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Original Article

Optimized biotransformation of acid-treated water melon peel hydrolyzate into ethanol

Biotransformação otimizada de hidrolisado de casca de melancia tratada com ácido em etanol

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Abstract

Today, global focus of research is to explore the solution of energy crisis and environmental pollution. Like other agricultural countries, bulk quantities of watermelon peels (WMP) are disposed-off in environment as waste in Pakistan and appropriate management of this waste is the need of hour to save environment from pollution. The work emphasizes the role of ethanologenic yeasts to utilize significant sugars present in WMP for low-cost bioethanol fermentation. Dilute hydrochloric acid hydrolysis of WMP was carried out on optimized conditions employing RSM (response surface methodology) following central composite design (CCD). This experimental design is based on optimization of ethanologenesis involving some key independent parameters such as WMP hydrolysate and synthetic media ratio (X1), incubation temperature (X2) and incubation temperature (X3) for maximal ethanol yield exploiting standard (*Saccharomyces cerevisiae* K7) as well as experimental (*Metchnikowia cibodasensis*Y34) yeasts. The results revealed that maximal ethanol yields obtained from *S. cerevisiae* K7 was 0.36±0.02 g/g of reducing sugars whereas *M. cibodasensis*Y34, yielded 0.40±0.01 g ethanol/g of reducing sugars. The yeast isolate *M. cibodasensis*Y34 appeared as promising ethanologen and embodies prospective potential for fermentative valorization of WMP-to-bioethanol.

Keywords: ethanologenesis, optimization, fermentation, response surface methodology, biodegradable waste, watermelon peels.

Resumo

Hoje, o foco global da pesquisa é explorar a solução da crise energética e da poluição ambiental. Como em outros países agrícolas, grandes quantidades de cascas de melancia (WMP) são descartadas como resíduos no meio ambiente no Paquistão, mas a gestão adequada desses resíduos é a mais recente solução para salvar o meio ambiente da poluição. O trabalho enfatiza o papel das leveduras etanologênicas para utilizar açúcares significativos presentes no WMP para fermentação de bioetanol de baixo custo. A hidrólise de ácido clorídrico diluído de WMP foi realizada em condições otimizadas empregando RSM (metodologia de superfície de resposta) e seguindo o projeto de composto central (CCD). Este projeto experimental é baseado na otimização da etanologenesis envolvendo alguns parâmetros independentes importantes, como hidrolisado de WMP e razão de meio sintético (X1), temperatura de incubação (X2) e temperatura de incubação (X3) para rendimento máximo de etanol explorando o padrão (*Saccharomyces cerevisiae* K7) também como leveduras experimentais (*Metchnikowia cibodasensis* Y34). Os resultados revelaram que os rendimentos máximos de etanol obtidos a partir de *S. cerevisiae* K7 foi de 0,36 ± 0,02 g / g de açúcares redutores, enquanto *M. cibodasensis* Y34 rendeu 0,40 ± 0,01 g de etanol / g de açúcares redutores. O isolado de levedura *M. cibodasensis* Y34 apareceu como um etanologeno promissor e incorpora um potencial prospectivo para a valorização fermentativa de WMP em bioetanol.

Palavras-chave: etanologenesis, otimização, fermentação, metodologia de superfície de resposta, resíduos biodegradáveis, cascas de melancia.

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1. Introduction

Currently many eco-friendly projects are going on in transportation sector for sustainable development. Rapid industrialization worldwide in last few decades resulted in the emission of harmful gases like CO_2, SO_2, NO and NO_2 associated with fossil-fuels combustion (Robak and Balcerek, 2018). If the consumption of fossil-fuels in transport and other sectors continues at high rate, there is a risk of depletion of fossil-fuels within fifty years (Sheehan et al., 2000). Now a days, advance techniques for efficient production of value added products are used globally. As lignocellulosic biomass is used currently as low cost substrate for biofuel production either liquid or in gaseous form. These biofuels are far way cheaper than fossil fuels (Reijnders, 2006; Waheed et al., 2021). There are multiple reasons which enable biomass to be a potential biofuels feedstock. For example, sustainable bioproduction employing abundant and renewable biomass resource, as well as less greenhouse gasses (GHGs) such as carbon dioxide released to environment (Cadenas and Cabezudo, 1998).

Being an agricultural country watermelon is a popular fruit in Pakistan that is extensively used as food due to being affordable to common man, good in taste and contains fibres, minerals and some essential vitamins important for body. However, the whole fruit is not consumed only the pulpy part of this fruit is being eaten and outer rind (peels) and seeds are discarded as wastes. Resultantly, large amount of agricultural waste i.e. waste watermelon is produced. The watermelon peels may pollute our surroundings which is a major challenge to handle (Gin et al., 2014; Ali et al., 2021; Yaseen et al., 2021). Numerous pathogens rapidly cultivate on watermelon waste that may result in production of hazardous chemicals, effecting the human health (Yaseen et al., 2021). Employment of this organic waste for valuable industrial productions will not only reduce the environmental pollution but also improve human health. In this regard, keeping in view the prevailing energy crisis, bioethanol production from waste biomasses e.g. watermelon waste (lignocellulose) can prove to be an approach for cost-effective production processes.

