Squares	df	Mean Square	F-Value	p-value
Reducing sugars	Model	1691.62	9	187.96	7.23	0.0035
	Residual	233.84	9	25.98		Significant
	Lack of fit	141.87	5	28.37	1.23	0.4313
	Pure Error	91.97	4	22.99		Not Significant
	Cor Total	1929.68	19			
Total sugars	Model	47713.74	9	5301.53	4.11	0.0235
	Residual	11608.33	9	1289.81		Significant
	Lack of fit	6440.30	5	1288.06	1.00	0.5156
	Pure Error	5168.03	4	1292.01		Not Significant
	Cor Total	59330.03	19			

Table 6
Quadratic regression analysis for optimization of reducing sugars and total carbohydrates investigated by Nitric acid PPWH.

Contents	C.V (%)	R-Squared	Adjusted R-Squared	Predicted R-Squared	Adequate Precision
Reducing sugar	12.71	0.8786	0.7571	0.1842	9.313
Total sugars	20.03	0.8043	0.6086	0.2446	6.796

sugar contents fluctuation and interrelationship of ethanol contents with yeast growth during fermentation was also noticed (Figs. 4, 5). FE% (Percent fermentation efficiency) was computed with respect to theoretical yield-0.5. Following values of 84.31, 80.39, 78.43 via *Metschnikowia* sp. Y31, *M. cibodasensis* Y34 and *S. cerevisiae* K7 was calculated after completion of fermentation study.

4. Discussion

The consumption of pomegranate has grown tremendously to cause a significant waste in the form of peels. Reducing sugars concentration can be released via hydrolysis as pre-treatment of lignocellulosic wastes. The significantly higher reducing sugars reported after hydrolysis were attributed similar results as recorded through various researches (Tewari et al., 1986; Gomathi et al., 2012; Bhandari et al., 2013). The compositional component of PPW was estimated following various analytical protocols. Different researchers found *Saccharomyces cerevisiae* as promising candidate for fermentation (Rizzello et al., 2019) employing varied substrates. In present investigation, *Saccharomyces cerevisiae* K7 was used to ferment PPWH as standard yeast isolate where as other yeast strains were *M. cibodasensis* Y34 and *Metschnikowia* sp. Y31.

Nitric acid was used as pretreatment agent to transform the lignocellulosic biomass into fermentable monomers which serve as feasible substrate for ethanologenic microbes. The current study investigated the optimized hydrolysis parameters by 2^3factorial design of RSM. The CCD of RSM also been reported for parameter optimization for biotransformation of ethanol (Walia et al., 2014; Adnan et al., 2014). Significance and validation of the model was analyzed by the F-value (7.23) and P-value (0.0035) as computed in Table 5. The F value greater than four and p values smaller than 0.0500 exhibited the significance of model. The non-significance of Lack of fit of model was evident with 1.23 F and 0.4313 p values. The coefficient R^2 value (0.8786) and R^2_{adj} value (0.7571) were mentioned in Table 6, which deliberated the reliability of model. A ratio of "Adeq Precision" of 9.313 and CV of 12.71% indicated the adequate signal.

