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ARTICLE

Ethanol Production from Orange Peels: Two-Stage Hydrolysis and Fermentation Studies Using Optimized Parameters through Experimental Design

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Orange peels were evaluated as a fermentation feedstock, and process conditions for enhanced ethanol production were determined. Primary hydrolysis of orange peel powder (OPP) was carried out at acid concentrations from 0 to 1.0% (w/v) at 121 °C and 15 psi for 15 min. High-performance liquid chromatography analysis of sugars and inhibitory compounds showed a higher production of hydroxymethyfurfural and acetic acid and a decrease in sugar concentration when the acid level was beyond 0.5% (w/v). Secondary hydrolysis of pretreated biomass obtained from primary hydrolysis was carried out at 0.5% (w/v) acid. Response surface methodology using three factors and a two-level central composite design was employed to optimize the effect of pH, temperature, and fermentation time on ethanol production from OPP hydrolysate at the shake flask level. On the basis of results obtained from the optimization experiment and numerical optimization software, a validation study was carried out in a 2 L batch fermenter at pH 5.4 and a temperature of 34 °C for 15 h. The hydrolysate obtained from primary and secondary hydrolysis processes was fermented separately employing parameters optimized through RSM. Ethanol yields of 0.25 g/g on a biomass basis (YP/X) and 0.46 g/g on a substrate-consumed basis (YP/S) and a promising volumetric ethanol productivity of 3.37 g/L/h were attained using this process at the fermenter level, which shows promise for further scale-up studies.

KEYWORDS: Ethanol; fermentation; orange peel; galacturonic acid; hydrolysis; response surface methodology

INTRODUCTION

Citrus fruits are among the most important fruits grown and consumed all over the world. Oranges alone account for about 55% of the global citrus fruit production. Most oranges are produced in tropical and subtropical regions across the globe. The United States is the second largest producer of oranges, accounting for about 11.5% of the total world production (*1*). Orange peel alone accounts for about 50% of the total fruit weight. Despite being rich in nutrients, citrus fruit residues do not find any commercial importance and are largely disposed of in municipal dumps or as underutilized cattle feed (*2*, *3*). Orange peels are rich in fermentable sugars, that is, glucose, fructose, and sucrose, along with insoluble polysaccharides cellulose and pectin (*4*). The presence of low lignin levels makes such substrates ideal for fermentation-based products, such as ethanol production; however, the presence of pectin requires either harsh pretreatment or application of enzymes for the release of sugars.

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Previous studies have reported the successful hydrolysis of citrus peels or citrus peel waste into sugars and their subsequent conversion into ethanol (*5*−*7*). Most of the studies on ethanol production from other lignocellulosic biomass resources employed simultaneous saccharification and fermentation (SSF) (*5*, *8*). Although SSF has several advantages, it needs the optimization of process parameters, including enzyme concentration, pH, and temperature, for efficient hydrolysis and fermentation. Optimization of enzyme mixtures including pectinase and cellulase is a complex process, as many enzyme activities are necessary for complete hydrolysis of pectin-rich cell walls to simple sugars (*9*, *10*). An incomplete understanding of interactions between the galacturonic acid (GA) units in pectin and the nature of the bonds between pectin and cellulose in fruit residues further accentuates this problem. Optimization of important fermentation parameters (pH, temperature, and fermentation time) and knowledge of the interaction between these variables are important for the successful economic production of ethanol. Response surface methodology (RSM) has been successfully employed for the optimization of parameters for the production of enzymes and ethanol in biological systems (*11*, *12*). In our studies, RSM was

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employed to optimize the pH, temperature, and fermentation time for ethanol production.

A pretreatment process is required for the hydrolysis of cellulosic and glycosidic bonds in pectin to release sugars for fermentation. Hydrolysis with dilute acids is less selective than enzymatic hydrolysis (*13*). Hydrolysis using steam or dilute acid under high temperature and pressure could solubilize most of the orange peel-soluble fractions, thereby concentrating the insoluble fractions for subsequent separation and bioconversion. Although primary and secondary hydrolysis processes require two steps, yeast cells could easily be recovered from the liquid medium in the first step for subsequent use. Because the cost of enzymes is an integral part of any SSF process, the primary and secondary hydrolysis steps could play a major role in reducing the cost if enzyme use could be reduced. Although the available literature (*14*) suggests that two-stage dilute acid hydrolysis is ideal for sugar production from lignocellulosic biomass, there are no published studies where two-stage hydrolysis using parameters optimized through statistical design has produced ethanol from fruit residues. The present study was conducted to optimize the primary and secondary hydrolysis for the production of sugars, to minimize the formation of inhibitory compounds [acetic acid, hydroxymethylfurfurals (HMFs), and phenolic compounds], and also to optimize ethanol productivity through statistical experiment design.

MATERIALS AND METHODS

Materials. Navel oranges were procured from a local supermarket in bulk, and the peels were removed, chopped, and dried to a constant weight at 70 °C in a hot air oven (Fisher Scientific Isotemp oven). The dried orange peel was ground using an electric mill (Perten Instruments AB, Sweden). Dried yeast powder was procured from Fleischmann's yeast (Fenton, MO). All of the media ingredients were procured from Difco, BD (United States), and analytical grade chemicals were obtained from Fisher Scientific Inc. (United States). RSM software was purchased from StatEase Inc. (Minneapolis, MN).