2. Materials and Methods

2.1. Bioethanol feedstock collection

For ethanologenesis *Citrullus lanatus* (Watermelon) peels were taken from different areas of Lahore, Pakistan. It have been confirmed that the experimental samples of plants, including the collection of plant material, complied with relevant institutional, national, and international guidelines and legislation with appropriate permissions from competent authorizes. Peels of the fruit (5 Kg, wet weight) were washed with distilled water and dehydrated in oven at 60°C for 48 hours. These dried peels were grinded and passed through sieve (stain less steel sieve. Diameter 10" with pore size 1mm), for obtaining fine powder.

2.2. Proximate analysis of watermelon peels (WMP)

In proximate analysis of different contents, specific methods were used including phenol sulfuric method of Dubois et al. (1956) to determine total sugars (carbohydrates). Method of Zollner and Kirsch (1962) was used to determine total lipid content, Lowry et al. (1951) protocol for estimating total protein content and for reducing sugars contents, DNS (3,5-Dinitrosalicylic acid) method were used devised by Miller (1959). AOAC (2012) procedures were used to determine moisture contents from peels. For quantification of extractives, hemicellulose, lignin and cellulose contents estimation, methods of Lin et al. (2010) was used. Experiment was carried out in laboratory, Zoology Department, Division of Science and Technology, University of Education, Lahore from April to June.

2.3. Acidic hydrolysis of WMP

Watermelon peels were hydrolyzed by dilute hydrochloric acid. Optimum parameters for hydrolysis were acid concentration, temperature and time period. The optimum conditions used for saccharification of WMP were 100°C hydrolysis temperature, 6% HCl concentration, and 60 minutes saccharification period. Reducing sugars were optimized up to 63.4±0.05 g/L (Batool, 2018).

2.4. Detoxification and neutralization of WMPH

To get rid of phenolic and toxic compounds produced during acid hydrolysis of WMP, the WMPH was detoxified. For this purpose, 2.5% activated charcoal was used. Folin-Ciocalteu method (Ghosh and Ghose, 2003) was used for the estimation of total phenolic contents. The supernatant obtained after filtering was neutralized till the pH 7.

2.5. Microorganisms

Two yeast cultures i.e. *Saccharomyces. cerevisiae* K7 (reference strain) and *Metchnikowia cibodasensis* Y34 (Experimental isolate) were selected for ethanologenesis and were collected from Mie University, Japan (Chaudhary and Karita, 2017). These yeast cultures were revived on MYGP media. The medium (g/L) was prepared using peptone 5 g, yeast extract 3g, Malt extract 3 g, and glucose 10 g, sterilized and inoculated by selected yeast strains and incubated for twenty four hours at 30°C shaking at 150 rpm.

2.6. Ethanologenesis from WMP hydrolysates (WMPH) from selected yeast isolates

2.6.1. Optimization Design for WMPH fermentation

Fermentation parameters were optimized by CCD following response surface methodology. A 20 runs experiment was conducted for ethanologenesis employing fermentation medium. The fermentation parameters optimized were WMPH: synthetic medium (X1), temperature (X2) and growth period (X3). In 20 runs experimental central composite design, different set compositions of WMPH: synthetic medium, temperature and incubation period with both ethanologenic yeast were employed (Tables 1, 2). Parameter optimization model was designed following design expert software (ver 6.0.9) at 2^3 factorial level. Model significance was calculated employing ANOVA and regression analysis. Three dimensional graphs were plotted to interpret the

Table 1. Coded variables for quadratic CCD fermentation model for HCl hydrolysed WMP.

Fermentation Parameters	Coded variables			
	Coded variable	Minimum variable	Middle variable	Maximum variable
WMPH (mL)	X_1	25	50	75
Incubation duration (days)	X_2	1	8	15
Incubation Temperature (°C)	X_3	25	32.50	40

Table 2. CCD Matrix presenting different conditions to optimize fermentation parameters for WMPH.

Experimental Runs	WMPH	Incubation days	Temperature °C
1	50	8	19.89
2	50	8	45.11
3	50	19.77	32.5
4	50	3.77	32.5
5	50	8	32.5
6	92.04	8	32.5
7	75	1	25
8	25	15	25
9	50	8	32.5
10	25	1	25
11	50	8	32.5
12	50	8	32.5
13	7.96	8	32.5
14	25	1	40
15	50	8	32.5
16	25	15	40
17	75	1	40
18	75	15	40
19	75	15	25
20	50	8	32.5

interaction within experimental parameters. Optimum points were predicted within the three dimensional graphs.

The association of all parameter to responses were illustrated by quadratic Equation 1 computed by regression analysis:

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

This equation presented the predicted response (Y), constant coefficient- β_0, linear coefficients (β_1, β_2, β_3), quadratic coefficients (β_{11}, β_{22}, β_{33}), cross products coefficients (β_{12}, β_{13}, β_{23}), input variables (X_1, X_2, X_3), residual error (e).