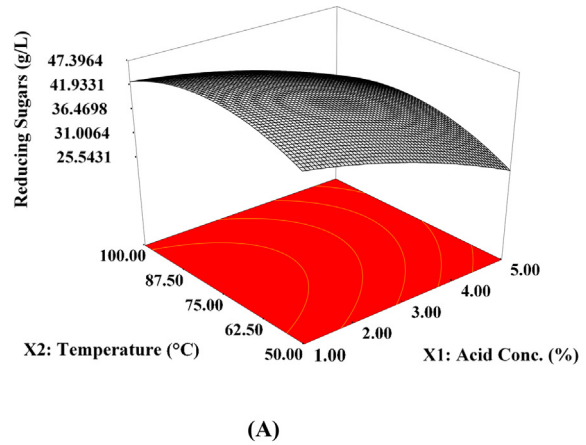
The optimum reducing sugars g/L (61.45 ± 0.01) were released at 3% HNO_3 concentration with 75 °C of temperature for an hour. The experimental value was better than predicted value of 50.2181 g/L for reducing sugars. Analysis by statistics revealed that this model was significant by means of F-value (7.23), p-value (0.0035) along with R^2 value (0.8786). Pomegranate peels were transformed by HCL (1%, 100 °C, half hour) and H_2SO_4 (3%, 100 °C, half hour) to release 48.02 ± 0.02 and 52.3 ± 0.10 g/L reducing sugars following same models (Saleem et al., 2020; Chaudhary et al., 2021). The increase was observed by nitric acid. Unhasirikul et al. (2012) reported the similar results with release of 56.07 g/liter sugars in durian peels. By reviewing the literature, increase in monomer sugars were observed by conversion lignoceelulosic sugars into monomeric sugars (El Asli and Qatibi, 2009; Unhasirikul et al., 2012).

Dilute acid pretreatment is proved to be an effective technique to modify the hemicellulose structure by making it porous. The porous structure facilitate enzymes to access the cellulose to improve the conversion into monomeric fermentable sugars (Toquero and Bolado, 2014; Loow et al., 2016). Nitric acid being costly is not used widely as hydrolysis catalyst to convert the cellulosic sugars to monomers (Zhang et al., 2011). The present study is cost effective by finding optimized nitric acid hydrolysis parameters to cut the cost and found better results as compared with hydrochloric and sulphuric acid pretreatment of PPW.

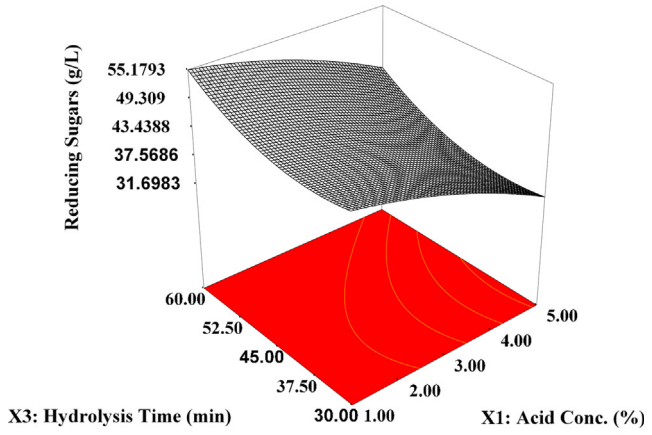
Optimum carbohydrate contents (3%, 75 °C, 60 min) were found to be 236 ± 0.01 while the predicted value was 228.041. The statistical approach revealed the significance of model with F-value and p-value of 4.11 and 0.0235 respectively while the R^2 value was 0.8043. Zhang et al. (2013) reported 96 to 98% yield of arabinose and xylose where as 18% for glucose by nitric acid pretreatment of corn stover.

After dilute HNO_3 hydrolysis, the PPWH were subjected to be detoxified through activated charcoal for eradication of inhibitors to improve fermentation conditions. Many phenolics that were generated during pre-treatment in current study were eliminated using activated charcoal (Yadav et al., 2011). The reduction in phenolic compounds was computed to 54% in PPWH. Various protocols and conditions for detoxification of phenolics and furfurals are widely used and reported (Baadhe et al., 2014; Castro et al., 2014). Acid saccharification resulted in production of phenolics by degradation of lignin contents. These inhibitors were a great challenge for microbes during fermentation process because these slowed down or blocked the microbial metabolism being a part of fermentation medium (Taherzadeh and Karimi, 2011; Kupiainen et al., 2014). Low temperature was considered better than high. Sugars were transformed in to furfurals along with lignin at high temperature when subjected to same hydrolysis time period (Łukajtis et al., 2018).

