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Biofuel production by *Candida tropicalis* from orange peels waste using response surface methodology

Noha Sorour, Saqer Herzallah, Nazieh Alkhalaileh, Amer Mamkagh, Ashraf El-Baz, Esraa Shalaby, Hani Dmoor, Rateb Abbas

ABSTRACT

Citrus fruits are widely consumed worldwide due to their nutritional and health benefits. However, the disposal of citrus waste poses significant environmental challenges. Orange peels (OP) are a substantial by-product of fruit processing and hold great potential as a source for bioethanol production, promoting investment in utilizing agricultural waste for biofuel purposes. OP offers a cost-effective substrate for producing value-added compounds, including bioethanol. Autoclaved-water treated OP biomass exhibited the highest release of reducing sugars (68.2%) this results supported by SEM images of that Autoclaving has definite effect on the structure of the OP particles. Among the five tested microbes, *Candida tropicalis* was selected as a promising bioethanol candidate due to its ethanol tolerance and ability to utilize xylose. Preliminary screening using Plackett-Burman Design (PBD) was conducted to identify six influential factors affecting the fermentation process at three levels, determining the optimum response region for bioethanol production by *C. tropicalis*. The significant variables were further investigated using Response Surface Methodology-Central Composite Rotatable Design (RSM-CCRD) at five levels, a novel approach in this study. The addition of cysteine and resazurin as reducing agents increased bioethanol production by 2.9 and 2.1 times, respectively, from the treated OP. Under the optimized conditions obtained from RSM-CCRD, bioethanol production reached 16.7 mg/mL per mg/ml reducing sugars. Implementing all the optimized conditions, including an initial pH of 5.75, 3% yeast extract, 2.25 g/L cysteine, 4% inoculum size, 0.6 g/L $ZnSO_4$, 0.29 g/L $MgSO_4$, 0.3 g/L $MnSO_4$, and substrate treatment with active charcoal before fermentation, the bioethanol yield increased by 2.2 times after three days of fermentation using co-cultures of *C. tropicalis* and *Kluyveromyces marxianus*. The fermentation process was conducted at 30 °C and 150 rpm. Exploring OP as a low-cost renewable substrate and employing efficient microorganisms open new avenues for bioethanol production.

Keywords: bioethanol, response surface, OP, submerged fermentation, SEM

INTRODUCTION

One of Biofuel production from agricultural waste materials is a highly effective solution for reducing both crude oil consumption and environmental pollution **[1]**. Each year, over 100 billion metric tons of biomass waste, including forestry residues, agricultural by-products, fruit processing waste, and other food processing waste, are generated globally **[2]**. Improper disposal of these waste materials can lead to severe health and environmental issues. Therefore, it is crucial to develop eco-friendly and efficient strategies for utilizing and managing various types of biomass waste. The increasing demand for alternative and sustainable energy sources, driven by concerns about energy security and environmental safety, has placed liquid biofuels, which account for approximately 40% of global energy consumption, among the prioritized renewable energies **[3]**, **[4]**.

Citrus fruits, particularly oranges, are widely cultivated and consumed worldwide, generating significant amounts of fruit waste. This waste, rich in sugars such as sucrose, glucose, and fructose, can be fermented to produce bioethanol. Oranges alone contribute to approximately 55% of global citrus fruit production. The potential of orange peels (OP) as a raw material for ethanol production has been extensively studied at both pilot plant and laboratory scales **[1]**. OP contains fermentable sugars like glucose, fructose, sucrose, and insoluble polysaccharides such as cellulose and pectin **[5]**. The low lignin content of OP makes it an ideal substrate for ethanol production; however, pectin requires pretreatment to release the sugars. Citrus-processing industries generate enormous amounts of waste yearly, with citrus peel waste accounting for nearly 50% of the wet fruit mass. Citrus waste holds significant economic value due to its abundance of flavonoids, carotenoids, dietary fiber, sugars, polyphenols, essential oils, ascorbic acid, and trace elements **[6]**. Hence, OP represents a promising substrate for numerous industrial applications.

Furthermore, environmental concerns, long-term economic sustainability, and national security have increased interest in renewable and domestically sourced fuels as alternatives to fossil fuels **[7]**. The depletion of global petroleum-based fuel reserves and the rising prices of such fuels have driven research on alternative fuel sources. In this context, bioethanol derived from renewable feedstock through bioconversion is widely recognised as a viable alternative fuel. The potential environmental benefits of replacing petroleum-based fuels with biofuels derived from renewable sources are significant driving factors for promoting biofuel production **[8]**. Orange peels can serve as a renewable source for bioethanol production, offering increased productivity and reduced processing costs while adding value to the orange juice industry waste. Most studies on bioethanol production through yeast fermentation have utilized Saccharomyces cerevisiae, which finds extensive applications in food and biofuels **[9]**, **[4]**. However, **[10]** reported a release of 10.924% using *S. cerevisiae* and *C. tropicalis* under optimum conditions, such as H_2O_2-pretreated corn stover (12%), inoculation (25%), pH of 5, and a temperature of 32 °C after 144 hours.

In biological experiments, it is important and advantageous to employ techniques that minimize costs by reducing the number of required experimental formulations to study specific characteristics **[11]**. The application of Response Surface Methodology (RSM) has demonstrated successful optimization of parameters for enzyme production, ethanol, and other bioprocesses **[12]**. Among the various second-order designs, Central Composite Design (CCD) is the most widely used class in RSM **[13]**. Surface plots, derived from fitting individual models to dependent variables, are created and overlaid to identify regions where acceptable predictions for independent variables coincide **[14]**. Thus, the primary objective of this study is to produce bioethanol from OP waste, starting with biomass pre-treatment and bio-treatment, followed by fermentation of liberated sugars using selected microorganisms. *Candida tropicalis* was investigated as a new biofuel candidate, exhibiting promising characteristics for bioethanol production from OP. The optimization of bioethanol production from pre-treated OP biomass was carried out using RSM-CCRD based on significant factors identified through preliminary PBD screening. Moreover, for the first time, the entire fermentation process was optimized using co-cultures of *Candida tropicalis* and *Kluyveromyces marixanus K77* as bioethanol producers from the OP substrate.

Scientific hypothesis

Production of bioethanol from treated orange peel is not less than or equal 20% using RSM-CCRD.

MATERIAL AND METHODOLOGY

Samples

The OP waste samples used in this study were collected from local household and orange juice shops present inside Sadat City, Minufiya Governorate. The collected fresh OP samples were washed with tap water and then dried in an oven at 60 °C for 24 hours till the final constant weight was achieved.

Chemicals

Chemicals used in this study were obtained from the following sources: Hydrochloric acid (HCl, 35%), sulfuric acid (H_2SO_4, 98%), sodium hydroxide (NaOH, 99%), calcium hydroxide ($Ca(OH)_2$, 85%), sodium potassium tartrate (99%), dextrose anhydrous(>99%), Folin–Ciocalteu reagent, sodium carbonate (Na_2CO_3, 99%), and 3,5-dinitrosalicylic acid (DNS, 99%) reagent were purchased from Central Drug House,India. Ammonium hydroxide (NH_4OH, 25%), gallic acid (98%), sodium citrate (99%), citric acid (99%), ammonium sulfate ($(NH_4)_2SO_4$, 99.5%), potassium dichromate($K_2Cr_2O_7$, 99.5%) and active charcoal powder were chemicals obtained from Biochem-Egypt. Cobalt chloride ($CoCl_2$, ATC) was obtained from Loba Chemie-India. Yeast extract, malt extract, agar, peptone, and Whatman filter paper (0.22µm) were obtained from El-Gomhouria Company Egypt. Ferrous sulfate ($FeSO_4.7H_2O$, 99%), manganese sulfate ($MnSO_4$, 98%), zinc sulfate ($ZnSO_4$, 99%), glycerol (98%), and potassium phosphate (K_2HPO_4, 99%) were purchased from El Nasr-Pharmaceutical CO. Egypt. Resazurin (75%) and cysteine(PTC) were obtained from Fisher Scientific.

Biological material

The microorganisms used in this study were obtained from GEBRI, University of Sadat City. They included *Geotrichum candidum*, *Rhizopus oryzae* NRRL 3563, *Candida tropicalis*, *Candida oleaphila* , *Kluyveromyces marixanus* K77, *Pichia anomala* J121, *Saccharomyces boulardii* CNCM I-745, and *Saccharomyces cerevisiae*.

Instruments

The MOD MARS 6 microwave sample preparation system (MARS 6 Synthesis, CEM) was used for sample preparation. The amino acid composition was determined using high-performance liquid chromatography "Agilent-1200", with a separating column InfinityLab Poroshell 120 HILIC 1.9 microns.

Laboratory Methods

Orange Peels Chemical Composition: Orange peels (OP) waste samples were collected from local households in Sadat City, Minufiya Governorate, Egypt, from 2016 to 2017. The proximate the proximate analysis of the collected OP that were used in this study is presented in Table 1. The moisture content of the dried samples was determined following the standard procedure of the US National Renewable Energy Laboratory **[15]**. The ash content was determined using AOAC methods **[16]**. Pectin analysis was conducted according to the method described by Sudhakar and Maini **[72]**. Acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) were determined following the method outlined by Cypriano **[17]**. All analyses were performed in triplicate, and the mean and standard deviation (SD) values were calculated using MS Excel.

Media and Microorganisms: Yeast Malt Peptone (YMP) and Potato-Dextrose Agar (PDA) media were utilized for maintaining fungal and yeast isolates. All media were prepared using double distilled water, and the pH was adjusted to 5.6 ±0.2. The fermentation media employed for bioethanol production **[18]** had the following composition (g/L): $(NH_4)_2SO_4$ 1, KH_2PO_4 1, Yeast extract 10. The fermentation medium was modified to include selected reducing agents such as resazurin and cysteine (0.5-1.5). All media were prepared as described, using double distilled water, and the pH was adjusted using NaOH (1N) or HCl (1N). The media were autoclaved at 121 °C for 20 minutes at 15 psi.

Physical, Chemical, and Water/Autoclaving Pre-treatment: The dried samples were subsequently ground using a spice-grinding machine and kept for all experiments (Figure 1).Alkaline hydrolysis using 2% NaOH was used with a biomass loading of 10% (w/v) in 250 mL screw-capped bottles. The bottles were then autoclaved for 30 minutes at 121 °C. After autoclaving, the treated samples were allowed to settle and cool. The biomass was then filtered and washed with distilled water. Ammonia pre-treatment was carried out using 15% ammonia with a solid-to-liquid ratio of 1:6 (g/mL; w/v) in 250 mL screw-capped bottles **[19]**. The biomass was placed in a water bath overnight at 60 °C.

Distilled water was hydrolysed, with a biomass loading of 10% (w/v) in 250 mL screw-capped bottles. The bottles were autoclaved for 30 minutes at 121 °C and a pressure of 1.5 atmospheres. The filtrate was allowed to settle and cool, and the biomass was filtered. Finally, the treated biomass was filtered, and the filtrate was adjusted to pH 4.5. The total reducing sugar content was measured using the DNS method with a spectrophotometer at λ540 nm **[20]**.

