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Original article

Punica granatum waste to ethanol valorisation employing optimized levels of saccharification and fermentation

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ABSTRACT

Pomegranate peels (PPW) as municipal waste is inexpensive biomass that could be a renewable source of sugars particularly rich in hemicellulosic contents. The subsequent conversion of available sugars in PPW can provide prospective strategy for cost-effective bioenergy production. In this study, an experimental setup based on CCD was implemented with the aim of bioconversion of biomass into bioethanol. The factors considered were Hydrochloric acid concentration (X_1), the hydrolysis temperature (X_2) and time (X_3) for optimization with dilute Hydrochloric acid (HCl) saccharification. The present study investigates the optimised level of bioethanol synthesis from acid pre-treated PPW explained by RSM. Subsequently, three yeasts viz. *Saccharomyces cerevisiae* K7, *Metschnikowia* sp. Y31 and *M. cibodasensis* Y34 were utilized for fermentation of acid hydrolysed and detoxified feed stocks. Optimum values of reducing sugars 48.02 ± 0.02 (gL^{-1}) and total carbohydrates 205.88 ± 0.13 (gL^{-1}) were found when PPW was hydrolyzed with 1% HCl concentration at 100C of temperature for 30 min. Later on, fermentation of PPWH after detoxification with 2.5% activated charcoal. The significant ethanol (g ethanol/g of reducing sugars) yields after fermentation with *Metschnikowia* sp. Y31 and *M. cibodasensis* Y34 found to be 0.40 ± 0.03 on day 5 and 0.41 ± 0.02 on last day of experiment correspondingly. *Saccharomyces cerevisiae* K7 also produce maximum ethanol 0.40 ± 0.00 on last day of incubation utilizing the PPWH. The bioconversion of commonly available PPW into bioethanol as emphasize in this study could be a hopeful expectation and also cost-effective to meet today energy crisis.

1. Introduction

Agro-industrial waste comprising of lignocellulosic substrate that is abundant as well as sustainable energy sources accumulated in large quantities every year (Mussatto et al., 2010). In Pakistan, fruit and vegetables contribute to thirty percent waste during harvesting and deprive to gain economic benefits. The annual yield of pomegranate reaches up to 0.5 million tons in 2010–11 in Pakistan. Pomegranate fruit consist of 50% exocarp or peels, 10% seeds and 40% aril (Aviram et al., 2000; Orzua et al., 2009; Christaki et al., 2011; Paul et al., 2019). Pomegranate fruit

Abbreviations: PPW, Pomegranate peel waste; PPWH, Pomegranate peel waste hydrolyzate; RT, Room temperature; MYG, Malt yeast glucose; DNS, Dinitrosalicyclic acid; ANOVA, Analysis of variance; CCD, Central composite design; RSM, Response surface methodology.

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is used as fresh and processed forms such as jams, marmalades and juices (Ay et al., 2012; Moghadam et al., 2013; Bhatnagar et al., 2015). The juice is extracted industrially as well as at domestic level. Massive waste in form of peels and seeds is produced by Industrial processing and will cause serious environmental issues when are not disposed of properly (Mohamad and Khalil, 2014). Pomegranate peels representing up to 40% of the whole fruit as the main by-product of pomegranate processing industries after production of pomegranate juice. This results a major waste disposal problem for the industry (Kanatt et al., 2010). To reduce the accumulation of agricultural waste, Strategy of 3 R i.e., reduce, reuse, recycle was suggested to improve energy and agro-industrial waste management. Other strategies include incineration and land filling. Though these strategies contribute to minimize the waste piles but they have some harmful ecological impacts, too (Forastiere et al., 2009; Mahmoudkhani et al., 2014). Such wastes can be consumed by biorefinerry technique to get the valuable products like bioethanol as they are chemically composed of sugars and other compounds of interests. The most investigated and optimistic replacement of cellulosic biomass is ethanologenesis. Ethanologenesis is appealing process due to consumption of cheap and incompatible biomass (Kabir et al., 2019). This waste with substantial sugars easily subsumes by microbes; proved it a very suitable raw material for ethanologenesis by fermentation (Reddy, 2019). According to Viuda-Martos et al. (2010) pomegranate arils consist of water (80–85%), hexose sugars such as fructose and sucrose, certain organic acid i.e., citric acid, malic acid, ascorbic acid, and other componds (Phenolics, pectin, antioxidants). The biomass based ethanol production enlarged in last decades but it represent around 40% – 70% of total cost of production (Claassen et al., 1999).

The usual and frequent technique for the lignocellulosic biomass is chemical as well as enzymatic hydrolysis. Hemicellulose is an amorphous component and required less extreme conditions for breakdown. Hydrolysis by dilute acid is well known technique could be appointed for hemicelluloses (Prasad et al., 2007). For ethanologenesis a pretreatment is mandatory step to proffer cellulose in further attainable forms. *Saccharomyces cerevisiae* frequently used microorganism for ethanologenesis from fermentable sugars. Broad ranges of celluolytic microorganism are known for dilute acid hydrolysis technique for the ethanologenesis (Berlin et al., 2005; Sanchez and Cardona, 2008).

Statistical advancement in experimental proposal provides controlling tool to swot numerous factors in a procedure at the same time. A CCD by RSM is accepted technique utilize for design interpretation and modelling (Talebnia et al., 2008). The PPW is composed of lignocellulosic waste that could process for bioethanol synthesis. Ethanologenesis from non edible and biodegradable waste render an acceptable solution towards waste management also energy resources (Dubois and Lasida, 2010).

2. Materials and methods

2.1. Biomass preparation for compositional analysis

PPW were accrued from numerous localities in Lahore and rinsed to get rid of dirt particles followed by dehydration in convection oven at 60 °C. The dried pomegranate substrate was crushed into powder of equal size (~1 mm).