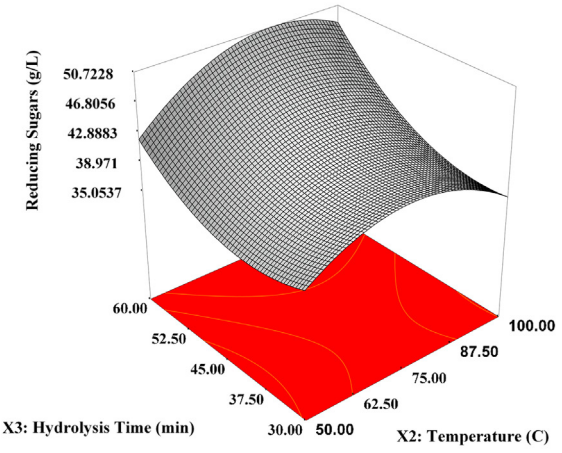
In nitric acid PPWH, the substantial ethanol yield (g/g) was 0.43 ± 0.04 (d7), 0.41 ± 0.03 (d7) and 0.40 ± 0.02 (d8) with *M. cibodasensis* Y34, *Metschnikowia* sp. Y31 as well as *S. cerevisiae* K7 cor-



(A)

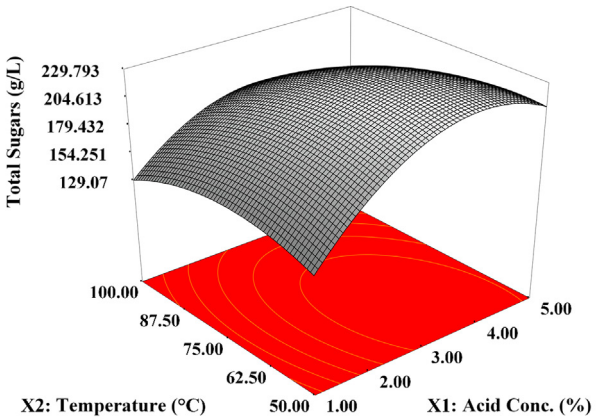


(B)

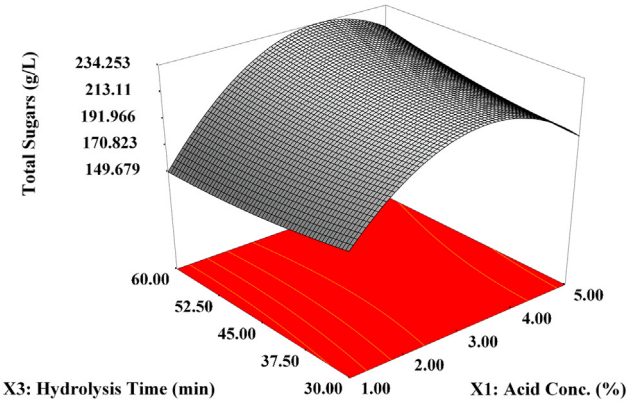


(c)

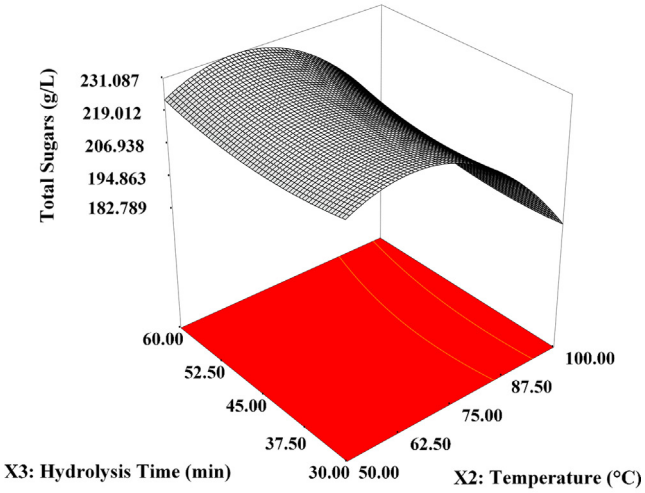
Fig. 1. Response surface Plots for g/L reducing sugars released from variable Nitric acid levels with varying hydrolysis temperature (A), time (B) and time with temperature (C) in PPWH.