Compositional Analysis. Moisture and ash contents in the orange peels were determined using AOAC methods (*15*). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed using the ANKOM 200 fiber analyzer (Ankom Technology, United States) as per the manufacturer's procedure (www.ankom.com). The difference between ADF and ADL and NDF and ADF was reported as cellulose and hemicellulose, respectively. The protein content ($N \times 6.25$) was determined by a Leco combustion method using a FP-2000 auto-analyser (*15*). Pectins were extracted and analyzed by the method devised by Sudhakar and Maini (*16*). The water-soluble fraction was extracted using 1 g of orange peel powder (OPP) in 30 mL of deionized water. The flasks containing the suspension were placed in a boiling water bath for 20 min. The suspension was filtered through cheese cloth, and the residue was subjected to the same treatment twice. The filtrate collected from all of the stages was mixed and concentrated using a rotary vacuum concentrator (Heidolph Collegiate Brinkmann, Germany). The extract was made up to 100 mL using deionized water. The final filtrate was centrifuged, and the supernatant was analyzed for sugars using high-performance liquid chromatography (HPLC; see the Analytical Methods section). The total phenolic concentration was analyzed using a previously described method (*17*). All of the analyses were carried out in triplicate, and the mean and standard deviation (SD) values were calculated using MS Excel.

Hydrolysis of Orange Peel. The 150 mL Erlenmeyer flasks (Fisher Scientific) containing 12% OPP (w/v) were pretreated using sulfuric acid at 0, 0.25, 0.5, 0.75, and 1.0% (w/v) concentrations at 121 °C for 15 min. The hydrolysate was collected in the receiver flask through vacuum filtration and was analyzed for sugars, HMF, furfurals, acetic acid, and total phenols. The experiment was performed in a completely randomized design (CRD), and data were analyzed using statistical analysis software 9.1 (SAS Inc., Cary, NC). The treatment resulting in the highest amount of sugars with relatively less inhibitory compounds was selected for fermentation. Secondary hydrolysis of the pretreated material used the same acid concentrations as used for primary hydrolysis. Secondary hydrolysis was performed at the same temperature and pressure as the primary treatment, except that the treatment time was increased from 15 to 30 min (*14*). The secondary hydrolysis treatment resulting in the highest sugar concentration with the lowest inhibitory and phenolic compounds was selected for fermentation.

Table 1. Response Surface Design Used for the Optimization of Parameters

				coded levels		
run	factor 1 X_1: temperature (°C)	factor 2 X_2: pH	factor 3 X_3: time (h)	X_1	X_2	X_3
1	32.50	5.00	15.00	0	0	0
2	32.50	3.32	15.00	0	−1.682	0
3	40.00	6.00	6.00	1	1	−1
4	40.00	4.00	24.00	1	−1	1
5	32.50	5.00	15.00	0	0	0
6	25.00	6.00	24.00	−1	1	1
7	32.50	5.00	15.00	0	0	0
8	40.00	4.00	6.00	1	−1	−1
9	40.00	6.00	24.00	1	1	1
10	25.00	4.00	24.00	−1	−1	1
11	32.50	6.68	15.00	0	1.682	0
12	19.89	5.00	15.00	−1.682	0	0
13	32.50	5.00	−0.14	0	0	−1.682
14	32.50	5.00	15.00	0	0	0
15	32.50	5.00	30.14	0	0	1.682
16	25.00	6.00	6.00	−1	1	−1
17	25.00	4.00	6.00	−1	−1	−1
18	32.50	5.00	15.00	0	0	0
19	45.11	5.00	15.00	1.682	0	0
20	32.50	5.00	15.00	0	0	0

Experimental Design. The optimization experiment involving pH, temperature, and fermentation time was carried out using the central composite design (CCD). The experiment was planned to obtain the optimized values for pH, temperature, and time for conducting fermentation studies. A three-factor and two-level CCD consisting of 20 experimental runs for ethanol production was employed. The experimental design based on the range of independent variables, generated by the Design Expert software, is presented in **Table 1**. The design consisted of a 23 CCD factorial design having six replicates at the central point and six axial points (α) to allow a better estimate of the experimental error and to provide extra information about the activities within the design space. The range for pH, temperature, and time was set at 4−6, 25−40 °C, and 6−24 h, respectively, based on previous studies (*5*, *18*). Twenty polycarbonate baffled flasks (Fisher Scientific), each containing 13 g of OPP (92% dry mass) in 87 g of deionized water to have a final concentration of 12% (w/v), were subjected to a sterilization pretreatment at 121 °C for 15 min followed by sudden depressurization in an autoclave (TONY SS-325 E Tokyo, Japan). The cotton-plugged receiver flasks (Pyrex filtering flasks), 10 N NaOH solution, and concentrated nutrient solution having 90 and 60 g/L yeast extract and peptone, respectively, were also sterilized. The polycarbonate flasks were removed from the autoclave while they were hot and opened in a biosafety cabinet (Labconco Corp., United States) under sterile conditions to remove volatile compounds (D-limonene), which is detrimental to the growth of yeast cells. The contents were vacuum filtered using a Buchner funnel lined with P-8 coarse filter paper (Fisher Scientific) aseptically in the biosafety cabinet for separation of the hydrolysate. The filtrate pH was adjusted as per the RSM design (**Table 1**), and each of the 20 flasks was supplemented with a concentrated nutrient solution to have a final concentration of 3 and 2 g/L of yeast extract and peptone, respectively. The flasks were inoculated with yeast cells at 1×10^9cells/mL and incubated on shakers at 100 rpm using the temperature and time specified by the RSM design (**Table 1**). The flask contents were centrifuged at 10000*g* at 4 °C for 10 min (Sorvall superspeed FCC B, Sorvall Inc., United States). The supernatant was collected and analyzed for sugar and ethanol using methods mentioned in the Analytical Methods section. Experimental data from CCD were analyzed using RSM algorithm

Design Expert 7.1 (Statease, MN) and fitted according to eq **1** as a second-order polynomial equation including main effects and interaction effects of each variable:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (1)$$

where y = predicted response, β_0 = constant coefficient, β_i = linear coefficient, β_{ii} = quadratic coefficient, and β_{ij} = interaction coefficient. The analysis of variance (ANOVA) and surface plots were generated using Design Expert 7.1, and the optimized values of three independent variables for maximum response were determined using the numerical optimization package of the same software.