To determine reducing sugar, total sugars and protein contents from biomass, the filtrate of PPW taking distilled water as solvent (1:100) was obtained after 24 h incubation (at 30 °C and 200 rpm) and processed by DNS, phenol–sulfuric acid and Folin-Ciocalteu methods (Layne, 1957; Marsden et al., 1982; Nielsen, 2010) while the filtrate of PPW in ethanol (1:10) incubated (at RT for 24 h) was used for the approximation of lipids by administrating Zolllner and Kirsch method (Zöllner and Kirsch, 1962). Ash and moisture were approximated by following the protocols of AOAC (2012). Intended for appraisal of extractives, hemicelluloses, lignin and cellulose the gravimetric method was used with a little change (Ayeni et al., 2013).

2.2. Biomass processing for ethanologenesis

2.2.1. Acid sacharrification and optimization of PPW by CCD

Dilute hydrochloric acid was used for PPW hydrolysis. Peels and acid were used with ratio of 1:10. Pomegranate peels and dilute hydrochloric acid mixture was dispensed in flask having capacity of hundred milliliter and covered by foil. Saccharification experiment was proceeded with three hydrolyzing parameters i.e. acid concentration, hydrolysis temperature and time. Experiment was executed into 20 runs with triplicates as designed by RSM. The PPW mixture with specified parameters was kept on shaking in incubator at 100 rpm. Filtration of hydrolysed PPW mixture was completed after specified time period followed by neutralization by NaOH pellets. Detoxification of PPWH (pomegranate peels waste hydrolyzate) was done after filtration of neutralized hydrolyzate

The PPW was hydrolyzed by hydrochloric acid due to which its cellulose and hemicellulose contents were broken down into fermentable sugars. The quantity of reducing sugars (Y) affected by some variables through hydrolysis was; HCl concentration X_1, hydrolysis temperature X_2 and time X_3. These variables were optimized pertaining CCD of RSM (Khuri and Mukhopadhyay, 2010). The experimental plan included 20 runs with three independent variables which describe their relationship with response variable (Y) (Dean et al., 2017). The range of parameters was set up with the assistance of CCD by RSM (Table 1). CCD provides matrix for three independent variables of responses that also expressed further (Table 2). The design of model comprised of low as well as high levels for hydrolysis parameters along with middle points as 3% X_1, 75∘C X_2 and 45 min. X_3. The model was designed on the basis of data obtained from previous research that emphasized the influence of some parameters in biomass hydrolysis (Akram, 2015; Siddique, 2016; Nasim, 2016; Pervaiz, 2016). The following study dealt with the optimization of certain key factors for acid hydrolysis employing Response Surface Methodology.

The quadratic general equation 'Y' (1) demonstrated the interrelationship of variables as well as responses viz reducing sugars (Y_1) and total sugars (Y_2) via RSM as:

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

In the equation, Y was predicted response whereas ß$_0$, ß$_1$-ß$_2$-ß$_3$, ß$_{11}$-ß$_{22}$-ß$_{33}$, ß$_{12}$-ß$_{13}$-ß$_{23}$, X_1-X_2-X_3 and e represented constant coefficient, linear coefficients, quadratic coefficients, cross products coefficients, input variables and error between the observed and predicted Y respectively. To investigate the suitability and reliability of model, ANOVA was used. The authenticity and model quality was evaluated by the coefficient of R^2 and Adjusted R^2. 3D surface graphs illustrated individual as well as interactive effect of the variables upon responses of polynomial equation. The optimum region was also pointed out through these graphs on parameters (Bashir et al., 2010).

2.2.2. Estimation of percent saccharification of PPWH

To calculate percent sacharrification, 5 g of PPW was added in 50 ml of 1% HCl at 100 °C for 30 min. To assess reducing sugars biochemically, DNS protocol was performed. The percent sacharrifica-

Table 1
Coded values of the variables for the central composite design of alkaline hydrolysis of PPW.

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

Table 2
Central composite design (CCD) matrix of three independent variables for responses using hydrochloric acid of hydrolysis of PPW.

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

tion (2) was evaluated by following formula (Alimon, 2011; Mithra et al., 2018).

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

2.2.3. Ethanologenesis from PPWH by using different yeast strains

Biethanol synthesis was performed using three yeasts isolated by first author in Advanced Fusion Technology Unit, Mie University, Mie, Japan (Chaudhary and Karita, 2017). The selected yeasts viz *Saccharomyces. cerevisiae* K7, *M. cibodasensis* Y34 and *Metschnikowia* sp. Y31 have dual cellulolytic and ethanologenic potential. *S. cerevisiae* K7 was arranged from Brewing Society, Tokyo, Japan.

2.2.4. Detoxification of PPWH by activated charcoal

During hydrolysis, certain inhibitors i.e. phenolic compounds were also formed with reducing sugars which hinder the efficiency of ehanol synthesis. For this purpose, detoxification of pomegranate peel waste hydrolyzate (PPWH) was performed by 2.5% activated charcoal. (2.5 g in 100 ml PPWH) and the supernatant was neutralized by standard solution of base or acid (Mussatto and Roberto, 2005). The hydrolyzate with charcoal was kept on shaking at 200 rpm (1 h at 30 °C) and removed by filteration. The clear supernatant was obtained by centrifugation (2500*g*, 25 min) followed by neutralization with the help of NaOH pellets. Total phenolic compounds were estimated in hydrolyzate via Folin-Ciocalteu method (González et al., 2003).

2.2.5. Ethanologenesis from PPWH by using different yeast strains

Three fermentation media prepared after neutralization with addition of inoculum (5 ml) of isolates separately. The MYG medium was prepared for inoculums then autoclaved at 121 °C temperature for 15 min. An addition of 500 μl of previously prepared yeast strains was done in medium and incubated for one day at 30 ± 0.2 °C.

In each flask, the detoxified hydrolyzate (50 ml) and synthetic media (45 ml) were added. The synthetic medium was prepared by adding minerals consisted of yeast extract (3.575 g), ammonium sulphate (1.43 g), monopotassium phosphate (1.496 g), magnesium sulphate (0.44 g), calcium chloride (0.165 g), citric acid (0.825 g), and sodium citrate (3.3 g) in distilled water (550 ml) and autoclaved.