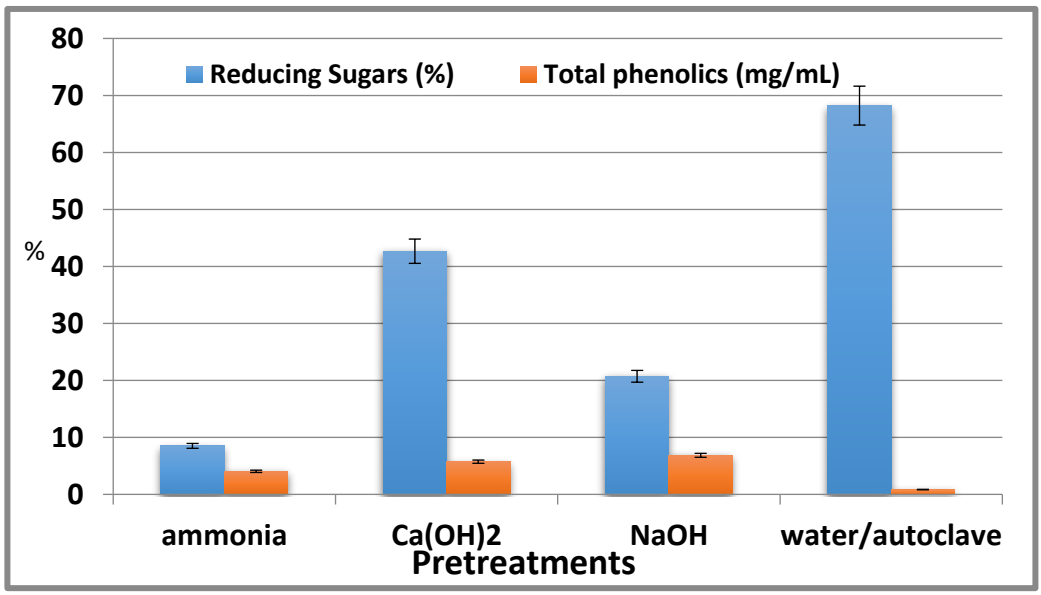

Figure 1 Pretreatments of orange-peels biomass (OP) showing released sugars yield (%), and total phenolic content (mg/mL) after treatment.

Bio-treatment of Orange Peel (OP) Biomass: Cultures of *Geotrichum candidum* and *Rhizopus oryzae* NRRL 3563 were grown on Potato-Dextrose Agar (PDA) plates for 5-6 days. One-week-old slants were mixed with a sterile solution of 0.05% Tween-80 in water to prepare the inoculum. The hydrolysis medium was then inoculated with four discs of *G. candidum* or *R. oryzae*, which had been freshly grown for 3-5 days at 30 °C **[21]**. All experiments were conducted in 250-mL conical flasks containing 5 g of OP biomass substrate and 100 mL of distilled water. The flasks were autoclaved at 121 °C for 20 minutes. The prepared microbial inoculum was added to the autoclaved OP biomass and incubated at 30 °C for 7 days on a rotary shaking incubator (New Brunswick, Canada) at 150 rpm. Samples were collected over a 6-day time course, and the biomass was separated by filtration using Whatman filter paper (No. 1). The filtrate was stored at -18 °C until it was assayed for total sugars using the DNS method.

Selection of Yeast for Fermentation: *Saccharomyces cerevisiae*, *Candida oleophila*, *Candida tropicalis*, *Kluyveromyces marixanus*, and *Pichia anomala* were assessed for their potential in producing bioethanol using the treated biomass over 4 days. To determine their ethanol tolerance, the selected strains were subjected to an ethanol tolerance test in glass-screw tubes containing YMP broth media with varying alcohol concentrations (1%, 2%, 3%, 4%, 8%, and 10%) **[22]**. The inoculated tubes were incubated under static conditions at 30 °C, and yeast viability was subsequently examined. After a 72-hour incubation at 30 °C, the microbial numbers were quantified as colony-forming units per milliliter (CFU/mL).

The chosen yeast strains were cultivated and maintained on YMP slants. After 48 hours of incubation at 30 °C, the grown cultures with an optical density of 0.55 at 600 nm (O.D.600 0.55) were utilized for inoculating the fermentation media at a volume of 2% (v/v). The fermentation medium was prepared in 100 mL screw-capped bottles using treated OP biomass. The medium composition consisted of a steam-autoclaved suspension of pretreated OP (50 mL), along with additional components such as KH_2PO_4 (0.1%), $(NH_4)_2SO_4$ (0.1%), and yeast extract (1%). The pH was adjusted to 5.5, and the bottles were autoclaved for 20 minutes at 121 °C **[23]**.

The autoclaved flasks were opened while still hot in a sterile environment within a biosafety cabinet to eliminate volatile compounds, such as D-limonene, which can hinder yeast growth. One of the screw-capped bottles served as a control without inoculation, while the others were inoculated with a seeding inoculum (inoculum load of 2%). Following inoculation, the screw-capped bottles were incubated at 30 °C in a shaking incubator with an agitation rate of 120 rpm for 5 days. On days 1, 2, and 3 of fermentation, the fermented broth was sampled and subsequently centrifuged for 15 minutes at 6000 rpm. The supernatant was collected, and the ethanol concentration was determined using the dichromate method.

Reducing Agent Selection: To optimize bioethanol production, two reducing agents, cysteine and resazurin, were evaluated following the method described by Anschau et al. **[24]**. In 100 mL screw-capped bottles, a solution of 50 mL treated OP biomass was combined with KH_2PO_4 (0.1%), $(NH_4)_2SO_4$ (0.1%), and yeast extract (1%). Various concentrations of cysteine and resazurin (0.5, 0.75, and 1 g/L) were added to the mixture, and the pH was adjusted to 5.5. The bottles were then autoclaved for 20 minutes at 121 °C. One bottle was left uninoculated as a control, and another without the reducing agent was included for comparison. All the inoculated bottles were incubated at 30 °C in a shaking incubator with an agitation rate of 120 rpm for 3 days. Daily samples were withdrawn for analysis.

Plackett-Burman Screening Design (PBD): Bioethanol production in the fermentation process is influenced by various factors, including nutritional and environmental conditions **[4]**. In the Plackett-Burman screening design (PBD), six variables were considered: initial pH, substrate concentration, reducing agent, inoculum size, agitation speed, and yeast extract concentration. Each variable was set at levels -1 and +1, representing low and high levels, respectively **[25]**. The PBD aimed to identify significant variables affecting bioethanol production, without considering the interaction effects between variables. The results of the PBD were analyzed using JMP version 8 software (SAS Institute Inc., Cary, NC, USA). All experiments were performed in triplicate, and the mean values of bioethanol production were recorded as the response (Plackett and Burman **[73]**). The experimental design consisted of 15 experiments, each with different settings for the six variables, as outlined in Table 2. The PBD utilized a first-order model, represented by Equation 1.

$$Y = \beta 0 + \Sigma \beta i x i \quad (1)$$

In the equation, Y represents the response (bioethanol production yield), β0 denotes the model intercept, βi represents the linear coefficient, and xi represents the level of the independent variable.

Central Composite Rotatable Design (CCRD): Building upon the results of the Plackett-Burman screening design (PBD), a Central Composite Rotatable Design (CCRD) was employed to further investigate the significant factors identified in the PBD, namely pH (X1), reducing agent (X2), and substrate concentration (X3). These factors were chosen as the main variables and were assigned five levels, coded as -2, -1, 0, +1, and +2. The

CCRD consisted of 17 trials, including three center points, and is presented in Table 3. A second-order polynomial function was used to predict the optimal conditions to establish the relationship between the independent variables and the bioethanol production as the response variable **[12]**. The function is represented by Equation 2:

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2 + \beta_{13}X_1X_3 +\beta_{23}X_2X_3 +\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2 \quad (2)$$

In the equation, Y represents the predicted response (bioethanol production), β0 is the model constant, X_1, X_2, and X_3 are the independent variables, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the cross-product coefficients, and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Regression analysis of the experimental data was performed using JMP version 8 software (SAS Institute Inc., Cary, NC, USA) **[26]**. The coefficient of determination, R^2, was used to assess the quality of fit of the polynomial model equation. The experiments were conducted in triplicate, and the mean values were calculated. Other factors were kept constant throughout all the response surface methodology (RSM) experiments.

Enhancement of bioethanol production (Active charcoal, Metals addition, and Cocultures addition): To enhance bioethanol production, C. tropicalis was inoculated into sterilized 250 mL Erlenmeyer flasks containing 100 mL of YMP broth and incubated at 30 °C for 48 h at 120 rpm on a shaking incubator to obtain the seeding culture. This seeding inoculum was then transferred to sterilized 500 mL Erlenmeyer flasks containing 200 mL of YMP broth. The fermentation media were prepared using the optimized conditions determined from RSM-CCRD, which included 3% yeast extract and an inoculum size of 4% of O.D600 = 1. Other factors such as a reducing agent at a concentration of 2.25 g/L and an initial pH of 5.75 were also included. Additionally, the effect of metal supplementation was investigated by adding 0.6 g/L of zinc sulfate, 0.29 g/L of magnesium sulfate, and 0.3 g/L of manganese sulfate **[27]**. The fermentation mixture was then transferred to 1 L screw capped bottles and incubated at 30 °C and 120 rpm for 2 days.

To further enhance bioethanol production, 1 g of active charcoal was added to 1 L of pretreated OP biomass based on the method described by Ma et al. **[28]**. The mixture was allowed to settle for 2 hours, filtered, and then used in fermentation. The initial reducing sugar concentration was increased to 21.2 mg/mL while maintaining the same conditions as described earlier.

For coculture experiments, 24-hour-old cultures of *C. tropicalis* and *K. marxianus* were separately grown on YMP agar media. They were then used to inoculate 100 mL of YMP broth medium, followed by incubation at 30 °C for 24 hours at 120 rpm. Two bottles were inoculated with the 24-hour-old C. tropicalis seeding broth, and another two bottles were inoculated with the 24-hour-old *K. marxianus* seeding broth. These bottles were incubated for 3 days at 30 °C. After 3 days, two different yeast cultures were frozen for 12 hours and then inoculated with the other yeast culture (24-hour-old). Additional bottles were inoculated directly with the yeast strains without freezing and incubated for 3 days at 30 °C. Samples were withdrawn daily and centrifuged at 6000 g for 15 minutes. The supernatants were stored at -18 °C for further analysis. The experiment was repeated twice, and the average values of bioethanol production were determined along with their standard error.

Scanning electron microscope (SEM): To analyze the treated and untreated OP samples, they were first fixed using 2.5% glutaraldehyde and then dehydrated using a series of ethanol dilutions with agitation in an automatic tissue processor (Leica EM TP, Leica Microsystems, Austria). The samples were then dried using a CO_2 critical point drier (Model: Audosamdri-815, Tousimis, Rockville, Maryland, USA). Finally, the samples were coated with gold using a sputter coater (SPI-Module) based on the method described by Tan et al. **[29]**. The coated samples were observed using a scanning electron microscope (JSM-5500 LV; JEOL Ltd, Japan) in high vacuum mode at the Regional Center of Mycology and Biotechnology in Cairo, Egypt. HPLC analysis: High-performance liquid chromatography (HPLC) was employed to analyze sugars such as glucose, xylose, fructose, and others. Isocratic Milli-Q degassed deionized water was used as the mobile phase, flowing at a 0.8 mL/min rate. The analysis was conducted using a Refractive Index (RI) detector, which was maintained at a temperature of 65 °C. Samples were withdrawn at specific reaction times, and then they were diluted, centrifuged, and filtered through 0.45 μm membranes into HPLC vials, which were stored at 4 °C. The peaks corresponding to the sugars were detected and quantified based on the area and retention time, utilizing standards of glucose, fructose, sucrose, xylose, mannose, and galactose obtained from Sigma Aldrich.