Preparation and Propagation of Yeast Cells. The dried yeast powder was aseptically inoculated into sterilized 150 mL Erlenmeyer flasks containing 50 mL of glucose yeast extract (GYE) broth in the P-II biosafety cabinet. The flasks were incubated at 30 °C for 48 h and 100 rpm on an incubator shaker. The inoculum was aseptically transferred to sterilized 250 mL Erlenmeyer flasks containing 100 mL of GYE broth. Fifty milliliters of prepared cultures was aseptically transferred to 1 L flasks containing 500 mL of sterilized GYE broth, and the flasks were incubated at conditions mentioned above for 24 h. The cells were concentrated by centrifugation in sterilized 50 mL centrifuge tubes at 10000*g* at 4 °C for 10 min. The cell count was determined using a hemocytometer (Hausser Scientific, United States). Cells were concentrated to the level of 1×10^9cells/mL for initial pitching purpose so as to have 1×10^8cells/mL in the final fermentation medium at a 10% (v/v) inoculum concentration.

Batch Fermenter Experiments. About 1.2 L of hydrolysate obtained from the selected primary pretreatment was collected in a 2 L batch fermenter (Biostat B, BBI Sartorius, United States). Hydrolysate was neutralized and supplemented with a concentrated nutrient solution to have a final concentration (w/v) of 0.3% yeast extract and 0.2% peptone, respectively, in the fermentation medium. The residual pretreated biomass was collected in sterile bags and stored frozen for secondary hydrolysis. The fermenter-containing hydrolysate was heated to a temperature of 80 °C for 30 min and agitated at 250 rpm, followed by exposure to UV light in the biosafety cabinet for about 30 min, prior to inoculation. This was done for uniform mixing of the nutrient solution with the fermentation medium and elimination of any contamination. Fermentation was performed at temperature, pH, and time optimized through RSM experiments. The fermenter was inoculated with 120 mL of yeast inoculum at a concentration of 1×10^9cells/mL. The agitation speed was maintained at 200 rpm, and the pH was maintained using sterilized 5 N HCl and 10 N NaOH solutions. Samples were drawn at 3 h intervals and analyzed for sugar, cell biomass, and ethanol concentration. The pretreated biomass was analyzed for moisture content using an IR-35 moisture balance (Denver Instruments, United States). Secondary hydrolysis of pretreated biomass used the acid level selected from the hydrolysis experiment. The cultural and operational parameters for secondary hydrolysate fermentation were the same as those for primary hydrolysis. The product yield based on sugar consumption was calculated using the following expression:

$$\frac{[EtOH_t - EtOH_0]}{0.511 \times (\text{carbohydrate concentration} + [\text{cellulose} + \text{hemicellulose}] \times 1.11)}$$

where $EtOH_t$ and $EtOH_0$ are ethanol concentrations at the sampling time and 0 h, respectively, 0.511 is the conversion factor for glucose to ethanol, and 1.11 is the conversion factor of cellulose and hemicellulose to simple sugars.

Cell Biomass Estimation. Ten milliliters of samples obtained during fermentation was transferred to 15 mL preweighed centrifuge tubes, the tubes were centrifuged at 10000*g* at 4 °C for 10 min, and the supernatant was collected and analyzed for sugar and ethanol. The pellet was repeatedly washed with 2 mL of deionized water, vortexed, centrifuged, and dried in a hot air oven at 60 °C until constant weight. The difference between the initial and the final weight was recorded as biomass and expressed in mg/mL.

Analytical Methods. Sugars were quantified by the binary HPLC system (Shimadzu Corp., Japan) using the refractive index detector (RID) and Rezex RCM monosaccharide column (300 mm × 7.8 mm) (Phenomenex, United States). Deionized water was from the Milli Q (Direct Q, Millipore Inc., United States) degassed using ultrasonicator (FS 60, Fisher Scientific) and used as a mobile phase at 0.6 mL/min. The column oven (Prominence CTD-20A) and RID were maintained at 80 and 65 °C, respectively (Prominence LC-20AB). Samples were diluted, centrifuged, and filtered (Phenomenex 0.45 μm RC membranes) into HPLC vials and maintained at 4 °C. The peaks were detected and quantified based on the area and retention time of standards (glucose, fructose, sucrose, xylose, arabinose, galactose, and rhamnose) procured from Sigma Aldrich. The ethanol and GA concentrations were measured using the quatenary gradient HP-1100 HPLC system (Agilent Technologies, United States) and the Phenomenex ROA organic acid column (150 mm × 7.8 mm). In this case, degassed 0.005 N sulfuric acid was used as a mobile phase at 0.4 mL/min. Diluted, filtered samples were stored in amber-colored glass vials at 4 °C. The column was maintained at 80 °C, and eluted compounds were detected using the RI detector. The photodiode array detector (PDA) was coupled with RID and used at 254 nm for detection of HMF, furfural, and acetic acid. The run time was increased from 20 to 36 min at the same conditions used for ethanol and GA analysis. The initial ethanol present in the inoculum was deducted from the final ethanol concentration to report the actual increase during fermentation.