For incubation, the flasks placed for 10 days without shaking at 30 ± 0.2 °C. Reducing sugars in acidic hydrolyzate provides carbon to yeast isolates whereas synthetic medium provides minerals, vitamins and water. The fermentation kinetics was studied at regular intervals of 24 h for 10 days. The assessment of reducing sugars, yeast growth and ethanol were performed subsequently. Optical densities (600 nm) of yeast were measured to assess the growth in fermentation medium spectrophotometerically (Yang et al., 2018). Fermentation efficiency (3) was computed (Mithra et al., 2018) as;

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

2.3. Statistical method

The experimental data for saccharification as well as fermentation experiments was obtained in triplicates. Design Expert (version 6.0.9 Software, Stat-Ease, Minneapolis, MN 55413) was the main software employing two statistical tools i.e., ANOVA and regression to analyze data obtained by CCD.

To examine the experimental data from fermentation experiment, one-way ANOVA by the Dunkun's Multiple Range test (SPSS ver. 17.0 Software) was used.

3. Results

3.1. Compositional data of PPW

The compositional analysis was designed to study various contents in PPW. Pomegranate peels waste gained the interest of researchers due to the presence of fermentable sugars to be helpful for ethanologenesis (Hasnaoui et al., 2014; Zhu et al., 2015). PPW have considerable amount of cellulose and hemicellulose that were hydrolyzed chemically as well enzymatically for bioethanol production employing microbes (Demiray et al., 2018, 2020). The calculations depicted the total amount of carbohydrates, proteins, lipids and reducing sugars contents (gL^{-1}) was 78.6 ± 0.01, 16.6 ± 0.005, 3.3 ± 0.001and 24.1 ± 0.01 correspondingly. As well as the extractive, hemicellulose, Crude cellulose + insoluble lignin and soluble lignin contents (%) were evaluated as 21.1 ± 0.11, 29.30 ± 1.26, 35.33 ± 0.30 and 14.30 ± 1.25 respectively. The study showed that the peels comprised of percent moisture (7.56 ± 0.08) and ash (12.4 ± 0.02) contents (Table 3).

Table 3
Compositional study of pomegranate peels.

Contents	Quantity
Moisture Contents (%)	7.56 ± 0.08
Reducing sugar Contents (**gL^{-1}**)	24.1 ± 0.01
Total Carbohydrate (**gL^{-1}**)	78.6 ± 0.01
Total Lipids (**gL^{-1}**)	3.3 ± 0.001
Total Proteins (**gL^{-1}**)	16.6 ± 0.005
Extractives (%)	21.1 ± 0.11
Hemicellulose Contents (%)	29.30 ± 1.26
Soluble lignin Contents (%)	14.30 ± 1.25
Crude Cellulose + insoluble lignin (%)	35.33 ± 0.30
Ash contents (%)	12.4 ± 0.02

All values represent means of triplicates ± S.E.M.

3.2. Optimization of saccharification parameters by CCD

Lignocellulosic biomass of PPW was hydrolysed by hydrochloric acid for release of reducing sugars to conduct fermentation for ethanologenesis. The values obtained through experiment for reducing sugars release by acid pre-treatment in 20 runs experimental design were tabulated (Table 4).

Here, the equation (4) represent reducing sugars (Y_1) by mean of ANOVA regarding coded factor was:

$$Y_1 = 52.072 + 4.31X_1 - 1.023X_2 + 1.068X_3 - 1.066X_1^2 + 0.0075X_2^2 - 0.0139X_3^2 - 0.052X_1X_2 + 0.108X_1X_3 + 0.0011X_2X_3 + 73.11 \quad (4)$$

The positive symbols designate synergistic interaction while the negative symbol identified antagonistic relatedness among variables. The optimum reducing sugars after hydrochloric acid hydrolysis were estimated with the help of CCD at 1% hydrochloric acid concentration with the hydrolysis temperature and time of 100C and 30 min consequently. The optimum value of percent reducing sugars in hydrochloric acid PPWH was calculated as 48.02 ± 0.02 while its predicted value was found to be 49.28.

Statistical methods ensured to interpret data correctly and to narrate apparent meaningful/significant relationships that are not due to chance occurrences. The computed significance by statistical tools interpreted the hypothesis of present study that PPW cellulose as well as hemicellulose was hydrolyzed into monomer (reducing sugars). The ethanol was the end product by fermenting monomeric sugars. Computed different values of F, p, R-squared and adequate precision by statistical tools will support to judge the appropriateness of hypothesis or model. The standard values to interpret the appropriateness of model will be more than four for adequate precision and F while near to one for R^2 and less than 0.05 for p. By analysing variance, the appropriateness of model was described by F-value of 4.71 and p-value of 0.0153. The not significant Lack of fit was good with the F-value of 1.89 and the p-value of 0.2787 (Table 5).

Regression was used as statistical tool to evaluate coefficient of R^2 and Adjusted R^2 as 0.8248 and 0.6495 correspondingly (Table 6). R-squared (R^2) explicated the variance proportion of dependent and independent variables. Usually, the fitness of observations were depicted by obtaining larger values of R^2 than 0.5. In present study the R^2 value was 0.8248 for Y1 response. Adequate precision compared predicted values range to present prediction error (average) at the design points. A ratio > 4 is desirable to interpret significance. The current investigation depicted ratios of 7.565 for response Y1 that is a better indicator of model to navigate the space (Table 6).

Response surface curves were conspired to give demonstration of the effect of each factors for the reducing sugar contents. In Fig. 1(A) graph showed the reaction of Hydrochloric acid concentration and temperature during hydrolysis experiment. It designated a diminutive decrease in the reducing sugars escorted by increase in acid concentration while increase was observed with increase in temperature up to 87.5 °C. In Fig. 1(B) plot was constructed among Hydrochloric acid concentration and time of hydrolysis. The graph indicated the decrease in reducing sugar by increasing both factors but the decrease in acid concentration is sharp. In Fig. 1(C) plot pointed out the relation of temperature and time during hydrolysis. The reducing sugars increase was traced by increasing both factors but decrease was noted between 87.5 and 100 °C

The percent saccharification yield (reducing sugars in hydrolyzate/reducing sugars in PPW) by the PPWH with Hydrochloric acid was found to be 2.00%.