Determination of reducing sugars: A DNS (3,5-dinitrosalicylic acid) reagent was prepared following the method described by Miller **[77]** to determine the content of reducing sugars. In this process, 1 g of DNS was dissolved in 20 mL of 2 M NaOH, and then 30 g of sodium potassium tartrate was slowly added. The mixture was diluted to a final volume of 100 mL using distilled water.

In a clean and dry test tube, 0.5 mL of the sample was combined with 0.5 mL of the DNS reagent. The resulting mixture was boiled for 5 minutes and then cooled to halt the reaction. The absorbance of the solution was measured spectrophotometrically at a wavelength of 540 nm, using a calibration curve prepared with a standard

glucose solution. The calibration curve was created using anhydrous glucose standard solutions with concentrations of 0.5, 1, 2, 3, 4, and 5 g/L. A blank solution prepared with distilled water was also used.The yield of reducing sugars (%) was calculated using the following formula, as outlined by Chen et al. **[30]**:

$$\textbf{Hydrolysis Yield } (\%) = \frac{(\textbf{Reducing sugars in mg/mL} * \textbf{0.9} * \textbf{100})}{\textbf{polysaccharide in substrate}}$$

Determination of total phenolics: In a clean and dry test tube, 3.16 mL of distilled water was combined with 40 µL of the sample. To this mixture, 200 µL of Folin-reagent was added and allowed to settle for 5 minutes. Next, 600 µL of a 20% Na_2CO_3 solution was added, and the mixture was left to settle for 2 hours in a clean, dark place. The absorption of the samples was measured using a spectrophotometer at a wavelength of 750 nm, as described by Blainski et al. **[31]**. All analyses were performed in triplicate, and the mean values and standard deviations (SD) were calculated using MS Excel.

Determination of bioethanol using the dichromate oxidation method: To prepare the potassium dichromate reagent, 62.5 mL of distilled water was slowly mixed with 162.5 mL of concentrated sulfuric acid. The mixture was then cooled under tap water. Subsequently, 17 g of potassium dichromate was added and diluted to a final volume of 250 mL. In a clean and dry test tube, 30 µL of the sample was diluted with 500 µL of distilled water. Then, 2 mL of the prepared potassium dichromate reagent was added, followed by 1 mL of 2 M sodium hydroxide. The mixture was incubated at 50 °C for 30 minutes. The samples were then measured using a spectrophotometer at a wavelength of 600 nm. The reagent was prepared by mixing 62.5 mL of distilled water with 162.5 mL of concentrated sulfuric acid. After cooling under tap water, 17 g of potassium dichromate was added, and the solution was diluted to a final volume of 250 mL. The samples were measured using a spectrophotometer at a wavelength of 600 nm, as described by Mushimiyimana et al. **[32]**.

Description of the Experiment: In accordance with the methodology outlined in the study, a total of 32 orange peel (OP) samples were meticulously prepared. These samples were further categorized into two groups: 15 derived from experimental sources and 17 from trials. Each of these samples underwent a chemical analysis, with three replicates conducted for every individual experiment, resulting in a total of 96 samples. The study rigorously adhered to the true experimental design framework, incorporating key elements such as randomization and control which involve three experimental treatments tested in BPD, RSM, and RSM-CCRD with the primary objective was to identify the treatment that yielded the highest efficiency in bioethanol production from OP.

Statistical Analysis

All experiments were performed in three replicates and the mean, standard deviation and analysis of variance (ANOVA) were used for statistical analysis of the experimental results. The significant differences between data obtained was tested at p <0. 05 using SPSS version 22 (IBM,USA).

RESULTS AND DISCUSSION

Chemical analysis of orange peels (OP) and its pre-treatment

The utilization of vegetable and fruit wastes for biofuel production has gained significant attention due to their abundance and high content of sugars, cellulose, and hemicellulose **[2]**. These wastes, including orange peels (OP), offer a cost-effective and sustainable alternative to fossil fuels. Previous studies have explored the potential of various agro-industrial wastes such as potato peel waste, rice straw, grape pomace, and apple pomace for bioethanol production using different microbial strains **[33]**.

In this study, the chemical composition of OP, based on dry weight, was analyzed (Table 1). The results revealed that OP contains substantial amounts of pectin, fiber, and saccharides, which can be effectively utilized in the fermentation process for bioethanol production. The presence of carbohydrates, cellulose, and pectin in OP was notable (Table 1). The relatively low lignin content of OP makes it easily hydrolysable. Fiber, pectin, cellulose, and total sugars collectively constitute approximately 71% of the total chemical composition. These findings align with the chemical analysis of OP conducted by **[5]**.

Table 1 Chemical composition of orange-peels biomass used in ethanol production[1].

Component	(%)
Moisture	12.1 ±0.21
Fiber	26.3 ±0.42
Total Sugars	12.6 ±0.90
Pectin	19.2 ±0.32
Lignin	0.4 ±0.02
Cellulose	13.07 ±0.35
Protein	7.8 ±0.32
Fat	12.3 ±0.15
Ash	2.1 ±0.07
Hemicellulose	1.4 ±0.04
Neutral detergent fibre	10.3 ±0.22
Acid detergent fibre	8.9 ±0.22
Total digestible nutrients	85.3 ±0.68
Net energy (Mcal/kg)	2.03

Note: [1]Values are means of three replicates (±SD) and analysis are measured on dry weight bases.

The chemical composition analysis highlights the potential of OP as a valuable feedstock for bioethanol production. The high content of sugars and cellulose indicates the availability of fermentable substrates for microbial conversion into bioethanol. Furthermore, the presence of pectin offers additional fermentable sugars, contributing to the overall bioethanol yield. The composition of OP supports its suitability as a renewable and sustainable resource for biofuel production, emphasizing its significance in waste valorization and energy sustainability.

The utilization of lignocellulosic biomass for producing value-added products holds great promise due to its abundant availability as unused biomass and its cost-effectiveness **[1]**. Importantly, this approach does not compete with food production or require additional land use, making it an environmentally sustainable option. Among the critical steps in the conversion of lignocellulosic biomass to bioethanol, pre-treatment plays a crucial role in enhancing the overall process efficiency by facilitating hydrolysis and increasing the yield of fermentable sugars, ultimately impacting ethanol production and production costs.

Various pre-treatment methods, including physical, chemical, and biological approaches, are currently employed in lignocellulosic biomass processing **[4]**. In the case of orange peels (OP), pre-treatment effects are believed to arise from the reduction of cellulose crystallinity and the increased porosity of the biomass, which promote easier hydrolysis and the release of sugars **[34]**. During pre-treatment, the complex and highly cross-linked structure of lignin, which consists of phenolic monomers, undergoes breakdown, producing different phenolic compounds **[35]**.

The pre-treatment step in the production of bioethanol from lignocellulosic biomass is crucial for maximizing the efficiency of subsequent hydrolysis and fermentation processes. By reducing cellulose crystallinity, increasing biomass porosity, and facilitating the breakdown of lignin, pre-treatment enables improved access to fermentable sugars and enhances overall ethanol yields. The selection of appropriate pre-treatment methods tailored to specific biomass feedstocks is essential for optimizing the efficiency and cost-effectiveness of the bioethanol production process.

This study investigated various pre-treatment methods, including water/autoclaved, NaOH, $Ca(OH)_2$, and ammonia (Figure 1). Each pre-treatment method exhibited distinct effects on the treated substrate. Mechanical size reduction, for instance, improved the efficiency of downstream processes by making the substrate biomass more susceptible to hydrolysis, thereby increasing the yield of monomeric sugars **[36]**.

The results indicated that the highest release of reducing sugars (68.2%) was achieved by subjecting 10 g of OP substrate to water/autoclaved pre-treatment for 30 minutes, resulting in a phenolic content of 0.84 mg/mL. Conversely, the lowest release of reducing sugars (8.5%) was observed with 15% ammonia pre-treatment in a water bath at 60 °C overnight (Figure 1). Steam-based hydrolysis, conducted under high temperature and pressure, led to the partial hydrolysis of soluble fractions, thereby concentrating the insoluble fractions for subsequent separation and bioconversion **[8]**.

Alkali and acid pre-treatments caused the degradation of ester and glycosidic bonds, resulting in the partial de-crystallization of cellulose and the liberation of individual monosaccharides. These treatments also induced alterations in the lignin structure and partial solvation of hemicellulose, enhancing cellulose digestibility and lignin solubilization **[37]**. On the other hand, water-autoclaved pre-treatment involved water penetration into

biomass cells, cellulose hydration, and the dissolution of hemicellulose and lignin, thereby facilitating the disruption of the lignocellulosic structure **[38]**.

Due to the complex nature of lignocellulosic biomass, various physical and chemical pre-treatment methods were investigated, followed by enzymatic hydrolysis to break down the biomass into simple sugars. These pre-treatments increased the material's porosity and reduced the crystallinity of cellulose **[38]**. de la Torre et al. **[39]** reported that orange waste contains several monosaccharides, and under specific conditions (pH 5.2, 50 °C, 300 rpm agitation speed, and enzyme concentrations of 6.7% w/w), glucose yields exceeding 80% were achieved. The pre-treatment method that yielded the highest amount of sugars while minimizing the presence of inhibitory compounds was selected for the subsequent fermentation process.

SEM analysis: The SEM analysis conducted at a scale of 500 μm (Figure 2) confirmed that autoclaving steam pre-treatment induced a significant change in the morphology of OP biomasses. A clear distinction can be observed between the morphology of the OP samples before (A, A') and after (B, B') the pre-treatment process. The pre-treatment increased the amorphous portion and a corresponding decrease in the crystalline part of the biomasses. The SEM micrographs reveal noticeable cracks on the surface of the fibers and splitting of the surface near the center.