Table 2. Compositional Analysis of Orange Peel on Dry Matter (DM) Basis[a]

parameters (%)	value
cellulose	14.17 ± 0.21
hemicellulose	5.7 ± 0.15
ADL	0.59 ± 0.03
water-soluble fraction[b]	45.93 ± 0.89
protein	6.89 ± 0.06
pectin	18.96 ± 0.90
ash	2.87 ± 0.07

[a] Fat, polyphenols, and other extractives make up the remainder of the composition. Because these were not relevant to the present study, they were not analyzed. Values reported are for means ± SDs for $n = 3$. The average moisture content of OPP was found to be 76%. [b] Comprised of (%) glucose, 16 ± 0.43; fructose, 16 ± 0.41; and sucrose, 11 ± 0.48.

RESULTS

Chemical Analysis of Orange Peel. It is clear from **Table 2** that carbohydrates, cellulose, and pectin are present in a significant quantity in orange peels. Relatively low levels of lignin make the substrate amenable to hydrolysis. Mamma et al. (*3*) had reported relatively higher values for cellulose and hemicellulose and a lower value for pectin in citrus peel waste.

Production of Sugars and Inhibitory Compounds during Primary and Secondary Hydrolysis. It is clear from the results depicted in **Table 3** that the initial sugar concentration of 50.8 g/L was produced during dilute acid hydrolysis at 0.50% (w/v), which was nearly 40% more than control. However, the sugar concentration declined when the acid level was increased beyond 0.5% (w/v), due to degradation of glucose into HMFs (**Table 3**). While the sugar concentration was 40% higher than control with use of 0.5% (w/v) sulfuric acid, the HMF concentration increased more than six times (**Table 3**). The additional sugars formed at 0.5% acid level (w/v) were also comprised of pentose sugars such as arabinose, whose concentration was about 10% of the total sugars (**Table 4**). There was a significant increase in the galactose concentration with an increase in the acid level to 0.75% (w/v), whereas arabinose was released when acid was used at 0.5% or higher (**Table 4**). This indicates that the acid initially attacks glycosidic bonds between the GA units in pectin and subsequently cleaves the bonds between cellulose and hemicellulose, thereby solubilizing some part of these fractions. Thus, after critical appraisal of all of the results, it was decided to carry out the primary hydrolysis using acid at the 0.5% (w/v) level.

Secondary hydrolysis also resulted in an increase in the overall sugar concentration at increased acid levels (**Table 5**) until 0.75%

Table 3. Effect of Primary Hydrolysis on Sugar and Inhibitory Compound Concentrations[a]

	g/L				
treatment	total sugars	HMF	acetic acid	GA	phenolics
control	35.13 e ± 1.29	0.043 d ± 0.001	0	0.52 e ± 0.005	1.61 e ± 0.02
0.25% (w/v) SA	37.55 d ± 1.23	0.05 d ± 0.02	0	0.86 d ± 0.01	1.78 d ± 0.04
0.50% (w/v) SA	50.81 a ± 1.53	0.37 c ± 0.03	0.11 ± 0.005 b	1.09 c ± 0.005	1.96 c ± 0.02
0.75% (w/v) SA	48.90 b ± 1.07	0.89 b ± 0.04	0.19 ± 0.005 b	1.62 b ± 0.005	2.29 b ± 0.03
1.0% (w/v) SA	47.90 c ± 1.36	1.12 a ± 0.01	0.23 ± 0.011 a	1.67 a ± 0.01	2.55 a ± 0.04
ANOVA, *P*(0.05)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
LSD (0.05)	0.75	0.05	0.01	0.01	0.05

[a] Values are means ± SDs, *n* = 3. Means that have the same letter are not significantly different.

Table 4. Sugar Profile of Different Sugars as a Result of Primary Hydrolysis[a]

	g/L				
treatment	glucose	fructose	galactose	arabinose	xylose
control	16.84 e ± 0.50	18.02 b ± 0.48	0.27 c ± 0.13	0	0
0.25% (w/v) SA	17.72 d ± 0.53	18.9 a ± 0.50	0.33 c ± 0.14	0	0
0.50% (w/v) SA	23.32 a ± 0.68	18.88 a ± 0.45	1.9 a ± 0.15	4.94 c ± 0.24	0.98 c ± 0.16
0.75% (w/v) SA	22.93 b ± 0.77	18.26 b ± 0.55	1.62 b ± 0.17	5.22 b ± 0.24	1.28 b ± 0.14
1.0% (w/v) SA	20.57 c ± 0.59	16.98 c ± 0.44	1.55 b ± 0.14	5.38 a ± 0.25	1.91 a ± 0.18
ANOVA, *P* (0.05)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
LSD (0.05)	0.23	0.56	0.094	0.06	0.05

[a] Values are means ± SDs, *n* = 3. Sugars like mannose or rhamnose were not detected. Means that have the same letter are not significantly different.

Table 5. Effect of Secondary Hydrolysis on Sugar and Inhibitory Compound Concentrations[a]

	g/L				
treatment	total sugars	HMF	acetic acid	GA	phenolics
0.25% (w/v) SA	26.56 c ± 0.13	0.08 d ± 0.005	0	0.27 c ± 0.011	1.96 d+0.02
0.50% (w/v) SA	27.54 b ± 0.16	0.18 c ± 0.015	0	0.59 b ± 0.015	2.07 c+0.02
0.75% (w/v) SA	29.65 a ± 0.15	0.35 b ± 0.05	0.15 b ± 0.02	0.71 b ± 0.005	2.35 b+0.02
1.0% (w/v) SA	29.52 a ± 0.04	0.54 a ± 0.02	0.52 a ± 0.005	0.84 a ± 0.01	2.55 a+0.06
ANOVA, *P*(0.05)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
LSD(0.05)	0.23	0.04	0.023	0.02	0.04

[a] Values are means ± SDs, *n* = 3. Means that have the same letter are not significantly different.