Table 4
Central Composite Design (CCD) matrix of three independent variables for reducing sugars, total carbohydrates, weight loss, extractives, hemicellulose, lignin and cellulose contents by hydrochloric acid hydrolysis in pomegranate peels.

Runs	Acid conc. (%)	Temp (C)	Time (min)	Red sugars (gL^{-1})	Total carbs (gL^{-1})	Wt loss (%)	Extractives (%)	Hemicellulose (%)	Soluble Lignin (%)	Crude Cellulose + insoluble lignin (%)
1	3	75	45	50.2 ± 0.06	236.3 ± 0.31	68.11 ± 0.22	19.87 ± 0.72	24.76 ± 1.25	26.61 ± 1.30	28.76 ± 1.86
2	5	50	30	39.1 ± 0.01	170.3 ± 0.18	66.00 ± 0.39	20.67 ± 4.17	20.35 ± 2.10	23.80 ± 1.52	35.18 ± 2.44
3	5	100	60	48.3 ± 0.00	205.2 ± 0.13	68.22 ± 0.73	15 ± 0.97	25.51 ± 0.21	28.72 ± 0.85	30.77 ± 1.97
4	5	100	30	44.1 ± 0.02	208.4 ± 0.18	71.89 ± 0.68	20.53 ± 1.18	15.91 ± 1.26	32.11 ± 0.84	31.44 ± 0.99
5	1	100	30	47.2 ± 0.01	171.4 ± 0.31	62.89 ± 0.40	17.95 ± 0.72	26.27 ± 1.57	23.79 ± 1.83	31.99 ± 2.70
6	3	75	45	34.1 ± 0.00	201.3 ± 0.27	73.89 ± 6.23	16.9 ± 0.40	19.09 ± 0.36	26.67 ± 1.06	37.34 ± 1.16
7	5	50	60	40.2 ± 0.01	220.5 ± 0.14	68.44 ± 0.62	17.59 ± 0.65	18.41 ± 1.09	29.34 ± 2.15	34.66 ± 1.84
8	3	75	45	47.2 ± 0.01	211.5 ± 0.07	68.56 ± 0.80	15.53 ± 0.33	19.72 ± 1.44	28.21 ± 1.07	36.54 ± 2.17
9	1	100	60	52.3 ± 0.01	195.6 ± 0.00	62.22 ± 0.95	16.83 ± 1.36	21.02 ± 2.24	24.19 ± 1.27	37.97 ± 2.04
10	1	50	30	56.1 ± 0.01	209.3 ± 0.08	64.78 ± 0.44	19.57 ± 1.41	20.74 ± 0.89	34.62 ± 1.54	25.07 ± 1.09
11	1	50	60	55.5 ± 0.00	197.5 ± 0.10	61.00 ± 0.19	15.39 ± 0.93	18.47 ± 1.79	35.47 ± 3.23	30.67 ± 2.34
12	3	75	45	39.5 ± 0.01	188.4 ± 0.28	74.11 ± 1.93	20.06 ± 0.81	23.13 ± 2.17	24.26 ± 1.91	32.55 ± 4.25
13	6.36	75	45	46.2 ± 0.01	193.8 ± 0.06	77.33 ± 0.19	20.09 ± 0.83	20.84 ± 1.50	24.85 ± 1.87	34.22 ± 3.09
14	0.36	75	45	22.5 ± 0.01	152.4 ± 0.15	56.11 ± 0.44	22.52 ± 0.90	17.97 ± 0.34	20.69 ± 3.12	38.81 ± 2.42
15	3	75	70.23	24.4 ± 0.00	176.3 ± 0.05	72.56 ± 0.44	19.85 ± 0.63	19.68 ± 0.47	23.87 ± 4.51	36.60 ± 4.48
16	3	75	45	26.3 ± 0.00	194.2 ± 0.13	69.00 ± 0.88	17.52 ± 0.95	19.56 ± 0.61	26.97 ± 3.74	35.95 ± 4.25
17	3	75	19.77	45.7 ± 0.00	203.2 ± 0.18	68.78 ± 0.11	21.36 ± 0.68	14.04 ± 0.62	25.28 ± 0.49	39.32 ± 1.77
18	3	75	45	44.7 ± 0.00	190.4 ± 0.16	68.00 ± 0.19	20.49 ± 0.70	17.46 ± 0.25	27.5 ± 0.86	34.55 ± 0.22
19	3	117.04	45	35.6 ± 0.01	192.4 ± 0.15	68.44 ± 0.73	18.31 ± 0.09	24.11 ± 1.47	21.45 ± 3.78	36.12 ± 2.52
20	3	32.96	45	56.8 ± 0.10	177.7 ± 0.07	67.56 ± 0.22	19.86 ± 0.30	26.07 ± 0.88	20.7 ± 3.70	33.37 ± 3.04

All values correspond to means of triplicates ± S.E.M.

Table 5
Single factor ANOVA ($p < 0.05$) of fitted quadratic regression model for reducing sugars and total carbohydrates examined by Hydrochloric acid treated PPWH.

Contents	Source	Sum of Squares	df	Mean Square	F Value	p value
Reducing sugars	Model	1156.44	9	128.49	4.71	0.0153
	Residual	245.73	9	27.30		Significant
	Lack of fit	172.62	5	34.52	1.89	0.2787
	Pure Error	73.11	4	18.28		Not significant
	Cor Total	1422.75	19			
Total carbohydrates	Model	18701.51	9	2077.95	4.63	0.0161
	Residual	4036.20	9	448.47		Significant
	Lack of fit	3547.22	5	709.44	5.80	0.0566
	Pure Error	488.99	4	122.25		Not Significant
	Cor Total	22944.17	19			

Table 6
Results of regression analysis for the optimization of reducing sugars and carbohydrates analysed by hydrochloric acid hydrolysis.