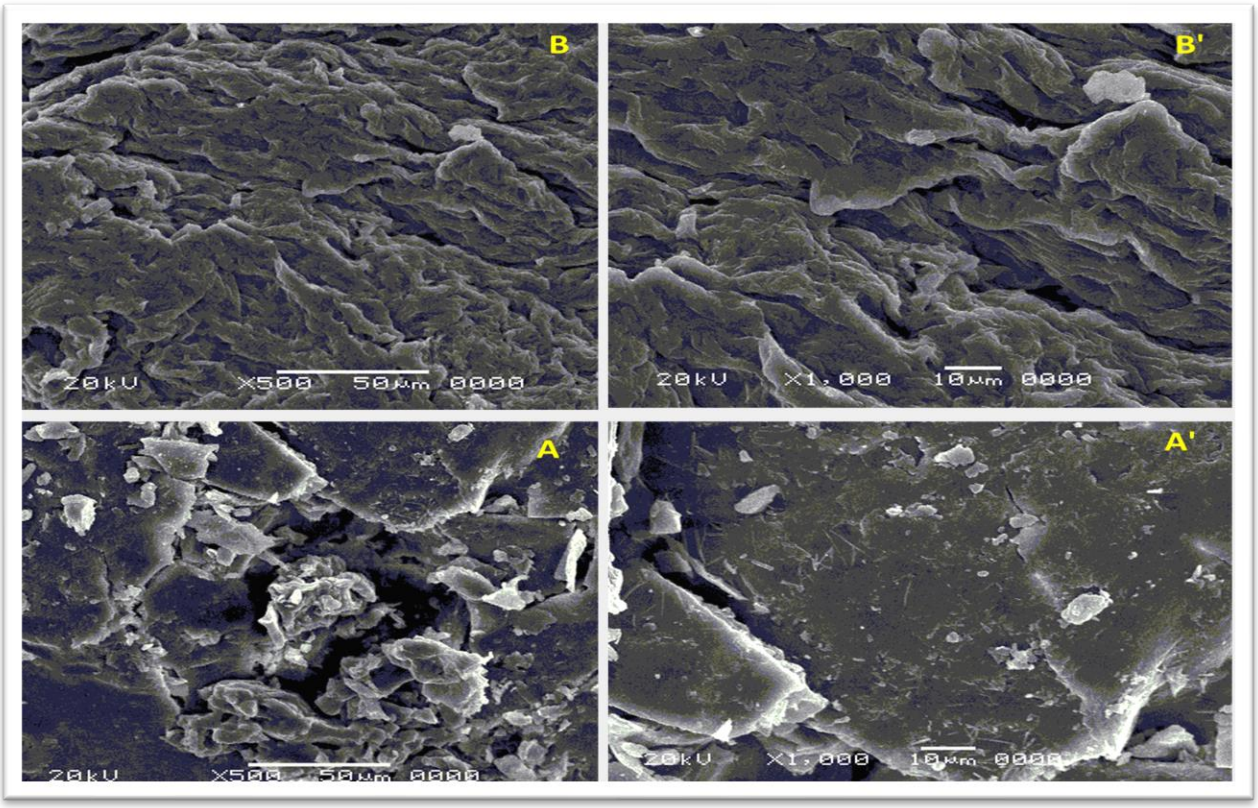

Figure 2 SEM micrograph of orange-peels biomass before (A, A') and after (B, B') water hydrolysis (autoclaving) treatment. Note: boundaries of the biomass after the pre-treatment (B, B'), while the untreated samples appear densely packed in comparison (A, A').

These observations are consistent with the findings of Li et al. **[40]**, who examined the effect of microwave treatment on OP morphology using SEM micrographs at a scale of 1000 μm before and after pre-treatment. They reported that OP exhibited a smooth surface before treatment, which transformed into a cracked and porous structure after microwave treatment. Similarly, Xu et al. **[41]** documented that steam explosion hydrolysis changed corn stover's morphology, transitioning from a smooth surface with a high crystal structure before treatment to a cracked and porous structure after treatment.

Fungal bio-treatment hydrolysis: The advancements in modern biotechnology have contributed significantly to reducing the cost of enzyme production, thereby enhancing the economic viability of the hydrolysis process **[74]**. As enzymes constitute a substantial portion of the production cost, minimizing their usage during hydrolysis can play a crucial role in cost reduction **[5]**. Filamentous fungi are a prominent source of cellulases and hemicellulases **[42]**. Fungi such as *Aspergillus niger*, *Geotrichium candidium*, and *Rhizopus oryzae*

have demonstrated the ability to produce cellulase and pectinase enzymes for the hydrolysis of lignocellulosic substrates **[43]**.

In this study, *R. oryzae* and *G. candidium*, previously recognized as producers of cellulase and pectinase, were inoculated into a medium containing untreated OP biomass. The cultures were incubated at 30 °C with an agitation rate of 150 rpm (Figure 3). For the incubation, both fungi exhibited distinct hydrolysis patterns, and the release of reducing sugars from the biotreated OP increased over time, reaching its peak values of 87.6% and 40.7% on the 2nd and 3rd days, respectively. The release of reducing sugars can be attributed to the enzymatic alteration or degradation of cellulose and pectin by the fungal enzymes, which act on cellulose's reducing and non-reducing ends. Additionally, the action of the enzymes opens up the cell wall structure, facilitating the subsequent hydrolysis of biopolymers **[6]**. Both fungal species are recognized for their ability to produce extracellular enzymes that degrade cell walls, making them valuable for industrial applications **[42]**. The genus *Rhizopus* includes several species employed for industrial enzyme production, such as glucoamylases, cellulases, and tannases, as well as organic acids **[44]**. Currently, this microorganism is primarily used to produce lactic and fumaric acid, as well as enzymes like lipases, amylases, and cellulases **[45]**. *Geotrichum sp*., on the other hand, has been reported as a proficient producer of active polygalacturonase among yeasts **[46]**.

However, the overall results depicted in Figure 3 indicate that *G. candidium* and *R. oryzae* require approximately 2 and 5 days to achieve a reducing sugar content of 51.9% and 43.1%. Consequently, water-autoclaving hydrolysis was employed before further optimization of bioethanol production.

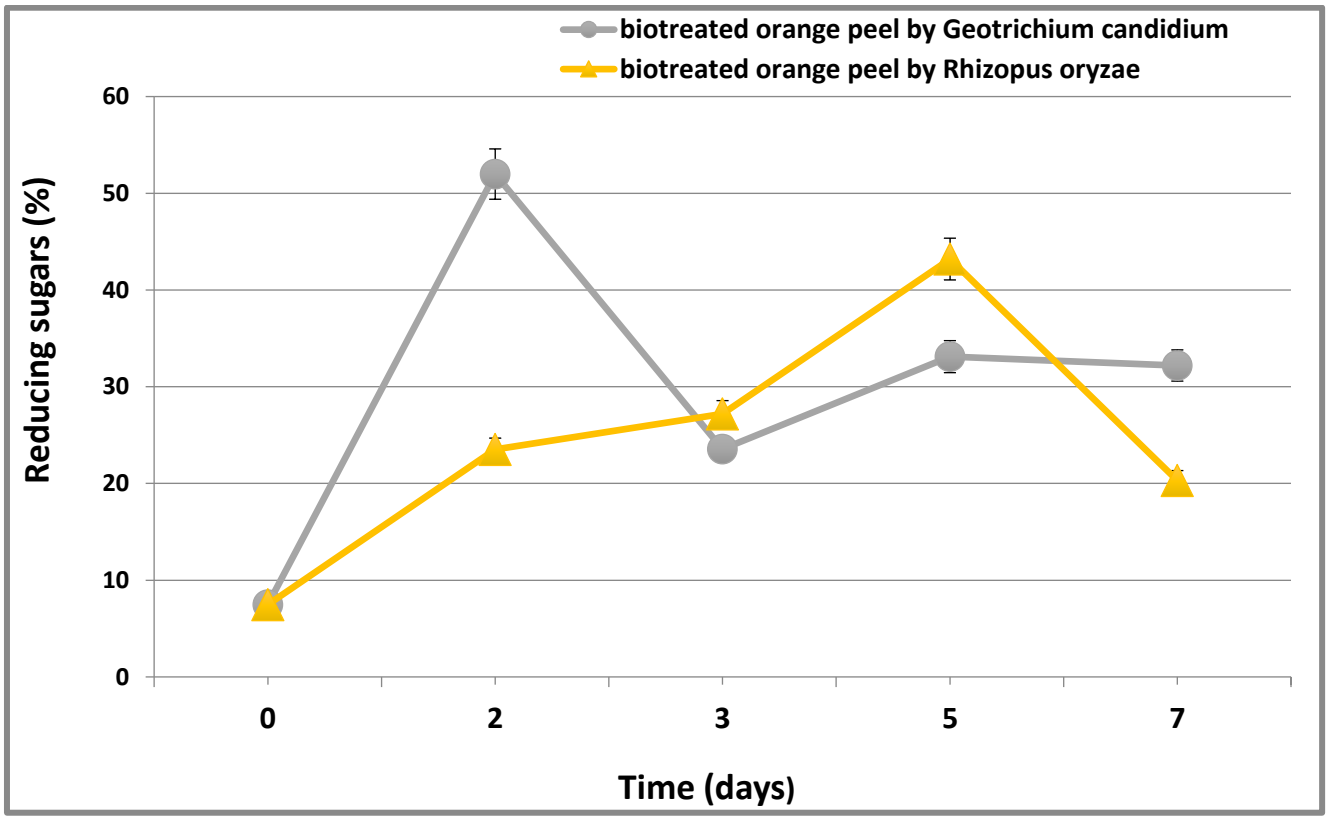

Figure 3 Hydrolysis of OP biomass using *Rhizopus oryzae and Geotrichium candidium* through time course of 7 days, incubated at 30 ºC and 150 rpm.

Selection of yeast used for bioethanol production: The selection of yeast for bioethanol production was crucial due to the specific requirements of the OP biomass, which contains xylose as one of its main sugars. *S. cerevisiae*, a commonly used microorganism for bioethanol production, was found to be unable to ferment xylose. Therefore, a preliminary experiment was conducted to evaluate different yeast species for their bioethanol production using treated OP biomass over a 4-day time course.

The results (Figure 4) showed that *C. tropicalis* exhibited the highest bioethanol production, with a 0.315 mg/mL concentration. *K. marxianus* and *C. oleophila* followed closely with bioethanol concentrations of 0.227 and 0.20 mg/mL on day 3, respectively. Industrial-scale fermentation of treated OP biomass requires microorganisms capable of functioning at high ethanol concentrations. Therefore, the alcohol tolerance of the newly identified candidates, *C. oleophila* and *C. tropicalis*, was compared with that of the well-known bioethanol producer *S. cerevisiae*, from 1 to 10%.

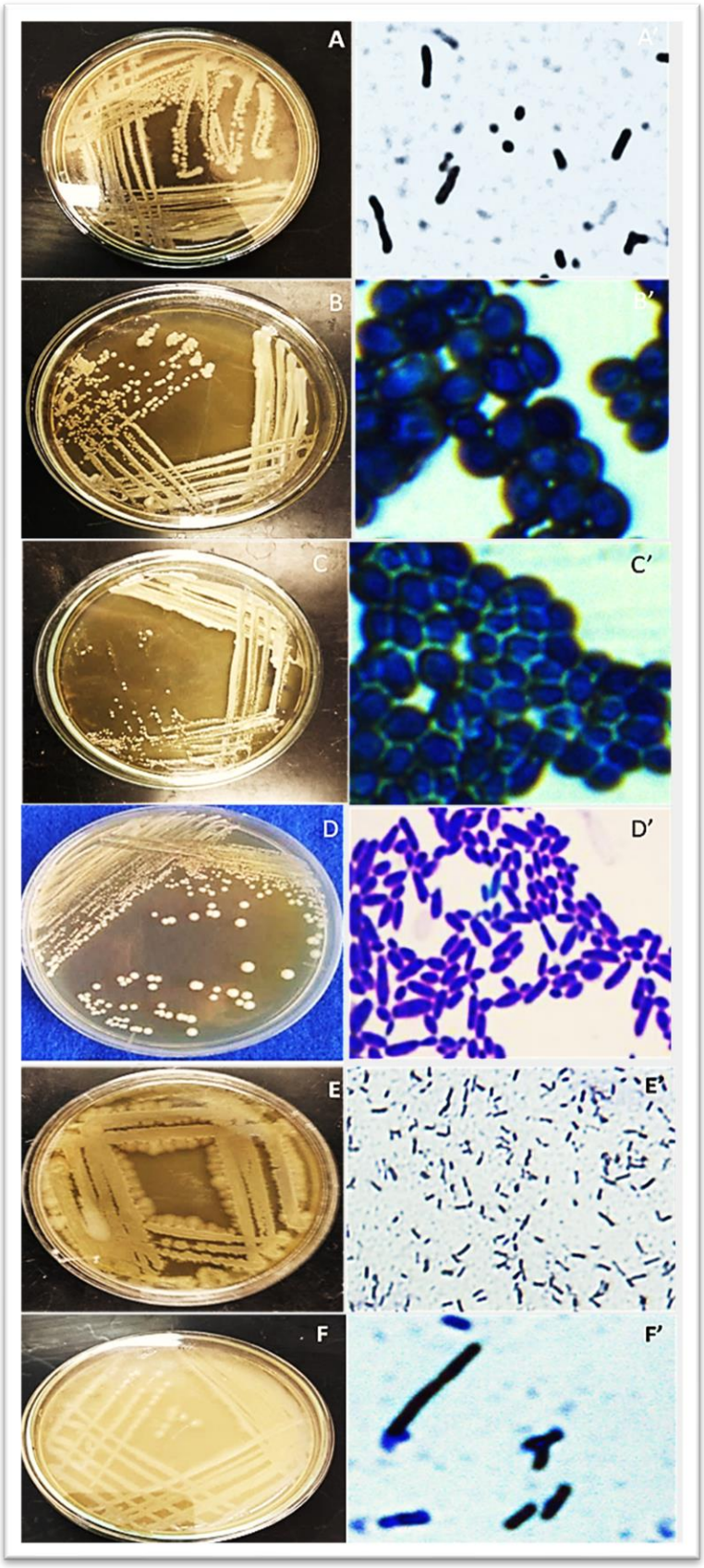

Figure 4 Yeasts used during the study, on YMP medium (A) and under light microscope stained with simple stain at 1000x magnification (A'); *C. oleophila* (A, A'); *S. cerevisiae* (B, B'); *S. boulardii* (C, C'); *Pichia anomala.* (D, D'); *C. tropicalis* (E, E') and *Kluyveromyces* marixanus (F, F').