Synthetic medium was used as mineral medium for fermentation experiment and was prepared (g/L) by mixing yeast extract 6.5, Glucose 50, Mg SO_4. 7 H_2O 0.8, $(NH_4)_2SO_4$ 2.6, $CaCl_2$ 0.3, KH_2PO_4 2.72, citric acid 1.5, sodium citrate 6, and zinc chloride 0.00042 in a narrow necked flask. It was then sterilized at 121°C for 15 min (Camelia et al., 2010).

Table 3. Compositional quantitative analysis of WMP.

WMP composition	Contents
Total sugars	92.9±0.03
Reducing sugars	25.8±0.06
Total lipids	3.4±0.01
Total proteins	3.9±0.02
Extractives*	20.96±0.05
Soluble lignin*	10.35±0.29
Hemicellulose*	30.90±2.26
Cellulose+insoluble lignin*	37.79±0.31
Moisture contents	8.3±0.04
Weight loss	17.99±0.47

*Crude cellulose + insoluble lignin (%) = 100 – (Extractive % + Hemicellulose % + Lignin %).

2.6.2. Analytical methods for WMPH fermentation

To study fermentation kinetics, reducing sugars and ethanol contents in fermentation medium was assessed. adopting protocols of DNS and acid dichromate (Miller, 1959; Bennett, 1971).Optical densities at 600 nm were measured to estimate the growth pattern of yeast during ethanologenesis.

2.6.3. Quantification of ethanol yield of WMPH

The ethanol yield obtained by fermentation of WMPH from 20 runs of design by standard as well as experimental yeast isolates was determined by the Formula 2:

$$Ethanol\ yield\ \left(g/g\right)=\frac{Ethanol\ titer\left(g/L\right)}{Reducing\ sugars\ consumed\left(g/L\right)}\times 100 \quad (2)$$

The potential of reference and experimental yeast isolates was measured by computing productivity of ethanol contents in optimized condition for 15 days. Daily samples were drawn to analyzed reducing sugars, yeast growth and ethanol titer.

2.7. Statistical analysis

Optimization of fermentative parameters were computed employing Design Expert Software (ver. 6.0.9)

adopting CCD with three factorial level in quadratic model. The data of analyzed responses were obtained by experiment with three replicates for confirmation and accuracy of predicted values. The fitness of model was predicted following ANOVA and regression tools.

3. Results

3.1. Proximate analysis of WMP contents

Proximate analysis of WMP waste was presented in Table 3. The analysis provided a base line about the nutrient profile of WMP waste that make them suitable for microbial activity. In WMP, it was found that the reducing sugar contents were 25.8±0.06 g/L, total carbohydrates were 92.9±0.03 g/L, total lipids were 3.4±0.01 g/L and total protein were 3.9±0.02 g/L. In WMP, percent extractives, hemicellulose, soluble lignin and cellulose+insoluble lignin contents were also determined that were 20.96±0.05, 30.90±2.26, 11.04±0.29 and 37.10±0.31 correspondingly (Table 3).

3.2. Hydrochloric acid saccharification of WMPH

The WMP waste was subjected to hydrolysis at optimized conditions viz 6% HCl concentration, 100 °C temperature and 60 minutes. WMPH was analyzed biochemically and the recorded contents (g/L) were 63.4±0.05 reducing sugars and 190.2±0.071 total sugars. The reported extractives, soluble lignin, hemicellulose, and cellulose+insoluble lignin were 26.96±0.05, 10.14±0.05, 24.62±0.05, 38.28±0.08 respectively (Batool, 2018).

3.3. Detoxification of WMPH

The estimated total phenolic contents in untreated WMPH were found to be 15.0 ± 0.32 g/L while after treating with activated charcoal, their value decreased to 94.0 ± 0.27 g/L resulting in overall 63% reduction in phenolic contents.

3.4. Fermentation of detoxified acidic WMPH for ethanologenesis

In present investigation, the designed quadratic model with 20 experimental runs was used to obtain experimental data for different responses. The responses were ethanol yield, ethanol titer and yeast growth pattern in fermentation medium. Selected fermentation parameters were percent WMPH volume in synthetic medium (X_1) along with incubation time (X_2) and temperature (X_3). The quadratic fermentation model was evaluated by statistical tools and analyzed data was tabulated in Table 4. The data about remining and consumed reducing sugars were recorded in Table 5.

Table 4. CCD matrix presenting different responses by yeast isolates with hydrochloric acid WMPH.