Contents	C.V	Press	R-Squared	Adjusted R-Squared	Predicted R-Squared	Adequate Precision
Reducing sugars	11.39	1890.53	0.8248	0.6495	0.3483	7.565
Total Sugars	11.16	36,594	0.8225	0.6450	0.6094	9.397

Total carbohydrates were also studied likewise after dilute acid hydrolysis of PPW with hydrochloric acid. The quadratic equation (5) was shown below.

$$Y_2 = +195.33 + 17.20X_1 + 15.35X_2 + 3.05X_3 - 16.97X_1^2 + 15.86X_2^2 - 7.92X_3^2 + 4.34X_1X_2 - 9.71X_1X_3 - 10.54X_2X_3 + 488.99 \quad (5)$$

The optimum value of total carbohydrates in PPWH was calculated as 205.88 ± 0.13 while its predicted value was found to be 177.89. The ANOVA (Table 5) indicated the model's significance with the F- 4.63 and p- 0.0161 values while lack of fit was non-significant having 5.80 F-value and 0.0566 p value. The estimated values of the coefficient of R^2, Adjusted R^2 and adequate precision were 0.8225 and 0.6450, 9.397correspondingly (Table 6).

In Fig. 2(A) the graph demonstrated an increase in carbohydrates by increasing temperature while reverse response was recorded with Hydrochloric acid concentration. The Fig. 2(B) showed a sharp decrease in carbohydrates by increasing acid concentration while slight decrease was observed with increasing in the hydrolysis time. The Fig. 2(C) graph depicted the decrease in carbohydrates with increase in both of temperature and time during hydrolysis.

3.3. Detoxification of chemically pre-treated biomass

The PPWH of hydrochloric acid was detoxified with the activated charcoal earlier than fermentation experiment to lessen the quantity of inhibitors. After detoxification, 51% reduction in phenol contents was reported. The percent phenolics in PPWH before fermentation was 1.32 ± 0.09 while after detoxification their amount was reduced to 0.68 ± 0.003.

3.4. Ethanologenesis of detoxified PPWH

The PPWH was prepared using the optimum conditions of acid pretreatment. Maximum ethanol (g ethanol/g of reducing sugars) was 0.40 ± 0.03 on day 5 and 0.40 ± 0.02 on day 10 noticed via *Metschnikowia* sp. Y31 and *M. cibodasensis* Y34 correspondingly while *S. cerevisiae* K7 synthesized maximum ethanol of 0.41 ± 0.00 (g ethanol/g of reducing sugars) on last day of incubation (Fig. 3). Ethanol contents (g/L), 10.77 ± 0.45, 11.04 ± 0.34 and 10.80 ± 0.18 were obtained from *Metschnikowia* sp. Y31, *M. cibodasensis* Y34 and *S. cerevisiae* K7 respectively. The fluctuation in the reducing sugar contents as well as growth of yeast was also observed during fermentation experiment (Figs. 4 and 5). Fermentation efficiency was calculated by dividing with theoretical ethanol yield (0.51) and was found 78.43% in *Metschnikowia* sp. Y31 and *S. cerevisiae* K7 while 80.39% in *M. cibodasensis* Y34 isolates in PPWH.

4. Discussion

Hydrolysis of PPW by dilute acid is an imposing treatment that permits hydrolysis of hemicellulose and cellulose into sugars for fermentation. An analysis of PPW composition was approximated by various analytical methods. Several researchers put efforts for ethanologenesis from waste by utilizing microorganisms. Amongst these microorganisms *S. cerevisiae* known as the baker's yeast that widely consumed during fermentation also known as model yeast (Rizzello et al., 2019). *Saccharomyces cerevisiae* K7 which is mostly used microorganisms for ethanol production used as standard yeast in present study. The challenge of this study was that *Metschnikowia* sp. Y31 and *M. cibodasensis* Y34 has been used as promising microorganisms for ethanologenesis.

The initial treatment of biomass is crucial to perform pre fermentation bioconversion that provides feasible substrate for the activity of microorganisms. In current study most employed technique of dilute acid hydrolysis for hemicellulosic and lignocellulosic breakdown was applied as pre-treatment. The CCD using RSM by software of Design Expert was used to examine individual effect of the hydrolysis factors and their independence effect. The design has also been reported by various studies for optimization of the bioethanol production (Pradeep et al., 2012; Adnan et al., 2014).

The optimum reducing sugar contents 48.02 ± 0.02 (gL^{-1}) were obtained at the 1% HCl concentration, 100 °C temperature with 30 min time for hydrolysis. The predicted value of reducing sugars (gL^{-1}) recorded during study was 49.28. Statistical analysis revealed the model's significance having 4.93-F, 0.0215-p and 0.3811-R^2 values. In another model, three percent sulphuric acid released 52.3 ± 0.10 gL^{-1} reducing sugars in PPW at 100 °C within 30 min with 4.71-F and 0.0153 p value (Saleem et al., 2020). The increase in reducing sugars was reported due to acidic pretreatment of PPW because acid convert the cellulose and hemicellulose into fermentable sugars (El Asli and Qatibi, 2009). Similar results with 56.07 g/liter release of reducing-sugar contents were recorded with 2.0% HCl concentration for 45 min in durian peels