In addition to alcohol tolerance, utilising xylose, which is released from hemicellulose during pretreatment, was a crucial factor. Based on its good ethanol tolerance and xylose utilization capabilities, *C. tropicalis* was selected for further investigations. Most published studies on bioethanol production predominantly focus on *S. cerevisiae* **[4]**. Therefore, *C. tropicalis* was chosen as a novel bioethanol producer, considering its bioethanol production of 0.315 mg/mL and its ability to utilize xylose. Subsequently, the strain was further adapted to alcohol concentrations ranging from 2 to 12%.

C. tropicalis is a species of yeast belonging to the *Candida genus*, while *S. cerevisiae* belongs to the *Saccharomyces* genus. Both are single-celled eukaryotes with various industrial applications. *S. cerevisiae* finds wide use in food, ingredients, biofuels, pharmaceuticals, and functional genomics, while *C. tropicalis* has diverse applications in industries such as food production **[47]**. *C. tropicalis* has been observed to produce high levels of xylitol, reaching 36 g/L in semi-synthetic conditions within 59 hours of fermentation **[48]**. Although *C. tropicalis* has not been extensively studied for biofuel production from OP biomass, it has been used in other applications, including the food industry.

Kluyveromyces, another genus of yeast in the *Saccharomycetaceae* family, has wide-ranging applications such as bioethanol production, low-lactose milk production, and heterologous protein production **[49]**. Studies involving simultaneous saccharification and fermentation using a co-culture of *S. cerevisiae* and *C. tropicalis* resulted in a bioethanol yield of 10.924% under optimal conditions, using H_2O_2-pretreated corn stover (12% concentration), 25% inoculum, pH 5, and 32 °C for 144 hours **[10]**. Additionally, *Candida tropicalis* MK-160 strain was found to produce higher titers of xylanase and 5.45% ethanol when grown on sugarcane bagasse and wheat bran **[50]**.

Since xylose constitutes a significant portion (30-40%) of the hemicellulose in OP biomass, efficient bioconversion of xylose is essential for economically feasible biomass conversion. Native xylose-utilizing yeasts such as *Candida shehatae*, *Scheffersomyces stipitis*, and *Spathaspora passalidarum* have been studied for their ability to convert xylose to xylulose through the oxidoreductase pathway **[51]**.

Candida tropicalis, an important *Candida* yeast species, can be found in plants and the digestive systems of mammals. It is considered an osmotolerant yeast due to its robust cell walls and broad environmental tolerance in terms of pH and ionic strength **[52]**. It possesses ascorbate oxidase enzyme activity and is amenable to biosensor construction due to its easy manipulation, rapid growth rate, and ability to utilize different carbon sources **[53]**.

HPLC analysis (Figure 5) revealed that different treatment methods resulted in distinct sugar profiles. Among them, the autoclaved water treatment exhibited higher levels of reducing sugars and relatively fewer inhibitory compounds, making it the preferred choice for fermentation purposes.

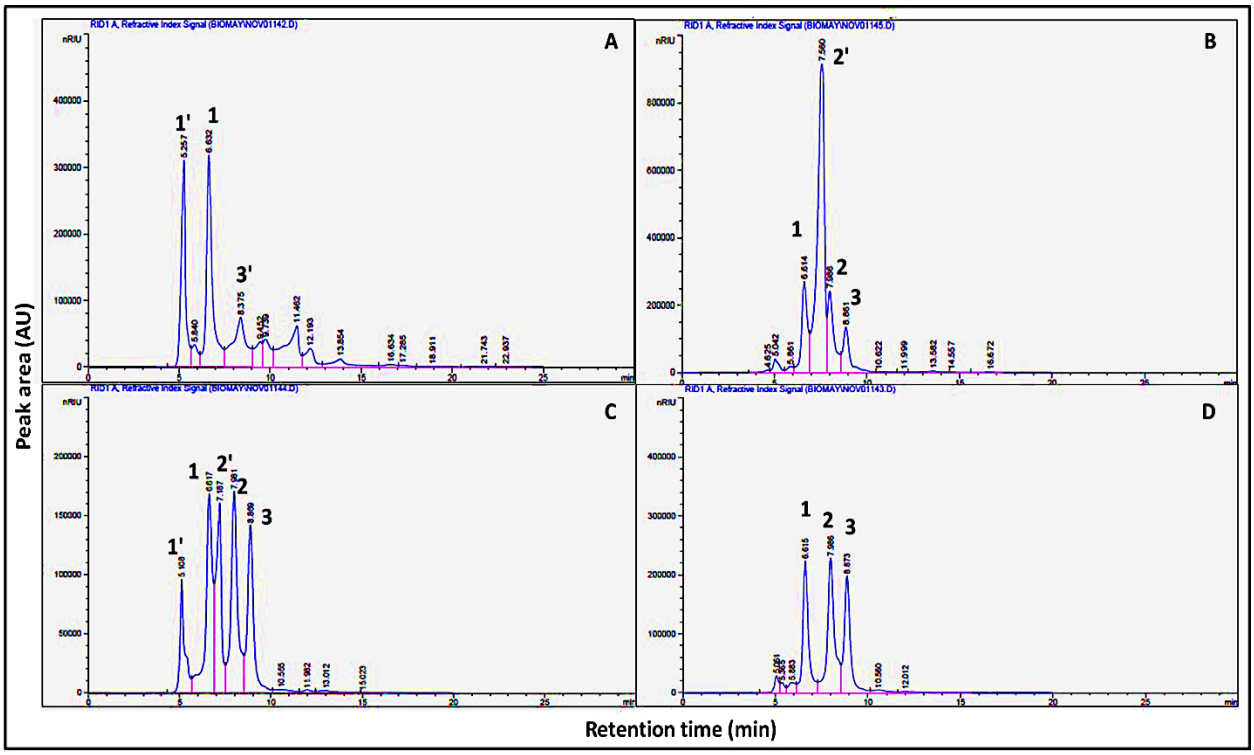

Figure 5 HPLC analysis of treated orange-peels biomass showing different sugar profiling of liberated sugars; biomass treated with calcium hydroxide (A), biomass treated with ammonia (B), autoclaved treated biomass with

water (C) and untreated biomass control (D); identified peaks are 1: sucrose, 1': xylose; 2: glucose; 2': galactose; 3: fructose; 3': mannose.

Effect of reducing agent on the fermentation process

The inclusion of reducing agents in the fermentation process plays a crucial role by reducing the redox potential of the cells. This, in turn, scavenges oxygen and alters the electron flow, ultimately influencing the production of bioethanol **[54]**. In this study, two reducing agents, cysteine and resazurin, were added to treated OP at concentrations of 0.5, 0.75, and 1 g/L. The addition of cysteine increased alcohol production from 0.31 mg/mL to 0.55 mg/mL, while resazurin resulted in a production of 0.41 mg/mL. These findings align with previous research conducted by Alriksson et al. **[55]**, who reported that the addition of 4 mM cysteine, glycine, and glutamic acid increased the fermentation rate from 119.6 mg/L to 236.1 mg/L of glutathione and reduced the biomass rate from 25.3 g/L to 25 g/L in cane molasses. Similarly, Hossain et al. **[56]** observed that the addition of 5 mM dithionite and 17.5 mM sulfite as reducing agents to sugarcane bagasse hydrolysate improved the SSF process from 0.9 to 3.9 g/L/h. Based on these results, cysteine at concentrations of 0.5 g/L and 1.5 g/L was selected for further optimization of the bioethanol production process from OP biomass.

Screening of bioethanol production using PBD

The production of bioethanol is influenced by various factors, including temperature, sugar concentration, fermentation time, pH, inoculum size, and agitation rate. This study employed a Plackett-Burman design (PBD) to identify the key factors affecting bioethanol production by *C. tropicalis* from treated OP biomass. Six variables were investigated, including initial pH, cysteine (reducing agent) concentration, inoculum size, yeast extract percentage, agitation speed, and substrate concentration. The fixed medium constitutions included temperature, KH_2PO_4, and ammonium sulfate.

The results in Table 2 and Figure 6 demonstrated a wide range of bioethanol production, from 1.93 to 6.03 mg/mL. This variation can be attributed to the interactions among the tested variables. The highest bioethanol production of 6.03 mg/mL was achieved at run number 4, with the following conditions: initial pH of 6, cysteine concentration of 1.5 g/L, agitation speed of 170 rpm, inoculum size of 3.5% (O.D600 0.9), yeast extract percentage of 3%, and substrate volume of 50 mL, after a fermentation time of 2 days.

Analysis of variance (ANOVA) using the Fisher test was conducted to assess the effects of the independent variables on bioethanol production, and statistically significant results were determined based on a p-value <0.05. The obtained model F-value of 8.3797 indicated the significance of the model for bioethanol production, with a p-value of 0.0108. A smaller p-value indicates a higher significance of the corresponding coefficient. The analysis suggested that pH, reducing agent concentration, and substrate concentration were significant factors with a positive effect, as illustrated in the pareto chart (Figure 6).

Using JMP version 8, the first-order model equation (Equation 3) for the PBD was determined as follows:

$$Y = -0.0287 + 0.00577X1 + 0.0075X2 + 0.00012X3 - 0.00137X4 - 0.00138X5 + 0.00028X6 \quad (3)$$

Y represents the bioethanol amount in the equation, while X1, X2, X3, X4, X5, and X6 correspond to pH, cysteine concentration, agitation speed, inoculum size, yeast extract percentage, and substrate concentration, respectively.

PBD has been widely employed for screening various parameters in bioprocesses, including enzyme production and ethanol production **[48]**, **[12]**. However, different authors have reported different results, which can be attributed to variations in yeast strains and substrates used. For example, El-Sayed et al. **[75]** utilized PBD to optimize ethanol production from *Ulva lactuca* using *S. cerevisiae*, and they found that inoculum size and sugar concentration significantly affected bioethanol production, resulting in a yield of 12 ±0.5 g/g of sugar/L. **[57]** employed PBD to investigate the effect of medium components on bioethanol production by *Wickerhamia* sp. from potato waste.