Table 6. Sugar Profile of Different Sugars as a Result of Secondary Hydrolysis[a]

	g/L			
treatment	glucose	fructose	galactose	arabinose
0.25% (w/v) SA	12.92 a ± 0.04	13.18 a ± 0.08	0.45 d ± 0.02	0
0.50% (w/v) SA	12.98 a ± 0.08	11.24 c ± 0.05	0.88 c ± 0.03	3.14 c ± 0.04
0.75% (w/v) SA	12.66 c ± 0.06	9.84 d ± 0.09	1.50 b ± 0.04	4.67 b ± 0.02
1.0% (w/v) SA	12.00 d ± 0.04	8.98 e ± 0.08	2.47 a ± 0.025	4.73 a ± 0.025
ANOVA, *P*(0.05)	<0.0001	<0.0001	<0.0001	<0.0001
LSD (0.05)	0.10	0.13	0.05	0.04

[a] Values are means ± SDs, *n* = 3. Sugars like xylose, mannose, or rhamnose were not detected. Means that have the same letter are not significantly different.

(w/v). However, a further increase in the acid level resulted in a decline in the sugar concentration. A significant increase in the arabinose concentration (10−12% of total sugars) was observed when the acid level was increased beyond 0.25% (**Table 6**), which indicates that an acid level of 0.25% or less was not effective in releasing sugars from the hemicellulosic fraction of orange peel biomass. This also shows the strong interaction of hemicellulose and pectin, which does not allow its solubilization under mild pretreatment conditions.

RSM Design and Its Evaluation. Table 7 depicts the ethanol concentration as a response to different combinations of three independent variables. The software analyzed the data and suggested both linear and quadratic models for this kind of interaction as significant. However, the regression coefficients and *P* value obtained through ANOVA indicated a higher significance for the quadratic model, which was subsequently used for evaluation purposes. The final response function to predict ethanol concentration after eliminating the nonsignificant terms was

$$Y = -76.18335 + 15.21413X_1 + 2.11948X_2 + 1.15254X_3 - 1.4119{X_1}^2 - 0.030776{X_2}^2 - 0.025410{X_3}^2 \quad (2)$$

where Y stands for ethanol and X_1, X_2, and X_3 stand for temperature, pH, and time, respectively.

The overall quadratic model was found to be significant with a R^2 value of 0.96 and an adjusted R^2 value of 0.95, indicating good agreement between the results obtained and the theoretical values predicted. While linear and quadratic terms of time had a significant effect, quadratic terms of temperature and pH had a significant effect on the ethanol production.

Model Graphs and Numerical Optimization. The response surfaces shown in **Figure 1A−C** were based on the final model in which one variable was kept constant at its optimum value and the other two were varied. It is clear from **Figure 1A** that at a constant pH of 5.0, maximum ethanol was produced in time and temperature of 15 h and 32.5 °C, respectively. However, the ethanol concentration was drastically lower at a temperature below 28 °C and a fermentation time of 10 h or less (runs 6 and 7, **Table 7**). The results indicate that pH 5.0, a temperature above

Table 7. Ethanol Production Using Different Combinations of Three Independent Variables as Per the Design Generated by RSM Software

run	ethanol concentration (g/L)
1	12.62
2	4.89
3	6.55
4	11.05
5	12.18
6	10.86
7	12.34
8	4.93
9	12.72
10	10.14
11	11.04
12	5.9
13	0
14	12.9
15	12.27
16	3.33
17	2.63
18	12.57
19	8.22
20	12.97

25 °C, and a fermentation time of 15 h or more favored ethanol production (**Figure 1A**). Ethanol production was highest when the pH was in the vicinity of 5.0 during fermentation for 15 h, at a fixed temperature of 32.5 °C (**Figure 1B**). During a fixed fermentation time of 15 h, a temperature of 32.5 °C and pH of 5.0 resulted in considerable ethanol production (**Figure 1C**), whereas it was reduced at a temperature and pH below 28 °C and 4.5, respectively. **Figure 1D** shows a positive correlation between the response predicted by the model equation and the actual results obtained through experimentation. The results presented in **Table 8** indicate that the independent terms of temperature, pH, and fermentation time and the square of their values were significant; however, the interactions between the factors were not found to be significant in this study.

Ethanol Production in a Laboratory Batch Fermenter. Fermentation of the hydrolysate obtained during primary hydrolysis proceeded vigorously during the first 6 h with nearly 90% of sugars getting consumed with a corresponding increase in cell biomass and ethanol concentration (**Figure 2A**). This could be attributed to the early entry of cells into the log phase because of use of high initial inoculum. It is possible that the cells might have reached the stationary phase around 6−9 h, after which the fermentation rate declined. Beyond 12 h, no significant increase in