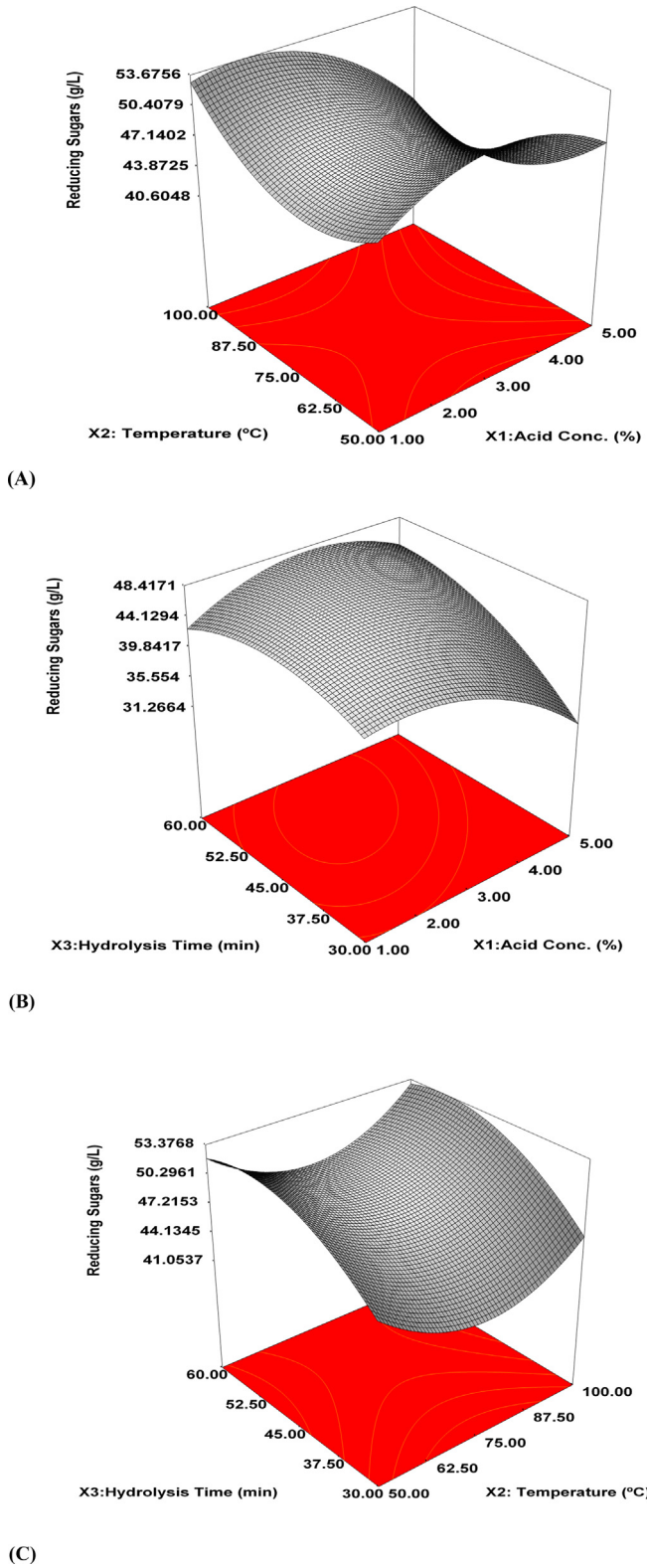

Fig. 1. Response surface graph for reducing sugars (g/L) from varying Hydrochloric acid concentrations with different temperatures (A), time (B) and temperature with time (C) in PPW.

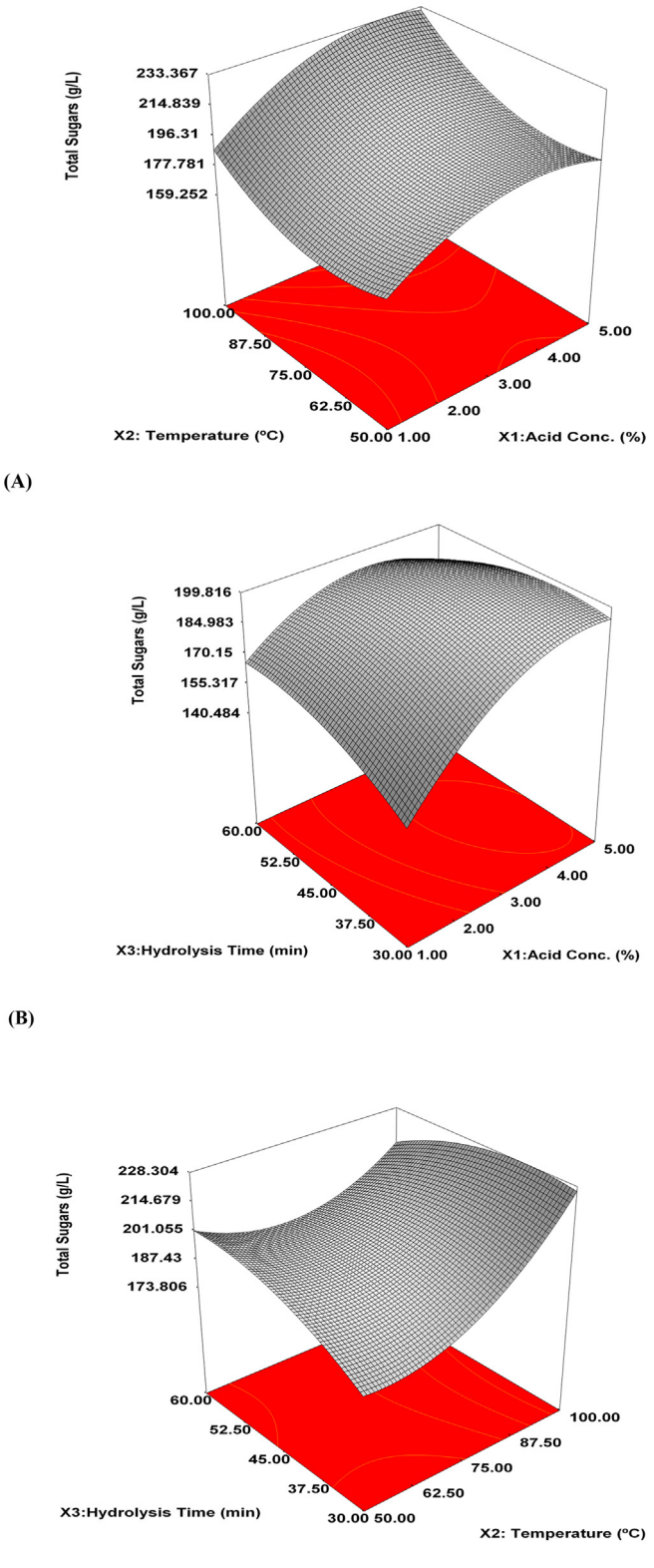

Fig. 2. Three dimensional plot for total carbohydrates (g/L) from varying temperatures (A), time (B) and temperature with time (C) by Hydrochloric acid hydrolysis in PPW.