Experimental Runs	Parameters			*S. cerevisiae* K7			*M. cibodasensis* Y34		
	WMP	Incubation days	Temp °C	Ethanol Yield g/g	Ethanol Titer g/L	Growth (O.Ds)	Ethanol Yield g/g	Ethanol Titer g/L	Growth (O.Ds)
1	75	15	40	23.6±0.03	19.6±0.03	4.0±0.03	27.4±0.06	24.8±0.21	2.6±0.02
2	50	8	32.5	27.8±0.06	23.9±0.01	3.9±0.03	28.7±0.06	26.2±0.10	2.5±0.04
3	75	15	25	31.3±0.03	31.2±0.04	0.1±0.09	35.4±0.36	35.3±0.03	0.1±0.03
4	75	1	40	33.0±0.03	32.9±0.04	0.1±0.13	34.3±0.06	32.5±0.03	1.8±0.03
5	25	1	40	10.9±0.09	6.9±0.18	4.0±0.11	11.1±0.06	7.6±0.11	3.5±0.19
6	50	8	32.5	28.0±0.06	23.9±0.01	4.1±0.02	27.3±0.06	23.4±0.03	3.9±0.02
7	25	15	40	19.4±0.09	19.3±0.11	0.1±0.02	19.7±0.06	19.6±0.06	0.1±0.02
8	7.96	8	32.5	15.5±0.04	15.3±0.05	0.2±0.08	16.1±0.01	15.7±0.02	0.4±0.10
9	50	8	32.5	27.5±0.06	23.9±0.01	3.6±0.05	27.4±0.06	23.2±0.07	4.2±0.04
10	50	8	19.89	24.5±0.06	15.6±0.07	8.9±0.03	25.8±0.06	22.3±0.10	3.5±0.05
11	50	19.77	32.5	24.0±0.06	17.6±0.06	6.4±0.03	20.3±0.06	17.8±0.07	2.5±0.01
12	50	8	45.11	26.1±0.06	25.4±0.01	0.7±0.03	28.2±0.06	27.8±0.02	0.4±0.03
13	50	8	32.5	25.3±0.06	23.9±0.01	1.4±0.05	27.8±0.06	23.7±0.06	4.1±0.08
14	50	3.77	32.5	25.4±0.06	14.7±0.23	10.7±0.15	25.4±0.06	19.4±0.08	6.0±0.04
15	92.04	8	32.5	31.7±0.16	26.3±0.18	5.4±0.05	35.4±0.05	32.9±0.09	2.5±0.09
16	50	8	32.5	28.4±0.06	23.9±0.01	4.5±0.04	28.4±0.06	24.7±0.08	3.7±0.03
17	75	1	25	23.0±0.03	15.8±0.05	7.2±0.09	29.6±0.06	15.9±0.29	13.7±0.28
18	25	1	25	18.9±0.09	17.4±0.04	1.5±0.03	19.7±0.06	8.1±0.12	11.6±0.19
19	25	15	25	27.6±0.04	27.5±0.02	0.1±0.02	28.1±0.06	28.0±0.05	0.1±0.14
20	50	8	32.5	28.3±0.06	23.9±0.01	4.4±0.02	28.6±0.06	26.7±0.02	1.9±0.11

Table 5. Reducing sugars (RS) contents in fermented hydrochloric acid WMPH.

Experimental Runs	Fermentation Conditions			*S. cerevisiae* K7			*M. cibodasensis*(Y34)		
	WMPH (mL)	Incubation days	Temp °C	Total RS (g/L)	Consumed RS (g/L)	Remaining RS (g/L)	Total RS (g/L)	Consumed RS (g/L)	Remaining RS (g/L)
1	75	15	40	23.6±0.03	19.6±0.03	4.0±0.03	27.4±0.06	24.8±0.21	2.6±0.02
2	50	8	32.5	27.8±0.06	23.9±0.01	3.9±0.03	28.7±0.06	26.2±0.10	2.5±0.04
3	75	15	25	31.3±0.03	31.2±0.04	0.1±0.09	35.4±0.36	35.3±0.03	0.1±0.03
4	75	1	40	33.0±0.03	32.9±0.04	0.1±0.13	34.3±0.06	32.5±0.03	1.8±0.03
5	25	1	40	10.9±0.09	6.9±0.18	4.0±0.11	11.1±0.06	7.6±0.11	3.5±0.19
6	50	8	32.5	28.0±0.06	23.9±0.01	4.1±0.02	27.3±0.06	23.4±0.03	3.9±0.02
7	25	15	40	19.4±0.09	19.3±0.11	0.1±0.02	19.7±0.06	19.6±0.06	0.1±0.02
8	7.96	8	32.5	15.5±0.04	15.3±0.05	0.2±0.08	16.1±0.01	15.7±0.02	0.4±0.10
9	50	8	32.5	27.5±0.06	23.9±0.01	3.6±0.05	27.4±0.06	23.2±0.07	4.2±0.04
10	50	8	19.89	24.5±0.06	15.6±0.07	8.9±0.03	25.8±0.06	22.3±0.10	3.5±0.05
11	50	19.77	32.5	24.0±0.06	17.6±0.06	6.4±0.03	20.3±0.06	17.8±0.07	2.5±0.01
12	50	8	45.11	26.1±0.06	25.4±0.01	0.7±0.03	28.2±0.06	27.8±0.02	0.4±0.03
13	50	8	32.5	25.3±0.06	23.9±0.01	1.4±0.05	27.8±0.06	23.7±0.06	4.1±0.08
14	50	3.77	32.5	25.4±0.06	14.7±0.23	10.7±0.15	25.4±0.06	19.4±0.08	6.0±0.04
15	92.04	8	32.5	31.7±0.16	26.3±0.18	5.4±0.05	35.4±0.05	32.9±0.09	2.5±0.09
16	50	8	32.5	28.4±0.06	23.9±0.01	4.5±0.04	28.4±0.06	24.7±0.08	3.7±0.03
17	75	1	25	23.0±0.03	15.8±0.05	7.2±0.09	29.6±0.06	15.9±0.29	13.7±0.28
18	25	1	25	18.9±0.09	17.4±0.04	1.5±0.03	19.7±0.06	8.1±0.12	11.6±0.19
19	25	15	25	27.6±0.04	27.5±0.02	0.1±0.02	28.1±0.06	28.0±0.05	0.1±0.14
20	50	8	32.5	28.3±0.06	23.9±0.01	4.4±0.02	28.6±0.06	26.7±0.02	1.9±0.11