(A)



(B)



(C)

Fig. 2. Response surface graphs for g/L total carbohydrates from varying treatments of acid concentrations comparable with various temperatures (A), saccharification time (B) and time versus temperature (C) in Nitric acid PPWH.

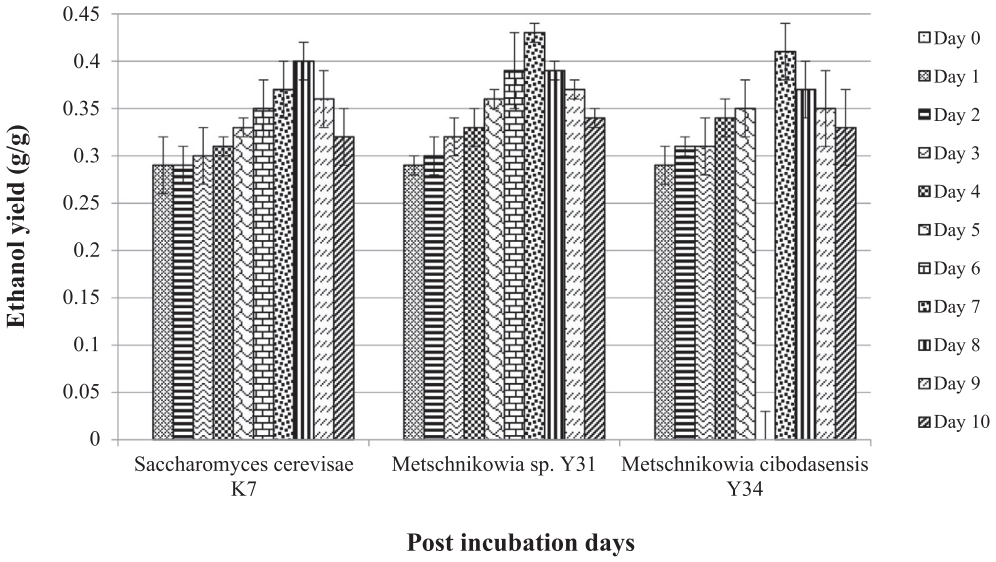


Fig. 3. Ethanol yield (g/g) via *Saccharomyces cerevisiae* K7, *Metschnikowia cibodasensis* Y34 as well as *Metschnikowia* sp. Y31 in nitric acid hydrolyzate of PPW.

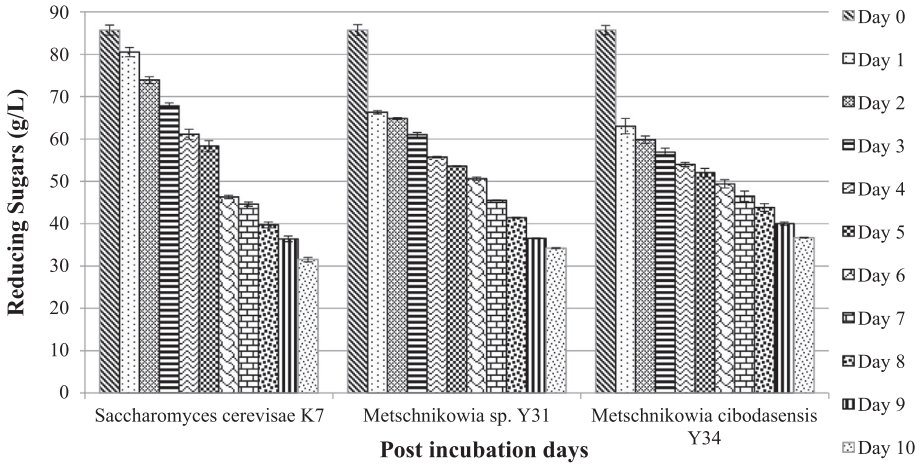


Fig. 4. Fluctuation in g/L reducing sugars in nitric acid treated PPWH fermented by *Saccharomyces cerevisiae* K7, *M. cibodasensis* Y34 and *Metschnikowia* sp. Y31.