(Unhasirikul et al., 2012). Acid pretreatment hydrolyzed durian peels into glucose as major sugar. Aguilar et al. (2002), reported the release of glucose and other sugars by the action of acid. Dilute acid is considered as promising pretreatment technique to convert hemicellulose into monomeric sugars by causing changes in the structure. Hollow pores are formed in lignocellulosic biomass that help the cellulose to be accessible for enzymatic treatment (Gil et al., 2010; Toquero and Bolado, 2014; Loow et al., 2016).

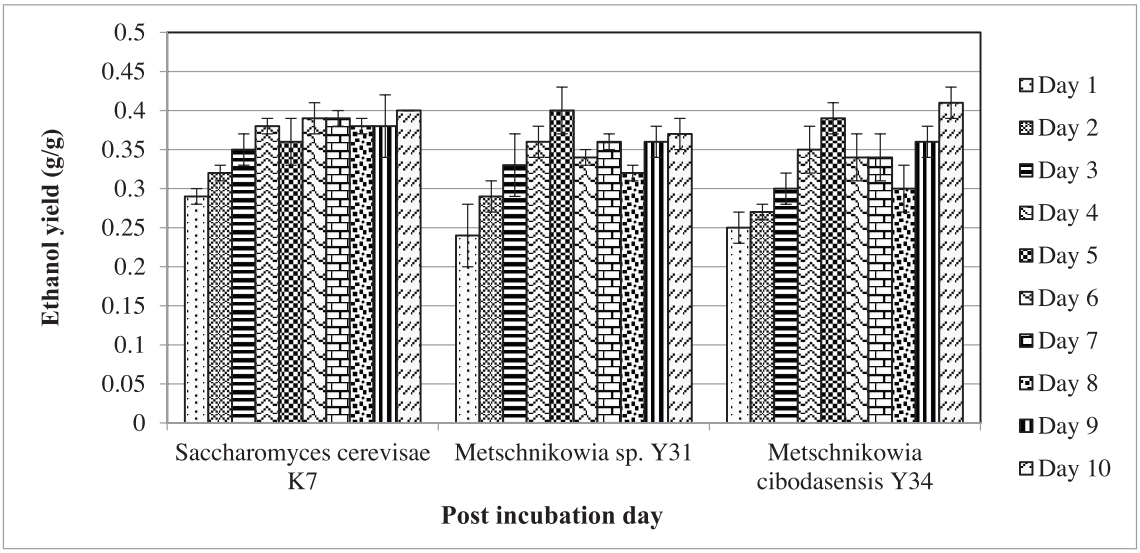

Fig. 3. Display of day wise ethanologenesis (g/g) by *Saccharomyces cerevisiae* K7, *Metschnikowia* sp. Y31 and *Metschnikowia cibodasensis* Y34 isolates by hydrochloric acid hydrolyzate. (Error bars represent SEM).

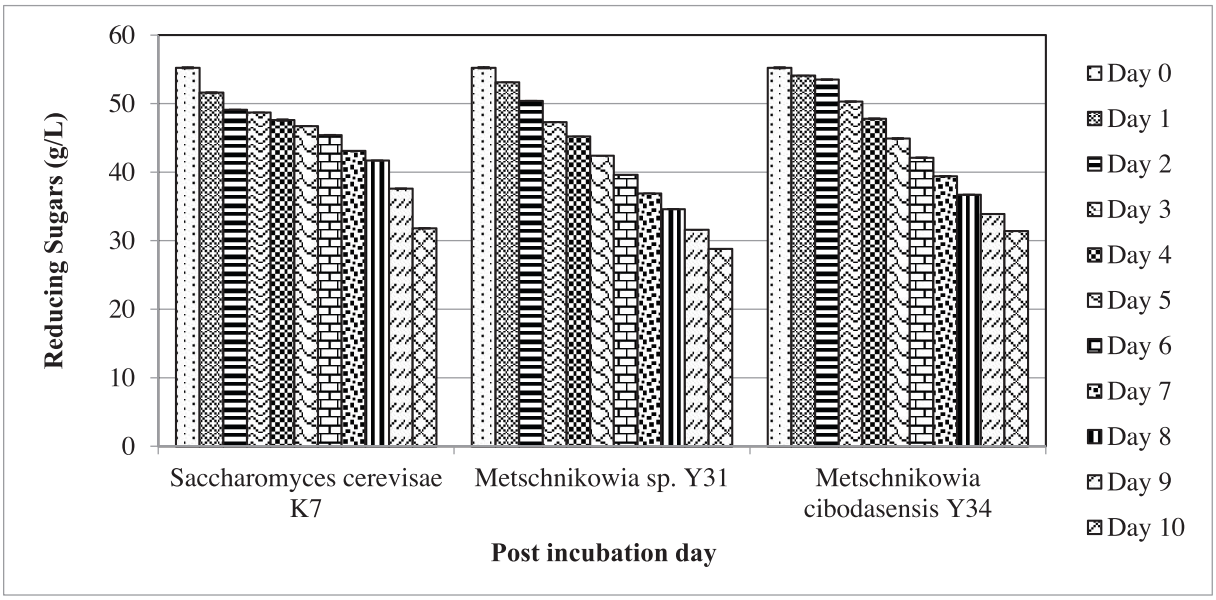

Fig. 4. Display of day wise reducing sugars (mg/ml) by *Saccharomyces cerevisiae* K7, *Metschnikowia* sp. Y31 and *Metschnikowia cibodasensis* Y34 isolates by hydrochloric acid hydrolyzate. (Error bars represent SEM).

For optimum carbohydrate contents, experimental value was found to be 205 ± 0.13 while the predicted value was 177. The model was significant with the F, p and R^2 values as 4.63, 0.0161 and 0.8225 correspondingly. Hydrochloric acid was used to enhance the conversion into reducing as well as total sugars and acid hydrolysis efficiency in durian peels (Unhasirikul et al., 2012).

In present study, more sugars (gL^{-1}) were released by increasing the acid concentration such as 47.2 ± 0.01 (1%, 100℃, 30 min) and 56.80 ± 0.10 (3%, 32.96℃, 45 min). By increasing acid concentration, more sugars are produced whereas sugars are converted into more inhibitors such as furfrals and phenolics (Chaudhary et al., 2012; Kupiainen et al., 2014; Baadhe et al., 2014; Castro et al., 2014).