3.5. Quadratic equation for ethanol yield from detoxified WMPH

The association of variables on yield by *S. cerevisiae* K7 was presented in following Equation 3;

$$Y1 = +0.20 - 0.024X_1 + 0.079X_2 + 0.0018X_3 + 0.015X_1^2 - 0.0047X_2^2 - 0.018X_3^2 + 0.0625X_1X_2 - 0.0125X_1X_3 - 0.059X_2X_3 + 0.0002 \quad (3)$$

Similarly, the variable inter relationship on ethanol yield response for *M. cibodasensis* Y34 (Equation 4) was;

$$Y2 = +0.25 - 0.017X_1 + 0.11X_2 + 0.022X_3 - 0.017X_1^2 - 0.0064X_2^2 - 0.0081X_3^2 + 0.015X_1X_2 - 0.015X_1X_3 - 0.037X_2X_3 + 0.0001 \quad (4)$$

3.6. Analysis of Variance (ANOVA) of Ethanologenesis from detoxified WMPH

The significance of responses was reported in Table 6 using ANOVA as statistical tool. The model for *S. cerevisiae* K7 was significant on the basis of F. p values as 8.21 and 0.002 having 0.21% chance with Lack of fit F value 60.47 (0.08% chance). The appropriateness of *M. cibodasensis* Y34 was described by 10.91 F, 0.0007 p (0.06% chance) and lack of fit 48.94 F-values (0.10% chance). Table 6 narrated data about ethanol titer and growth of yeast.

Table 7 interpreted regression data of model elucidating adequate precision, R^2, and adjusted R^2 as 10.211, 0.8961, 0.7823 (*S. cerevisiae* K7), and 0.9146, 0.823 and 12.573 indicating navigation of model inside design space.

3.7. Validation experiment

The software calculated the predicted and experimental fermentation conditions on the basis of optimum responses. The data of performed experiment following predicted conditions were documented in Table 8. The optimum value of ethanol yield in detoxified WMPH was calculated as 0.37±0.01 which is concordant with the predicted value i.e. 0.39.

3.8. Three dimensional Response Surface Plots (RSM) of ethanol yield from detoxified WMPH

The software interpreted the interaction of experimental variables on ethanol yield response in WMPH in form of graphs. For *S. cerevisiae* K7, three dimensional plot showed positive interaction of WMPH and incubation period on ethanol yield. Slight and sharp elevation in ethanol yield was observed by increasing hydrolyzate volume in fermentation medium and incubation days respectively (Figure 1). Increasing temperature led to slight while Increasing WMPH ratio in fermentation medium boosted the yield while slight elevations were noted by raising temperature

Table 6. Analysis of Variance of designed quadratic model for different responses in hydrochloric acid WMPH.

Responses	Source	Sum of Squares	DF	Mean Square	F value	p value
Ethanol Yield (*S. cerevisiae* K7)	Model	0.17	9	0.019	8.21	0.002 Significant
	Residual	0.021	9	0.002		
	Lack of Fit	0.021	5	0.004	60.48	0.008 Significant
	Pure Error	0.0002	4	0.00006		
	Core Total	0.19	19			
Ethanol Yield (*M. cibodasensis* Y34)	Model	0.17	9	0.019	10.97	0.0006 Significant
	Residual	0.016	9	0.001		
	Lack of Fit	0.015	5	0.003	48.93	0.0010 Significant
	Pure Error	0.0001	4	0.00006		
	Core Total	0.19	19			
Ethanol Titer (*S. cerevisiae* K7)	Model	1.07	9	0.017	3.40	0.0412 Significant
	Residual	0.36	9	0.025		
	Lack of Fit	0.31	5	0.063	209.69	<0.0001 Significant
	Pure Error	0.0012	4	0.0003		
	Core Total	1.42	19			
Ethanol Titer (*M. cibodasensis* Y34)	Model	1.40	9	0.016	3.40	0.0414 Significant
	Residual	0.41	9	0.046		
	Lack of Fit	0.40	5	0.080	31.15	0.0027 Significant
	Pure Error	0.010	4	0.002		
	Core Total	1.89	19			
Yeast Growth (*S. cerevisiae* K7)	Model	1.64	9	0.18	24.13	<0.0001 Significant
	Residual	0.068	9	0.007		
	Lack of Fit	0.064	5	0.013	13.20	0.0135 Significant
	Pure Error	0.003	4	0.0009		
	Core Total	1.76	19			
Yeast Growth (*M. cibodasensis* Y34)	Model	1.78	9	0.20	12.58	0.0004 Significant
	Residual	0.14	9	0.016		
	Lack of Fit	0.14	5	0.028	28.46	0.0032 Significant
	Pure Error	0.003	4	0.0009		
	Core Total	1.98	19			