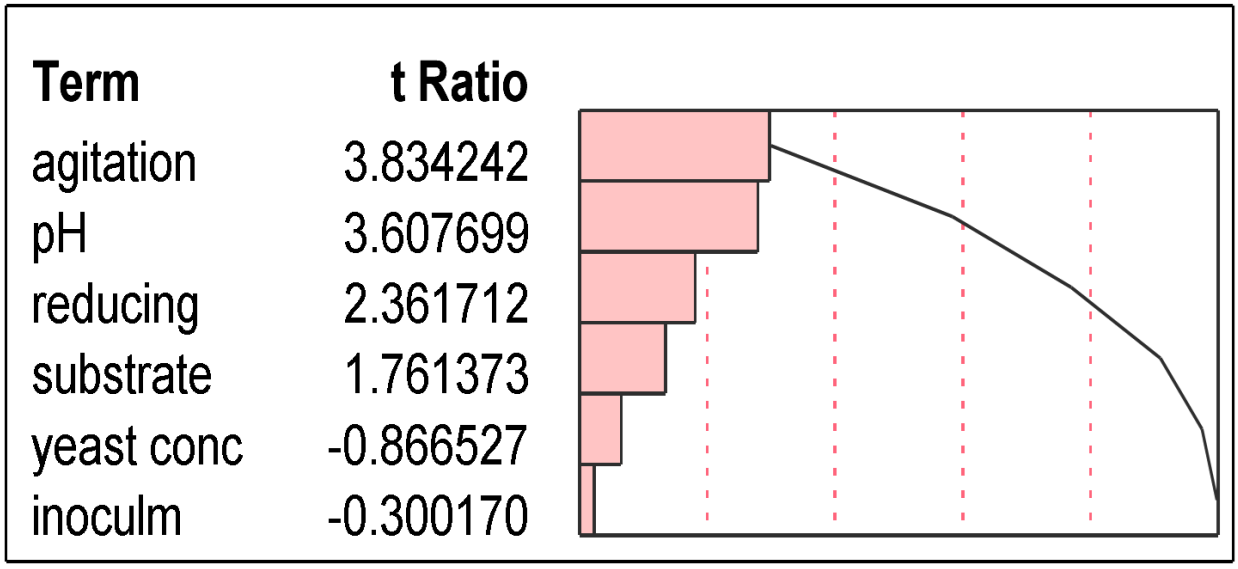

Figure 6 Pareto-chart rationalizing the effect of each variable on bioethanol production by *Candida tropicalis* using treated orange-peel biomass as substrate by PBD. The calculated F-ratio was higher than the theoretical one for the regression model (ANOVA), indicating its significance.

Table 2 Plackett-Burman experimental design matrix, and the actual values of bioethanol production by *C. tropicalis* using pretreated orange peel as substrate, after 2 days of fermentation at 30 °C.

Exp.no.	Pattern	Initial H	Cysteine (g/L)	Agitation speed (rpm)	Inoculum (O.D)	Yeast extract (%)	Substrate (mL)	Actual Bioethanol (mg/mL)	Predicted Bioethanol (mg/mL)
1	−−−+−−	4	0.5	70	0.9	1	30	1.937266	1.454627
2	+−−−+−	6	0.5	70	0.2	3	30	2.654305	2.580505
3	−+−−+−	4	1.5	70	0.2	3	30	2.000164	2.119252
4	−−+−−+	4	0.5	170	0.2	1	50	4.176442	3.637194
5	+++−−−	6	1.5	170	0.2	1	30	5.434407	5.194974
6	++−−−+	6	1.5	70	0.2	1	50	3.861951	4.427616
7	+−−+−+	6	0.5	70	0.9	1	50	2.968797	3.44221
8	−+++−−	4	1.5	170	0.9	1	30	2.968797	3.748315
9	−−+−++	4	0.5	170	0.2	3	50	2.591407	3.316414
10	+−+++−	6	0.5	170	0.9	3	30	3.434243	3.888788
11	−+−+++	4	1.5	70	0.9	3	50	2.704624	2.660176
12	++++++	6	1.5	170	0.9	3	50	6.03823	5.415118
13	0	5	1	120	0.55	2	40	3.861951	3.490432
14	0	5	1	120	0.55	2	40	3.861951	3.490432
15	0	5	1	120	0.55	2	40	3.861951	3.490432

Note: **: Significant p <0.01.

They achieved a bioethanol yield of 33.1 mg/mL under 30 °C, 150 rpm for 96 hours. Factors such as malt extract, tryptone, KH_2PO_4, Na_2HPO_4, NaCl, and $CaCl_2.2H_2O$ showed positive effects on the fermentation process, while $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$ showed negative effects. Similarly, Yu et al. **[58]** used PBD to optimize bioethanol production from potato waste using *S. cerevisiae*, and they obtained a bioethanol yield of 36.85 mg/mL at 30 °C, 150 rpm after 48 hours. In their study, yeast extract, malt extract, and $MgSO_4.7H_2O$ positively affected bioethanol production, while KH_2PO_4 and $CaCl_2.2H_2O$ had negative effects.

In another study, **[1]** utilized Taguchi orthogonal array statistical design to optimize parameters for biomass pretreatment of sweet lime peel biomass, including solid loading, time of exposure, and sulfuric acid concentration. The optimized parameters of 17% (w/v) solid loading, 0.25% (v/v) acid concentration, and 60 minutes of steam exposure were used for enzymatic hydrolysis of the pretreated sweet lime peel. Subsequent fermentation using baker's yeast resulted in the release of 7.09 mg of reducing sugar/mL of hydrolysate, with a

bioethanol yield of 18% at 30 °C after 72 hours. However, Oberoi et al. **[5]** conducted primary hydrolysis of OP using acid concentrations ranging from 0 to 1.0% (w/v) at 121 °C and 15 psi for 15 minutes. The hydrolysate obtained from hydrolysis was then fermented using parameters optimized through response surface methodology (RSM), resulting in an ethanol yield of 0.25 g/g on a biomass basis.

Overall, PBD and other statistical designs have proven valuable in exploring and optimizing the parameters involved in bioethanol production from different substrates, leading to improved yields and process efficiency.

Optimization of Bioethanol production using RSM

RSM has proven to be effective in optimizing parameters for the production of enzymes and ethanol in biological systems **[5]**. To successfully produce bioethanol from OP biomass, optimising important fermentation parameters such as pH, substrate concentration, reducing agent, and agitation rate is crucial. Agitation rate plays a role in nutrient permeability and ethanol removal in the fermentation broth, with 150-200 rpm being the common range for yeast cell fermentation. However, excessive agitation can hinder yeast cell metabolic activities **[59]**, so an agitation rate of 150 rpm was selected for subsequent experiments. Similarly, sugar concentration affects fermentation, but excessive levels can lead to steady rates due to the limited uptake capacity of microbial cells **[4]**.

After identifying the most significant variables influencing bioethanol production using PBD, a CCRD was conducted, with day 2 chosen as the optimal day. The major variables identified from PBD were pH (X1), reducing agent (X2), and substrate concentration (X3), while the agitation speed remained at 150 rpm. These factors were tested at five levels (-2, -1, 0, +1, and +2) using 17 trials and three center points (Table 3). The significance of the model was assessed using the F-test and ANOVA, and the response surface quadratic model demonstrated statistical significance with a *p*-value of 0.011. The signs and statistical significance of coefficients ($p < 0.05$) were used to interpret the data, considering the positive or negative effects on the response and the presence of antagonistic or synergistic interactions between factors. The model achieved a determination coefficient (R^2) of 0.92, explaining 92% of the total variations and showed excellent agreement between experimental results and predicted values. A second-order polynomial model (Equation 4) was fitted to the experimental bioethanol production results to predict the optimal point within the experimental constraints.

Run 8 represented the optimum conditions for bioethanol production, including 3% yeast extract, 3.5% inoculum size with OD600 of 0.9, initial pH of 5.75, 2.25 g/L cysteine, initial reducing sugar concentration of 2.56 mg/mL, 85 mL reaction volume, 30 °C temperature, 2-day fermentation time, and agitation rate of 150 rpm. Under these optimized conditions, bioethanol production increased from 6.03 to 16.71 mg/mL. The model proved statistically valid in explaining all bioethanol production within the investigated experimental ranges (Table 3, Figure 7). The Pareto chart provided insights into the magnitude and ranking of factor estimates. Regression coefficients for the tested variables indicated positive and negative effects for pH and reducing agent concentration, respectively. Three-dimensional response surface plots based on the model equation were used to understand the interaction among variables and determine the optimal levels of each factor for bioethanol production from OP biomass (Figure 7). A more horizontal 3D surface and perfect interaction indicate the highest significant effect **[11]**.

Researchers have widely utilized RSM for optimizing bioethanol production from various substrates, leading to different yields depending on the substrate and yeast used **[60]**. For instance, **[23]** employed RSM to optimize bioethanol production from pineapple peels using *S. cerevisiae*. The optimum conditions were determined as pH 6, 5 g/L ammonium sulfate, and 6 g/L yeast loading, resulting in a maximum bioethanol yield of 5.82%.

In another study, **[61]** applied RSM to optimize bioethanol production from cellulase-treated sugarcane bagasse using *Candida wickerhamii*. Under the optimal conditions of pH 5.7, 33 °C temperature, and 50 mg/mL substrate concentration for 104 hours, a bioethanol yield of 4.28 mg/mL was achieved.

Sharma et al. **[62]** investigated the fermentation parameters influencing bioethanol production from kinnow waste and banana peels using simultaneous saccharification and fermentation with cocultures of *Pachysolen tannophilus* MTCC 1077 and *S. cerevisiae*. They determined that an inoculation rate of 6% (v/v) *S. cerevisiae*, incubation time of 48 hours, agitation time of 24 hours at 30 °C, and 4% (v/v) *P. tannophilus* resulted in optimal conditions, yielding 26.84 mg/mL of bioethanol.

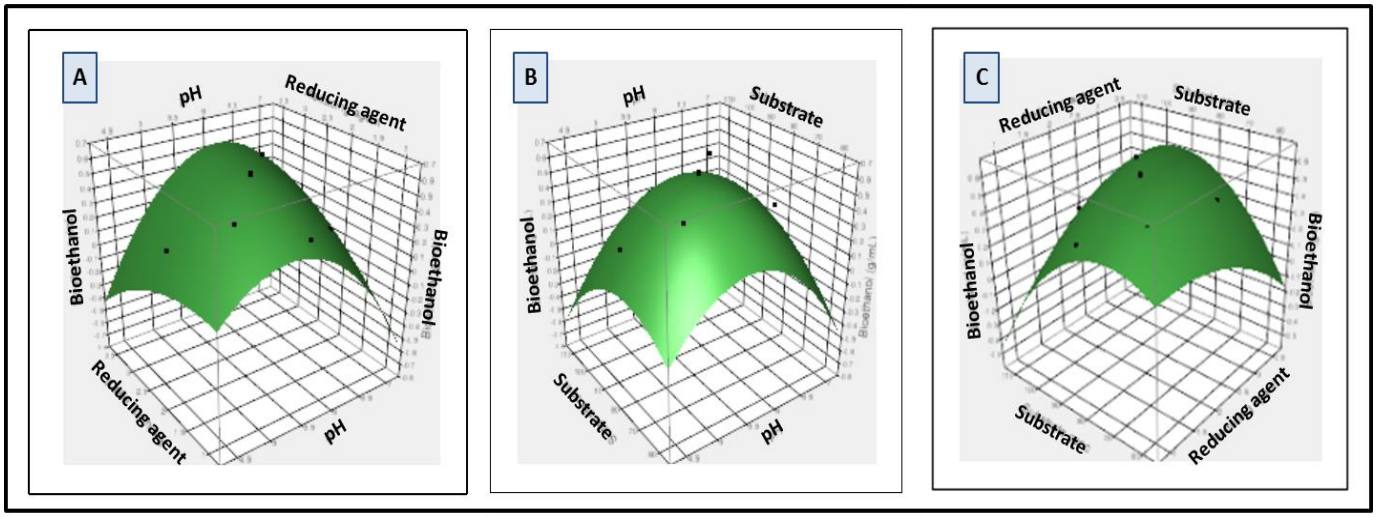

Figure 7 Response surface plot showing the effect of initial pH and reducing agent (A), pH and substrate conc. (B), reducing agent and substrate conc. (C) on the production of bioethanol by *Candida tropicalis* under three variables using pre-treated orange-peel biomass as substrate, other variables are held at zero level.