Many detoxification methods have been reported as functional to alleviate the inhibitors of hydrolysis for improving microbial growth. Numerous phenolics that were generated at the time of pre-treatment in present study were eradicated using 2.5% activated charcoal. The amount of phenolics was reduced to 51% in hydrochloric acid PPWH. Lignin is transformed partially into phenolic compounds by acid saccharification. Remaining part of lignin became the part of fermentation medium which leads to the formation of inhibitors to slow down the microbial metabolism. The inhibitors produced during fermentation not only perturbed the microbial metabolism but cause blockage of the whole process (Taherzadeh and Karimi, 2011; Kim et al., 2013). High temperature contributed to produce more inhibitors than low temperature because sugar degrading rate into phenolics and furfural is high for same hydrolysis period. As temperature exceeded to 100 °C, the inhibitors as well as sugars are degraded as same rate (Łukajtis et al., 2018).

During ethanologenesis by dilute hydrochloric acid hydrolyzate the log phase for *Metschnikowia* sp. Y31 ends on day 7. While with

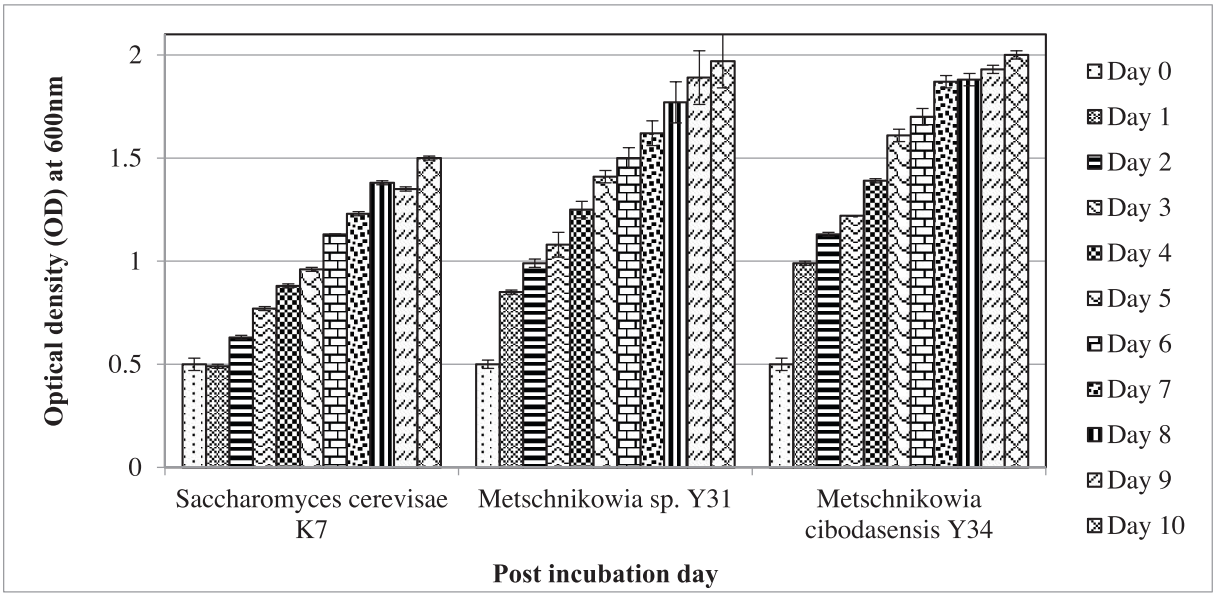

Fig. 5. Display of day wise yeast growth in *Saccharomyces cerevisiae* K7, *Metschnikowia* sp. Y31 and *Metschnikowia cibodasensis* Y34 isolates in hydrochloric acid hydrolyzate. (Error bars represent SEM).

M. cibodasensis Y34 and standard yeast *S. cerevisiae* K7, it was observed on day 8. The significant amount of ethanol was evaluated in

log phase for *Metschnikowia* sp. Y31 and in stationary phase for *M. cibodasensis* Y34 and standard yeast *S. cerevisiae* K7. With dilute Hydrochloric acid hydrolyzate the significant ethanol yield (g ethanol/g of reducing sugars) was 0.40 ± 0.03 with *Metschnikowia* sp. Y31 on day 5. While on last day of incubation *M. cibodasensis* Y34 showed maximum ethanol yield (g ethanol/g of reducing sugars) of 0.41 ± 0.02 and by *S. cerevisiae* K7 it was 0.40 ± 0.00. The current study was close to findings that reported the ethanol production using Pineapple, Watermelon and Muskmelon rinds (Walia et al., 2014). The ethanol titer (g/L), 10.77 ± 0.45, 11.04 ± 0.34 and 10.80 ± 0.18 were obtained from three yeast strains i.e. *Metschnikowia* sp. Y31, *M. cibodasensis* Y34 and *S. cerevisiae* K7 respectively. These results are comparable with the finding of 14.3 g/L ethanol by *Kluyveromyces marxianus* on acid treated pomegranate peels (Demiray et al., 2020) while Demiray et al. (2018) reported 5.5 g/L ethanol contents on acid tread pomegranate peels with *S. cerevisiae.* Improved ethanol contents were observed in present study as compared with other fruit waste such as date palm, orange, mango, and banana peels waste (Arumugam and Manikandan, 2011; Boulal et al., 2016; Maina et al., 2017).

5. Conclusion

The investigation was concluded as dilute hydrochloric acid hydrolysis factors contributed for maximum reducing sugars (g/L) 56.80 ± 0.10 with decrease in hemicellulose as well as cellulose contents in pomegranate peels waste. Ethanol yield recorded in current study proved that the experimental strains *Metchnikowia* sp. Y31 (0.40 ± 0.03) and *M. cibodasensis* Y34 (0.41 ± 0.02) appeared as promising candidates for bioethanol production and biorefinery of 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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Ethical statement

This article does not deal the experiments regarding animal models and human participants. All standard guidelines for the conduction of experiments on microbes were strictly followed.

Consent statement

Informed consent was obtained from all authors involved in the study.

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