Table 7 Computed regression data for responses in hydrochloric acid WMPH

Responses	C.V %	R-Squared	Adj R-Squared	Pred R-Squared	Adeq Precision
Ethanol yield (*S. cerevisiae* K7)	26.51	0.8916	0.7832	0.1073	10.210
Ethanol yield (*M. cibodasensis* Y34)	17.80	0.9164	0.8329	0.1074	12.537

(Figure 2). Raising temperature and incubation period tends to increase the ethanol yield (Figure 3).

The interaction of different parameters on ethanol yield during fermentation of WMPH employing *M. cibodasensis*Y34 yeast were interpreted by Figure 3-6. Fermentation of WMPH for more days by *M. cibodasensis* Y34 boosted the ethanol yield (0.367929) while minor increase was showed in case of hydrolyzate ratio (Figure 4).

Table 8. Validation for optimized Fermentation in Hydrochloric Acid Hydrolysate

Responses	Predicted Value	Experimental Value	Residual	Error (%)
Ethanol yield (*S. cerevisiae* K7)	0.34	0.36±0.02	0.02	5.88
Ethanol yield (*M. cibodasensis* Y34)	0.39	0.40±0.01	0.01	2.56
Ethanol titer (*S. cerevisiae* K7)	9.5	9.4±0.03	-0.1	-1.057
Ethanol titer (*M. cibodasensis* Y34)	11.4	11.5±0.04	0.1	0.87
Yeast growth (*S. cerevisiae* K7)	0.65	2.50±0.05	1.85	28.4
Yeast growth (*M. cibodasensis* Y34)	0.84	2.59±0.01	1.75	20.8

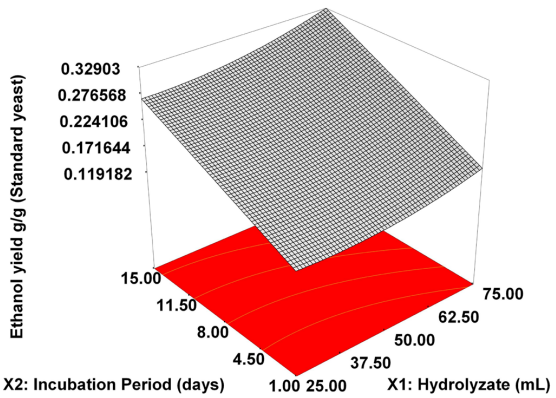

Figure 1. Three dimensional graph presenting ethanol yield by *S. cerevisiae* K7 in WMPH for different incubation periods.

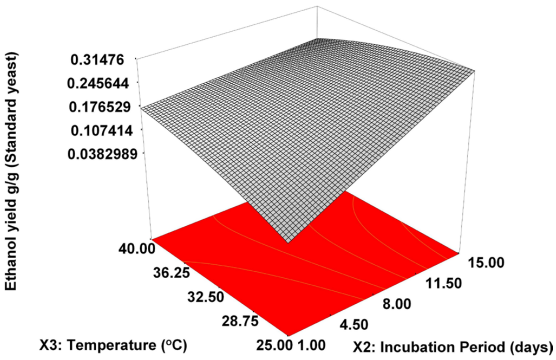

Figure 3. Three dimensional graph presenting ethanol yield by *S. cerevisiae* K7 in WMPH with different incubation periods and temperature.

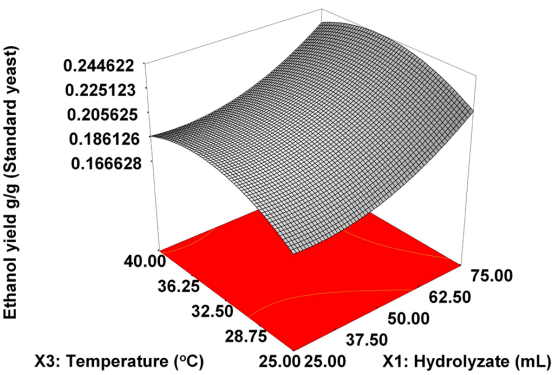

Figure 2. Three dimensional graph presenting ethanol yield by *S. cerevisiae* K7 in WMPH at different hydrolysis temperatures.