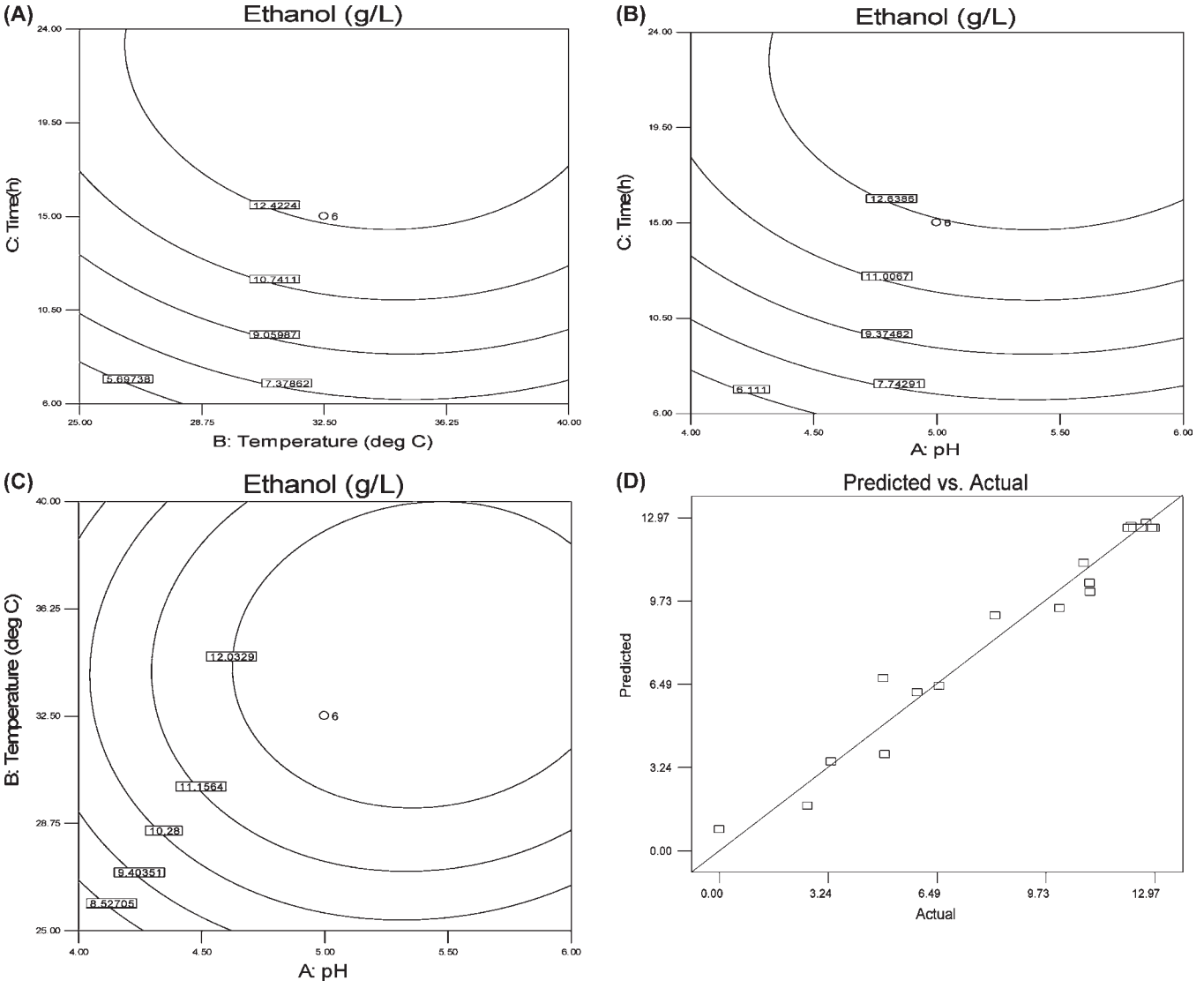

Figure 1. Model graphs showing interactions between the independent variables (**A**) time and temperature at fixed pH, (**B**) time and pH at fixed temperature, and (**C**) temperature and pH at fixed time. (**D**) Diagnostic plot showing the distribution of observed and predicted values of ethanol production.

ethanol concentration was observed, while a decline in cell biomass was seen, primarily due to lack of nutrients and production of toxic metabolites, resulting in death of a few cells. Similar trend in ethanol production and sugar consumption were observed during fermentation of the hydrolysate obtained during secondary hydrolysis (**Figure 2B**).

DISCUSSION

The presence of fermentable sugars (**Table 2**) in a significant amount and low lignin levels offer a good potential for use of orange peel as a substrate for fermentation-based products, such as ethanol. Proteins serve as organic nutrients for the growth of micro-organisms, thus supporting the fermentation process.

Primary hydrolysis using a combination of acid and steam resulted in the production of sugars in high concentrations, as compared to control. However, inclusion of acid, accompanied with high temperature, also resulted in the formation of compounds known to be inhibitory to yeast growth. HMFs and furfurals are formed during degradation of hexose and pentose sugars, respectively (**Table 3**). Acetic acid, a known inhibitor to yeast growth and metabolism, is generally formed due to break down of the acetyl bonds in hemicellulose, when subjected to high pressure and temperature under acidic conditions (*20*). Most of the pentose sugars are concentrated in the hemicellulosic fraction of the cell wall (*4*). Delegenes et al. (*21*) had reported that growth of *Pichia stipitis*, a xylose-fermenting yeast strain, declined by about 43% when the HMF concentration was around 0.5 g/L. At a higher HMF concentration of 1 g/L, the growth of *Saccharomyces cerevisiae* and its fermentation ability were drastically affected (*22*). Although the GA concentration also increased with an increase in the acid concentration (**Table 3**), it would not have had a major affect on ethanol production as samples were neutralized prior to fermentation. An increase in the acid level to 0.5% (w/v) effectively released both hexose and pentose sugars, indicating that the 0.5% (w/v) acid level was effective in releasing sugars from both hemicellulosic and cellulosic fraction of orange peel (**Table 4**). There was a significant increase in the HMF, GA, and phenolic concentrations when the acid level increased beyond 0.5% (**Table 5**), indicating degradation of hexose sugars and solubilization of pectin. Degradation of fructose was more pronounced than glucose (**Table 6**) due to its early release from the substrate; the galactose concentration, however, increased with an increase in acid level. This could be explained by the fact that at a higher acid concentration, effective disruption of