Table 3 Optimization of bioethanol production using RSM by *C. tropicalis* under three variables by CCRD-design matrix, with actual and predicted values from pretreated orange peel as substrate, after 2 days of fermentation at 30 °C.

Trial	Pattern	Initial pH	Reducing agent (mg/mL)	Reaction Substrate (mL)	Actual Bioethanol (mg/mL)	Predicted Bioethanol (mg/mL)
1	−−−	5	1.5	70	14.3807	14.50829
2	+−−	6.5	1.5	70	8.999076	8.206792
3	−+−	5	3	70	8.210543	8.604322
4	−−+	5	1.5	100	5.296981	7.063348
5	++−	6.5	3	70	12.96179	10.55903
6	+−+	6.5	1.5	100	5.858309	4.828137
7	−++	5	3	100	8.339737	8.495629
8	+++	6.5	3	100	15.28061	14.51662
9	0	5.75	2.25	85	16.71066	15.73255
10	0	5.75	2.25	85	14.1535	15.73255
11	0	5.75	2.25	85	16.48791	15.73255
12	00a	5.75	2.25	59.77	9.94487	11.22784
13	00A	5.75	2.25	110.23	8.678316	8.295341
14	0a0	5.75	0.99	85	12.04183	11.69251
15	0A0	5.75	3.519	85	13.62558	14.8749
16	a00	4.49	2.25	85	8.678316	6.91852
17	A00	7.019	2.25	85	4.022854	6.682645

Note: *: Significant p <0.01.

Furthermore, Raja Sathendra et al. **[60]** utilized RSM to optimize bioethanol production from palm wood using *Kluyveromyces marxianus*. The optimal conditions were found to be pH 5, temperature 45 °C, agitation rate of 156 rpm, 3.2% inoculum size, and 8% (v/v) substrate concentration, leading to a maximum bioethanol yield of 22.90 mg/mL. Similarly, Jambo et al. **[63]** employed RSM to optimize bioethanol production from *Eucheuma cottonii* using *S. cerevisiae*. The optimal conditions were determined as a 12% (v/v) inoculum size, pH 5.2, temperature of 32 °C, and a fermentation time of 72 hours, resulting in a bioethanol yield of 9.77 mg/mL.

Likewise, Sininart Chongkhong **[64]** utilized RSM to optimize bioethanol production from banana peels using *S. cerevisiae*. The optimal conditions were identified as pH 4.8, 28 °C temperature, and a yeast loading of 4% (w/w) for a fermentation duration of 192 hours, achieving a bioethanol yield of 9.2%.

Enhancement of the bioethanol production using successive cocultures and metal addition

The bioethanol production was significantly improved by implementing a series of enhancements, including successive cocultures and metal addition. Initially, bioethanol production increased from 6.03 to 16.71 mg/mL under optimized conditions determined by response surface methodology (RSM). These conditions included 3% yeast extract, a 3.5% v/v, O.D600 (0.9), inoculum size, initial pH of 5.75, 2.25 g/L cysteine, and 2.56 mg/mL initial reducing sugar. This fermentation was carried out by *C. tropicalis* at 30 °C and 150 rpm for 2 days.

Further optimization was conducted by introducing selected metals into the fermentation process. Studies have shown that certain metal ions, such as Zn+2, Mg+2, and Mn+2, have the potential to enhance bioethanol production and the fermentation process **[76]**. For example, Alamri et al. **[65]** demonstrated that the addition of zinc, magnesium, and manganese at their respective optimum concentrations of 0.6 g/L, 0.2-0.3 g/L, and 0.03 g/L induced ethanol production from date molasses. Specifically, *Hanseniaspora guilliermondii* KKUY-0036 and *H. uvarum* KKUY-0078 achieved ethanol yields of 6.48% and 6.11%, respectively. This enhancement can be attributed to the activation of alcohol dehydrogenase enzymes by zinc ions, which are essential for ethanol formation. Magnesium ions directly influence various aspects of yeast physiology, including sugar consumption, ethanol production, yeast growth, and responses to environmental stress. They act as size transducers, growth enhancers, and stress protectants during the fermentation process **[65]**.

To further improve bioethanol production, the substrate filtrate was treated with activated charcoal before fermentation. Activated charcoal is commonly used as an adsorbent to remove inhibitors, such as furans and phenolic compounds, from lignocellulosic hydrolysates due to its high adsorption capacity and strong hydrophobicity **[66]**. This treatment helps to create a more favorable environment for bioethanol production.

Additionally, successive coculturing of *C. tropicalis* and *K. marxianus* was employed as a strategy for enhancement. Co-culture processes involve the simultaneous cultivation of two different yeast strains in the same reaction mixture, leading to improved ethanol production compared to pure cultures. The use of co-cultures, such as combining pentose-utilizing yeasts like *Pichia stipitis* and *Pichia fermentans* with *S. cerevisiae*, allows for the utilization of both hexose and pentose sugars, thereby enhancing ethanol production **[4]**, **[67]**.

Overall, by combining these optimization strategies, the bioethanol yield was increased to 87.66% after 3 days of fermentation, as shown in Table 4. This represents a significant improvement compared to the initial conditions and demonstrates the effectiveness of successive cocultures and metal addition in enhancing bioethanol production.

Activated charcoal is commonly used as an adsorbent in lignocellulosic hydrolysates to remove inhibitors with higher hydrophobicity than sugars and aliphatic carboxylic acids, such as furans, furfurals, and phenolic compounds. This is due to its strong hydrophobicity and high adsorption capacity **[66]**. The effectiveness of active charcoal treatment in enhancing butanol production was investigated by Zhang et al. **[68]**. They found that the addition of 5.0% (w/v) active charcoal removed 77.9% of furan derivatives and 98.6% of aromatic monomers, resulting in an increased butanol yield of 0.22 g per g sugar.

Co-culture processes involve the simultaneous cultivation of two different yeasts in the same reaction mixture, and they have shown to enhance ethanol production compared to pure cultures **[69]**. In the case of bioethanol production, co-cultures often combine pentose-utilizing yeasts like *Pichia stipitis* and *Pichia fermentans* with *S. cerevisiae* to utilize both hexose and pentose sugars **[67]**, **[4]**.

In the present study, the overall results (Table 4) demonstrated that under the optimized conditions of an initial pH of 5.75, 3% yeast extract, 4% inoculum size (O.D600 1), 2.25 g/L cysteine, 0.6 g/L $ZnSO_4$, 0.29 g/L $MgSO_4$, and 0.3 g/L $MnSO_4$, along with treating the OP filtrate with active charcoal before fermentation, the bioethanol yield was increased to 87.6%. This represents a 4-fold increase compared to the PBD method and a 1.84-fold increase compared to RSM. Notably, this enhancement was achieved through the successive co-culture of *C. oleophila* and *K. marixanus*, which was applied for the first time in this study.

Similar approaches utilizing co-cultures have been explored in previous studies. For example, **[33]** produced bioethanol from apple pomace hydrolysate by co-culturing *Trichoderma harzianum*, *Aspergillus sojae*, and *S. cerevisiae*. They found that the optimal conditions for maximum bioethanol production included a 4% (v/v) inoculation rate of *S. cerevisiae* and 6% (w/v) inoculation rates of *T. harzianum* and *A. sojae*, along with vented aeration and 200 rpm agitation speed. Patle and Lal **[70]** reviewed various bioethanol-producing strains isolated from raw honey, molasses, grapes, and apple, and demonstrated that mixed cultures of *Zymomonas mobilis* and *Candida tropicalis* using these substrates were promising for bioethanol production. Wu et al. **[71]** reported a maximal ethanol concentration of 48.98 mg/mL and productivity of 2.23 g/L/h under optimal conditions of 5% *Kluyveromyces marxianus* K21 inoculum at 40 °C after 22 hours.

Furthermore, Sindhu et al. **[2]** highlighted the limitation of pentose fermentation by yeasts such as *Z. mobilis* and *S. cerevisiae*, which efficiently ferment glucose but cannot consume xylose. However, yeasts like

Scheffersomyces stipitis, *Pichia stipitis*, and *Candida shehatae* can consume xylose and produce bioethanol. This limitation can be overcome by using genetically modified yeast or a co-culture of two yeast strains. For example, co-culturing *S. cerevisiae* and *S. stipitis* using a rice husk hydrolysate containing pentose and hexose sugars resulted in an ethanol yield of 0.42 $g.g^{-1}$ **[2]**. Co-culturing *S. cerevisiae* with *A. niger* was also considered a cost-competitive method for simultaneous saccharification and fermentation, resulting in the production of 35.2 mg/L of bioethanol using potato waste as a substrate **[57]**, **[2]**.

Table 4 Optimization of bioethanol production from treated OP using *C. tropicalis* under all detected optimizedconditions[1].

Experiment	Treatment Conditions of OP	Reducing sugars (g/L)	Ethanol (mg/mL)	Ethanol Yield[2] (%)
Plackett-Burman Design (PBD)	pH of 6, yeast extract (3 g/L), cysteine (0.5 g/L), substrate volume of 30 mL, inoculum size (3.5%), and OD_{600} (0.9), kept at 30 °C for 2 days fermentation	**1.30**	**6.03**	**21.55**
Response surface methodology (RSM)	pH of 5.57, yeast extract (3 g/L, cysteine 2.25 g/L, substrate volume 85 mL, inoculum size 3.5%,) OD_{600} of 0.9, kept at 30 °C for 2 days fermentation.	**5.80**	**16.7**	**34.73**
Successive Co-Culture addition *Kluyveromyces marxianus* was incubated 3 days, frozen, then *C. tropicalis* was added and incubated for 3 days at 30 °C	Treated with 1 g/L active charcoal before fermentation for 2h. Then Addition of metals**;** $ZnSO_4$ (0.6 g/L), $MgSO_4$ (0.29 g/L) and $MnSO_4$ (0.3 g/L), yeast of 24 h age and similar optimized condition as in RSM for inoculum size, and microbial obtical density, pH, and incubation temperature.	**0.93** **1.12**	**2.44** **2.26** **4.70**	**38.11** **49.55** **87.66**

Note: [1]Values are means of triplicates, [2]ethanol yield calculated based on reducing sugar (reducing sugar/ethanol)*100.