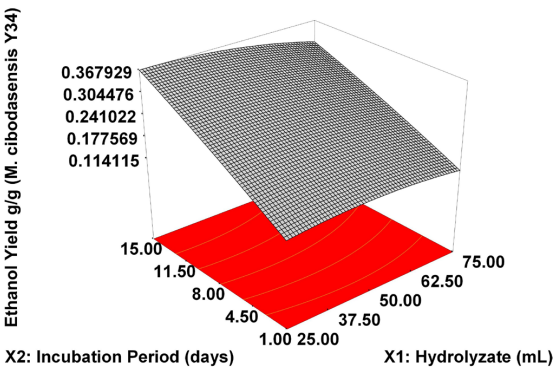

Figure 4. Three dimensional graph presenting ethanol yield by *M. cibodasensis* Y34 in WMPH for different incubation periods.

Same trend was shown in Figure 5 with raised temperature. Minor increased yield was observed by increasing WMPH volume up to 50 ml followed by slight decrease (Figure 5). By incubating for more days, yield of ethanol tends to enhance sharply. Minor increasing trend was presented by raising temperature (Figure 6).

3.9. Fermentation kinetics of ethanologenesis

Study of fermentation kinetics for ethanologenesis employing *S. cerevisiae* K7 and *M. cibodasensis* Y34 were performed under optimized conditions (i.e., hydrolysate concentration (75 ml), incubation temperature (25 °C) up to 15 days) as elucidated by CCD. On 15th day. The association of ethanol production with yeast growth was presented in Figure 7. Both yeast isolates showed exponential growth with slight increase of ethanol upto day 8. Stationery phase of both strains led to sharp increase till the end of experiment. Highest yield (g/g) was computed as 0.40 g/g (*M. cibodasensis* Y34) and 0.36 (*S. cerevisiae* K7) as presenting in Figure 8.

4. Discussion

Concept of bioethanol production is very attractive to researchers to conduct different research projects to make it feasibly practical at large scale. In this regard, lignocellulosic

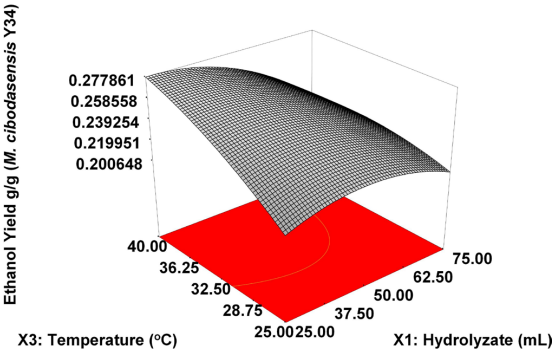

Figure 5. Three dimensional graph presenting ethanol yield by *M. cibodasensis* Y34 in WMPH at different hydrolysis temperatures.

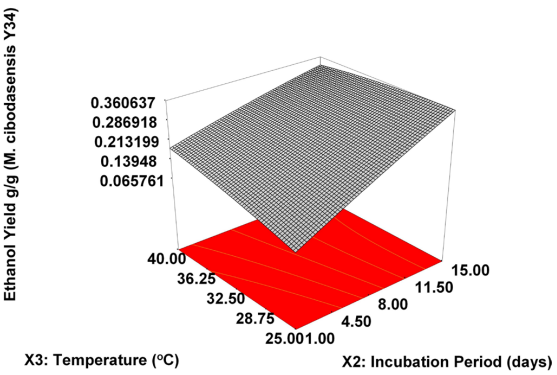

Figure 6 Three dimensional graph presenting ethanol yield by *M. cibodasensis* Y34 in WMPH with different incubation periods and temperature.

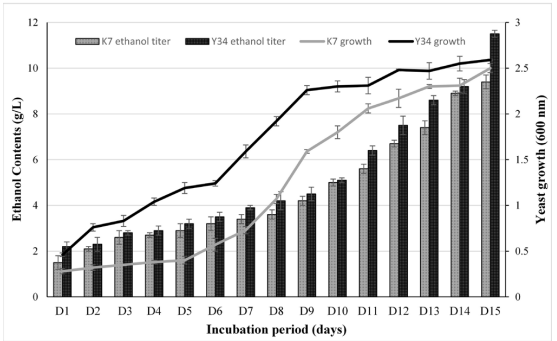

Figure 7. Association of ethanol contents and yeast growth under optimized conditions (WMPH 75 ml, 25 °C, and 15 days) elucidated by CCD from *S. cerevisiae* K7 and *M. cibodasensis* Y34.

biomass (LCB) e.g. watermelon peels for ethanol production can provide a viable substitute as substrate for renewable, sustainable biofuel production that is non-petroleum based and non-polluting for the environment (Goh et al., 2010; Mahmood et al., 2021). So this study relies on potential practicality of ethanol production by using watermelon peels (WMP). At first, dilute hydrochloric acid (HCl) was used for pretreatment of WMP and then the WMP