Table 8. ANOVA for Ethanol Production as a Function of Three Independent Variables[a]

source	sum of squares	degrees of freedom	mean square	*F* ratio	*P* value
X_1	10.88	1	10.88	12.74	0.0034
X_2	16.59	1	16.59	19.43	0.007
X_3	168.46	1	168.46	197.26	<0.0001
X_1^2	43.19	1	43.19	50.57	<0.0001
X_2^2	28.70	1	28.70	33.60	<0.0001
X_3^2	61.05	1	61.05	71.48	<0.0001
residual	11.10	13	0.85		0.9805
total	318.79	19			<0.0001*

[a] X_1, X_2, and X_3 stand for temperature, pH, and time, respectively. $R^2 = 0.96$, and adjusted $R^2 = 0.95$.

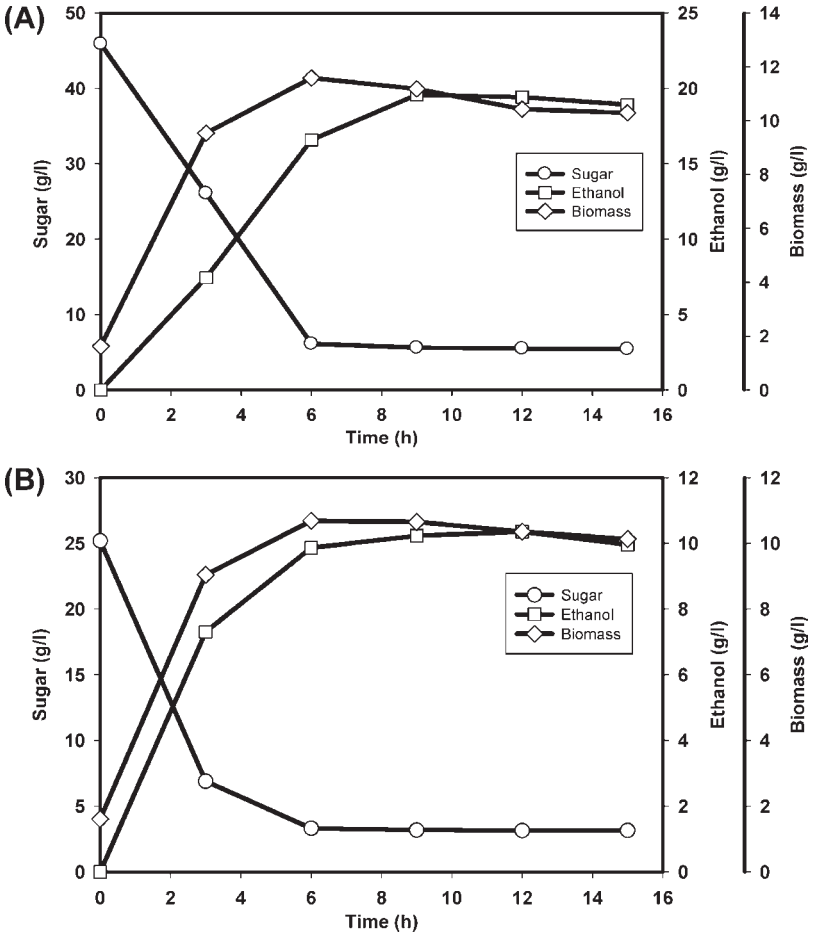

Figure 2. Relation between sugar consumption, ethanol production, and cell biomass during fermentation of the filtrate obtained during primary (**A**) and secondary (**B**) hydrolysis.

Table 9. Ethanol Productivity Parameters for Fermentation of the Filtrate Obtained through Primary and Secondary Hydrolysis Using RSM-Optimized Parameters[a]

time (h)	residual sugars (g/L)	volumetric ethanol productivity (g_p/L/h)	ethanol yield on biomass basis (g/g)	ethanol yield ($Y_{P/S}$)
3	32.81 a ± 0.22	4.90	0.11	0.40
6	9.23 b ± 0.10	4.44	0.22	0.42
9	8.36 c ± 0.20	3.37	0.25	0.46
12	8.27 c + 0.22	2.52	0.25	0.46
15	8.25 c + 0.17	2.02	0.25	0.46
ANOVA, *P*(0.05)	<0.0001			
LSD (0.05)	0.20			

[a] Means that have the same letter are not significantly different. Although the fermentation of the filtrate obtained during primary and secondary hydrolysis was separately carried out, the results presented in the table represent productivity of the overall process.

glycosidic bonds results in pectin-releasing galactose and GA. Pectins in citrus fruit peels are comprised mainly of GA, galactose, rhamnose, and glucose (*4*). A steep increase in the phenolic concentration was observed when acid was used. Unlike lignocellulosic biomass, where phenolics derived from lignin degradation have an adverse effect on the cell membrane integrity (*23*), most of the phenolics present in the citrus peel are comprised of flavonoids, such as naringin, hesperidin, isoflavanone, etc., which at low concentration are not known for antimicrobial activity. Although a significant increase in hexose sugar concentration at an acid level beyond 0.50% was observed, a substantial increase in HMF and acetic acid concentration was also observed at such acid levels. Hence, the 0.50% (w/v) acid level was selected for carrying out secondary hydrolysis of the pretreated biomass, too. Xiang et al. (*24*) have reported that in a lignocellulosic biomass, during acid hydrolysis, xylose and arabinose are the first sugars to be degraded followed by mannose, galactose, and glucose. However, we observed a significant degradation of fructose and glucose at an increased acid level. The sugar degradation pattern depends upon the structure of polysaccharides, intra- and intermolecular interactions between insoluble polysaccharides, and the kind and concentration of sugars present in cellulose and hemicellulose. There are structural differences between lignocellulosics from crop residues, which are rich in hemicellulose and lignin, and fruit residues, which are rich in fructose (*3*) and pectin and low in lignin.