respondingly. The yield data was similar to the results obtained using Pineapple, Watermelon and Muskmelon rinds as well as PPW (Bhandari et al., 2013; Saleem et al., 2020; Chaudhary et al., 2021). The considerable g/L ethanol titer was recorded as 12.99 ± 0.40 (*Metschnikowia* sp. Y31), 11.56 ± 0.31 (*M. cibodasensis* Y34) and 10.72 ± 0.38 (*S. cerevisiae* K7). Kim et al. (2014), reported the ethanol contents (g/L) 10.92–14.50 in nitric acid pretreated rice straw by *Pichia stipitis*. These contents were also comparable with 14.3 g/L ethanol titer obtained from acid hydrloysed PPW with *Kluyveromyces marxianus* (Demiray et al., 2020). Current findings were far better for ethanol titer when compared with data obtained from different fruit waste viz mango, orange, date and banana (Arumugam and Manikandan, 2011; Boulal et al., 2016; Maina et al., 2017).

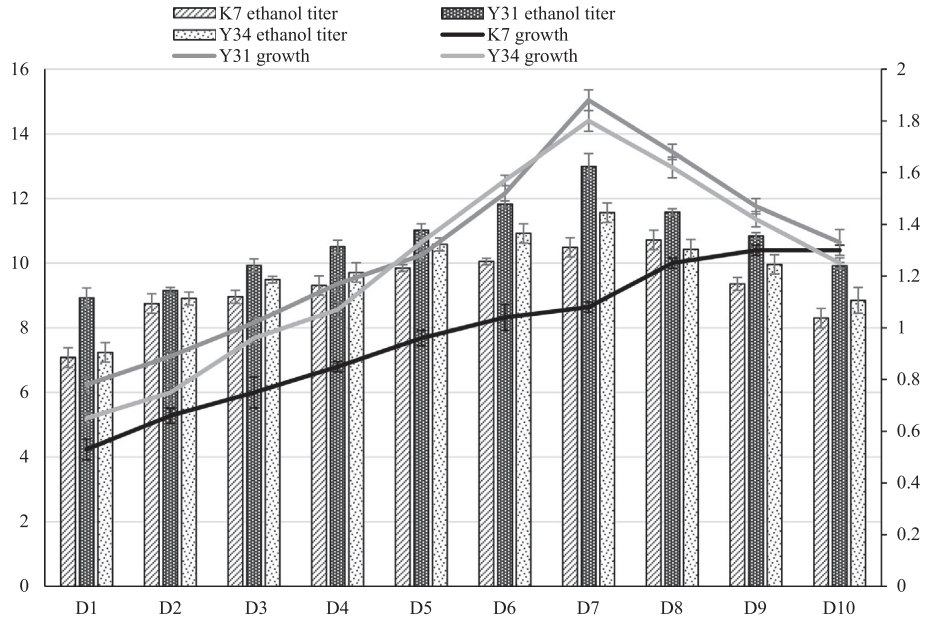


Fig. 5. Interrelation of ethanol titer (g/L) and growth (Optical densities) of *Saccharomyces cerevisiae* K7, *Metschnikowia cibodasensis* Y34 and *Metschnikowia* sp. Y31 during nitric acid PPWH fermentation.

5. Conclusion

Dilute nitric acid is proved as efficient and cost effective pretreatment catalyst to release maximum reducing sugars (g/L) 61.45 ± 0.01 from pomegranate peels waste by decreasing cellulosic contents. *Metchnikowia* sp. Y31 appeared as substantial yeast isolate to yield 0.43 ± 0.04 g/g ethanol with 84.31 percent fermentation efficiency to manage fruit waste.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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