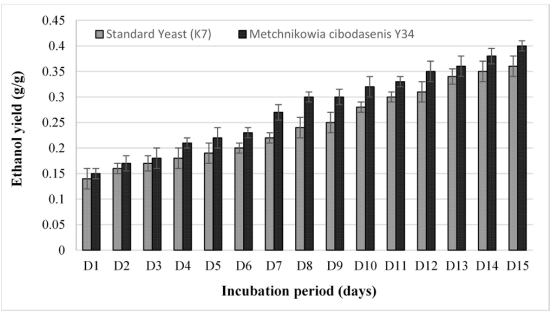

Figure 8. Ethanol yield (g/g of reducing sugar) under optimized conditions (WMPH 75 ml, 25 °C, 15 days) elucidated by CCD from *S. cerevisiae* K7 and *M. cibodasensis* Y34.

hydrolyzates (WMPH) were fermented employing *S. cerevisiae* K7 and *M. cibodasensis*Y34 yeast isolates in comparitive manner.

WMP contains substantial amounts of different contents (g/L) e.g. 25.8±0.06 reducing sugars, 92.9±0.03 total sugars, 3.4±0.01 total lipids and 3.9±0.02 total proteins whereas 20.96±0.05, 30.90±2.26, 11.04±0.29 and 37.10±0.31 percent extractive, hemicellulose, soluble lignin and crude cellulose+insoluble lignin contents were recorded respectively. Calculated percent moisture contents (0.83±0.04) in WMP were corroborated with findings (1.1%) of Zubairu et al. (2018), and contrary to the values reported by Hoque and Iqbal (2015) and Al-Sayed and Ahmed (2013). The total carbohydrate, lipids and protein contents in WMP were less than the values (56.02, 2.44, 11.17 and %) noted by Al-Sayed and Ahmed (2013). WMP consisted of crude cellulose, hemicellulose and lignin contents in greater quantity (9.99, 33.98, 6.93%) recorded by Rivas- Cantu et al. (2013) in citrus waste. Optimization of saccharification for subsequent ethanologenesis was elucidated by CCD following response surface methodology (RSM). RSM reduces experimental trial runs and interprets the inter relationship of parameters (Bitaraf et al., 2012). Pretreatment is considered as vital factor to breakdown the inner bonds of LCB, thus exposing the fermentable sugars for fermentation reactions. The procedure helped the free cellulose to be available for chemical/enzymatic hydrolysis. The saccharification converted the polymeric cellulose and hemicellulose to monomeric fermentable sugars. In present study, dilute HCl was used to hydrolyze WMP. Acids solubilize polymeric hemicellulose contents to make cellulose accessible for further processing by making changes in structure (Alvira et al., 2010; Ejaz et al., 2020; Loow et al., 2016). Glucose as main reducing sugar was released when WMP was subjected to acid hydrolysis (Aguilar et al., 2002).

Previously, it has been reported that in case of pretreatment with dilute HCL, optimized saccharification parameters were 6% HCl hydrolysis for 60 minutes at 100°C (Batool, 2018). While the optimum conditions for switch grass 1.2% HCl hydrolysis for 30 sec at 180°C and for popular, it was 1% dilute sulfuric acid hydrolysis for 0.56 min at 180°C (Chung et al. (2005).

By following the optimized conditions for pretreatment i.e. 6% HCl concentration, 100 °C temperature and 60 minutes (Batool, 2018) reducing sugars were released up to 63.4±0.05 g/L. Subsequent ethanol fermentation using WMPH was studied for 15 days at 25°C. Results revealed that 0.40±0.01 g ethanol/g of reducing sugars was observed using *M. cibodasensis*Y34 whereas 0.36±0.02 g ethanol/g reducing sugars was obtained from *S. cerevisiae* K7 that was comparable with findings of Roukas, (1996). The temperature and incubation time has been proved to effect directly on the growth of yeast. At start of experiment yeast isolates growing exponentially showed slight increasing trend in ethanol production. Sharp ethanol production observed by yeasts in stationary phase (with stability in ethanol productivity) for several days to complete the normal growth cycle. Increasing temperature shortens the exponential growth phase of microbes cause increase in ethanologenesis as supported by previous finding (Lin et al., 2012).

Evaluating ethanol titer, it was observed that 11.5±0.04 and 9.4±0.03 g ethanol/L was found when 75mL WMPH was used in fermentation medium at 25°C for 15 days employing *M. cibodasensis*Y34 and *S. cerevisiae* K7, respectively. Previosly, 14.3 g/L ethanol has been produced produced by *Kluyveromyces marxianus* from pomegranate hydrolyzates (Demiray et al., 2020) whereas 5.5 g/L contents were found by *Saccharomyces cerevisiae* (Demiray et al., 2018). So the results are analogous to the previous finding and embodies great potential for scale-up study.

Conclusively, bioethanol production from waste biomass proved as effective low cost technique in agrarian countries especially Pakistan. Fermentative ethanologenic yeast *M. cibodasensis*Y34 gave 0.40±0.01 g of ethanol yield per g of reducing sugars on 15th day post-inoculation from WMP. The ethanologenisis as well as implicated ethanol tolerance exhibited by *Metchnikowia* species elucidate its high bio-converting potential for WMP to ethanol at large scale.

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