Although the conventional yeast strains can operate in a wide temperature range, they usually perform better in a temperature range of 30−35 °C (*18*). At a fixed temperature of 32.5 °C, fermentation time of 15 h, and pH of 5.0, higher ethanol production was favored. Fermentation was adversely affected when the pH was below 4.5 during 6−10 h of fermentation (runs 8 and 17, **Table 7**). Russell (*19*) has also reported that a pH in the range of 5.0−5.2 is ideal for fermentation and higher ethanol production. Because the RSM experiments were carried out in shake flasks with no control over pH, CO_2 may have accumulated during fermentation, resulting in lower pH, affecting the fermentation ability of yeast. Previous research suggested that ethanol production from enzyme hydrolyzed orange peel using *S. cerevisiae* at 35 °C, and a pH of 5.0 did not show any increase beyond 12 h (*6*). After the model graphs and the combinations suggested by the numerical optimization package were evaluated, validation experiments in a batch fermenter were conducted at 34 °C and a pH of 5.4 for 15 h.

After the elimination of nonsignificant terms, the *p* value for all of the independent variables and their squared values was found to be significant at a 99% confidence level (**Table 8**). The significance of data is judged by its *p* value being closer to 0. The *p* value should be less than 0.1, 0.05, and 0.01 for a 90, 95, and 99% confidence level for the factors to be termed significant. Because the interaction between variables was not found to be significant at the 95% confidence level, it indicates that all three factors independently affect ethanol production.

Fermentation beyond 9 h led to a significant reduction in volumetric productivity (**Table 9**). The ethanol product yield and volumetric productivity play a decisive role in commercial adoption of any process. Grohmann et al. (*6*), while using enzymatic hydrolysis for orange peels and fermentation by *S. cerevisiae*, reported that a yield of 55−65 gallon/ton could be obtained from dried orange peel biomass. In one of our earlier studies, we obtained an ethanol concentration of 26 g/L in 48 h using crude cellulase enzyme and a combination of hexose and pentose-fermenting yeasts from a mixture of kinnow waste and banana peel (*2*). Grohmann et al. (*7*) reported an ethanol concentration of 35−38 g/L in 48−72 h using orange peel hydrolysate and recombinant *Escherichia coli* capable of fermenting both hexose and pentose sugars. Wilkins et al. (*5*) reported an ethanol concentration of 42 g/L from citrus peel waste in 27 h employing SSF using cellulase, pectinase, and *S. cerevisiae*. In comparison with all of the other studies conducted so far using citrus fruit residues, we could achieve a higher product yield on a biomass basis ($Y_{P/X}$) of 0.25 g/g, on a substrate consumed basis ($Y_{P/S}$) of 0.46 g/g, and a high volumetric productivity of 3.37 g/L/h after 9 h of fermentation time. This indicates that the results obtained through the present study are encouraging in terms of product yield and volumetric productivity for further scale-up studies and commercial exploitation of such a process. Because considerable arabinose was produced during both hydrolysis stages and remained unutilized, future studies should be directed toward exploiting the use of mixed cultures and recombinant yeasts or in the development of robust strains that will simultaneously ferment hexose and pentose sugars for the production of ethanol. However, in our studies, because a robust pentose sugar-fermenting yeast was not used, arabinose inclusion in the medium would have only resulted in substrate utilization without any significant increase in ethanol concentration, as most of the yeasts follow a diauxic growth pattern. Hahn-Hagerdal et al. (*25*) had reported that the pentose sugar-fermenting yeasts were relatively slow growing as compared to hexose-fermenting yeasts, and even the recombinants are not able to produce a substantial amount of ethanol exclusively from pentose sugars. It has also been observed that the pentose sugar-fermenting yeast strains are highly sensitive to the concentration of inhibitory compounds, besides having a different oxygen requirement as compared to conventional hexose-fermenting yeasts (*26*).

The present study demonstrated that primary hydrolysis using 0.5% (w/v) acid level followed by dilute acid hydrolysis of the pretreated biomass at 121 °C and 15 psi resulted in significant sugar release from orange peels. Because the sugar concentration in hydrolysate obtained from primary and secondary hydrolysis was significantly different, separate fermentation was performed. Fermentation beyond 9 h in both cases did not result in any significant increase in ethanol concentration. Fermentation variables optimized through RSM were successful in producing ethanol with a high volumetric productivity of 3.37 g/L/h in a batch fermenter. This indicates potential for such a process to commercially produce ethanol from orange peels. However, use of a robust strain capable of fermenting both hexose and pentose sugars is needed to improve the final ethanol concentration and productivity, since a significant quantity of pentose sugars was left unutilized in hydrolysates. This study evaluated the use of

orange peels as a resource to produce ethanol; sugar generated from this hydrolysis process can be effectively utilized for the production of many other value-added products, particularly specialty chemicals, flavors, and fragrances via microbial fermentation.

ABBREVIATIONS USED

RSM, response surface methodology; CCD, central composite design; CRD, completely randomized design; OPP, orange peel powder; GYE, glucose yeast extract; HPLC, high-performance liquid chromatography; GA, galacturonic acid; HMFs: hydroxymethylfurfurals; RID, refractive index detector; PDA, photodiode array detector.

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