CONCLUSION

In conclusion, this study explored bioethanol production from agro-industrial waste, specifically OP wastes, using selected microorganisms. The findings suggest that OP wastes, which have a high cellulose content, hold promise as a low-cost substrate for bioethanol production. Water/autoclaving pre-treatment was found to be the most effective, resulting in the lowest phenolic content and the highest sugar yields of 68.2%. *C. tropicalis* was investigated as a potential candidate for bioethanol production, and two reducing agents, cysteine and resazurin, were introduced for the first time. The addition of cysteine and resazurin increased bioethanol production by 2.9 and 2.2 times, respectively. By using a combination of Plackett-Burman design (PBD) and response surface methodology-central composite rotatable design (RSM-CCRD), the significant factors influencing bioethanol production were identified and optimized. The optimized conditions included an initial pH of 5.75, 3% yeast extract, 2.25 g/L cysteine, 4% inoculum size, 0.6 g/L $ZnSO_4$, 0.29 g/L $MgSO_4$, and 0.3 g/L $MnSO_4$. Under these conditions, a maximum bioethanol yield of 87.6% was achieved using successive co-culturing of *C. tropicalis* and *K. marxianum*, with an incubation period of 3 days at 30 °C. To the best of our knowledge, this is the first study to utilize both *C. tropicalis* and *K. marxianum* for bioethanol production from OP. These co-cultures demonstrated their effectiveness in bioethanol production. Overall, this study serves as an important initial step in utilizing OP wastes for bioethanol production, and it highlights the potential of co-cultures as an efficient approach in bioethanol production. Further research in this field can build upon these findings and contribute to the development of sustainable and cost-effective bioethanol production processes.

REFERENCES

1. John, I., Yaragarla, P., Muthaiah, P., Ponnusamy, K., & Appusamy, A. (2017). Statistical optimization of acid catalyzed steam pretreatment of citrus peel waste for bioethanol production. In Resource-Efficient Technologies (Vol. 3, Issue 4, pp. 429–433). National Research Tomsk Polytechnic University. https://doi.org/10.1016/j.reffit.2017.04.001
2. Sindhu, R., Gnansounou, E., Rebello, S., Binod, P., Varjani, S., Thakur, I. S., Nair, R. B., & Pandey, A. (2019). Conversion of food and kitchen waste to value-added products. In Journal of Environmental Management (Vol. 241, pp. 619–630). Elsevier BV. https://doi.org/10.1016/j.jenvman.2019.02.053
3. Tan, K. T., Lee, K. T., & Mohamed, A. R. (2008). Role of energy policy in renewable energy accomplishment: The case of second-generation bioethanol. In Energy Policy (Vol. 36, Issue 9, pp. 3360–3365). Elsevier BV. https://doi.org/10.1016/j.enpol.2008.05.016
4. Mohd Azhar, S. H., Abdulla, R., Jambo, S. A., Marbawi, H., Gansau, J. A., Mohd Faik, A. A., & Rodrigues, K. F. (2017). Yeasts in sustainable bioethanol production: A review. In Biochemistry and Biophysics Reports (Vol. 10, pp. 52–61). Elsevier BV. https://doi.org/10.1016/j.bbrep.2017.03.003
5. Oberoi, H. S., Vadlani, P. V., Madl, R. L., Saida, L., & Abeykoon, J. P. (2010). Ethanol Production from Orange Peels: Two-Stage Hydrolysis and Fermentation Studies Using Optimized Parameters through Experimental Design. In Journal of Agricultural and Food Chemistry (Vol. 58, Issue 6, pp. 3422–3429). American Chemical Society (ACS). https://doi.org/10.1021/jf903163t
6. Sharma, K., Mahato, N., Cho, M. H., & Lee, Y. R. (2017). Converting citrus wastes into value-added products: Economic and environmently friendly approaches. In Nutrition (Vol. 34, pp. 29–46). Elsevier BV. https://doi.org/10.1016/j.nut.2016.09.006
7. Liu, Z.-H., Qin, L., Li, B.-Z., & Yuan, Y.-J. (2014). Physical and Chemical Characterizations of Corn Stover from Leading Pretreatment Methods and Effects on Enzymatic Hydrolysis. In ACS Sustainable Chemistry & Engineering (Vol. 3, Issue 1, pp. 140–146). American Chemical Society (ACS). https://doi.org/10.1021/sc500637c
8. Tiwari, S., Jadhav, S. K., & Tiwari, K. L. (2015). Bioethanol production from rice bran with optimization of parameters by Bacillus cereus strain McR-3. In International Journal of Environmental Science and Technology (Vol. 12, Issue 12, pp. 3819–3826). Springer Science and Business Media LLC. https://doi.org/10.1007/s13762-014-0746-1
9. Satyanarayana, T., & Kunze, G. (Eds.). (2009). Yeast Biotechnology: Diversity and Applications. Springer Netherlands. https://doi.org/10.1007/978-1-4020-8292-4
10. Liu, L., Zhang, Z., Wang, J., Fan, Y., Shi, W., Liu, X., & Shun, Q. (2019). Simultaneous saccharification and co-fermentation of corn stover pretreated by H2O2 oxidative degradation for ethanol production. In Energy (Vol. 168, pp. 946–952). Elsevier BV. https://doi.org/10.1016/j.energy.2018.11.132
11. Nemeth, M. A. (2003). Response Surface Methodology: Process and Product Optimization Using Designed Experiments, 2nd Edition. In Journal of Quality Technology (Vol. 35, Issue 4, pp. 428–429). Informa UK Limited. https://doi.org/10.1080/00224065.2003.11980243
12. Nuanpeng, S., Thanonkeo, S., Klanrit, P., & Thanonkeo, P. (2018). Ethanol production from sweet sorghum by Saccharomyces cerevisiae DBKKUY-53 immobilized on alginate-loofah matrices. In Brazilian Journal of Microbiology (Vol. 49, pp. 140–150). Springer Science and Business Media LLC. https://doi.org/10.1016/j.bjm.2017.12.011
13. Mäkelä, M. (2017). Experimental design and response surface methodology in energy applications: A tutorial review. In Energy Conversion and Management (Vol. 151, pp. 630–640). Elsevier BV. https://doi.org/10.1016/j.enconman.2017.09.021
14. Wang, Y., Qiu, L., Zhang, T., Yang, X., & Kang, K. (2019). Optimization of Carbonization Process for the Production of Solid Biofuel from Corn Stalk Using Response Surface Methodology. In BioEnergy Research (Vol. 12, Issue 1, pp. 184–196). Springer Science and Business Media LLC. https://doi.org/10.1007/s12155-018-9955-7

15. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. L. A. P. (2008). Determination of structural carbohydrates and lignin in biomass. In Laboratory analytical procedure (Vol. 1617, Issue 1, pp. 1–16). National Renewable Energy Laboratory.
16. Paez, V., Barrett, W. B., Deng, X., Diaz-Amigo, C., Fiedler, K., Fuerer, C., Hostetler, G. L., Johnson, P., Joseph, G., Konings, E. J. M., Lacorn, M., Lawry, J., Liu, H., Marceau, E., Mastovska, K., Monteroso, L., Pan, S.-J., Parker, C., Phillips, M. M., … Coates, S. G. (2016). AOAC SMPR® 2016.002. In Journal of AOAC INTERNATIONAL (Vol. 99, Issue 4, pp. 1122–1124). Oxford University Press (OUP). https://doi.org/10.5740/jaoacint.smpr2016.002
17. Cypriano, D. Z., da Silva, L. L., & Tasic, L. (2018). High value-added products from the orange juice industry waste. In Waste Management (Vol. 79, pp. 71–78). Elsevier BV. https://doi.org/10.1016/j.wasman.2018.07.028
18. Zabed, H., Sahu, J. N., Boyce, A. N., & Faruq, G. (2016). Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. In Renewable and Sustainable Energy Reviews (Vol. 66, pp. 751–774). Elsevier BV. https://doi.org/10.1016/j.rser.2016.08.038
19. Kang, K. E., Jeong, G.-T., Sunwoo, C., & Park, D.-H. (2011). Pretreatment of rapeseed straw by soaking in aqueous ammonia. In Bioprocess and Biosystems Engineering (Vol. 35, Issues 1–2, pp. 77–84). Springer Science and Business Media LLC. https://doi.org/10.1007/s00449-011-0606-z
20. Negrulescu, A., Patrulea, V., Mincea, M. M., Ionascu, C., Vlad-Oros, B. A., & Ostafe, V. (2012). Adapting the reducing sugars method with dinitrosalicylic acid to microtiter plates and microwave heating. In Journal of the Brazilian Chemical Society (Vol. 23, Issue 12, pp. 2176–2182). FapUNIFESP (SciELO). https://doi.org/10.1590/s0103-50532013005000003
21. El-Tayeb, T. S., Abdelhafez, A. A., Ali, S. H., & Ramadan, E. M. (2012). Effect of acid hydrolysis and fungal biotreatment on agro-industrial wastes for obtainment of free sugars for bioethanol production. In Brazilian Journal of Microbiology (Vol. 43, Issue 4, pp. 1523–1535). FapUNIFESP (SciELO). https://doi.org/10.1590/s1517-83822012000400037
22. Fakruddin, Md. (2013). Characterization of Stress Tolerant High Potential Ethanol Producing Yeast from Agro-Industrial Waste. In American Journal of BioScience (Vol. 1, Issue 2, p. 24). Science Publishing Group. https://doi.org/10.11648/j.ajbio.20130102.11
23. Oiwoh, O., Ayodele, B. V., Amenaghawon, N. A., & Okieimen, C. O. (2018). Optimization of bioethanol production from simultaneous saccharification and fermentation of pineapple peels using <i>Saccharomyces cerevisiae</i> In Journal of Applied Sciences and Environmental Management (Vol. 21, Issue 7, p. 1256). African Journals Online (AJOL). https://doi.org/10.4314/jasem.v21i7.5
24. Anschau, A., Santos, L. O. dos, & Alegre, R. M. (2013). A cost effective fermentative production of glutathione by Saccharomyces cerevisiae with cane molasses and glycerol. In Brazilian Archives of Biology and Technology (Vol. 56, Issue 5, pp. 849–857). FapUNIFESP (SciELO). https://doi.org/10.1590/s1516-89132013000500017
25. Yong, X., Raza, W., Yu, G., Ran, W., Shen, Q., & Yang, X. (2011). Optimization of the production of poly-γ-glutamic acid by Bacillus amyloliquefaciens C1 in solid-state fermentation using dairy manure compost and monosodium glutamate production residues as basic substrates. In Bioresource Technology (Vol. 102, Issue 16, pp. 7548–7554). Elsevier BV. https://doi.org/10.1016/j.biortech.2011.05.057
26. Le Man, H., Behera, S. K., & Park, H. S. (2009). Optimization of operational parameters for ethanol production from Korean food waste leachate. In International Journal of Environmental Science & Technology (Vol. 7, Issue 1, pp. 157–164). Springer Science and Business Media LLC. https://doi.org/10.1007/bf03326127
27. Palukurty, M. A., Telgana, N. K., Bora, H. S. R., & Mulampaka, S. N. (2008). Screening and optimization of metal ions to enhance ethanol production using statistical experimental designs. In African Journal of Microbiology Research (Vol. 2, Issue 4, pp. 87–94). Academic Journals.

28. Ma, Y., Gao, N., Chu, W., & Li, C. (2013). Removal of phenol by powdered activated carbon adsorption. In Frontiers of Environmental Science & Engineering (Vol. 7, Issue 2, pp. 158–165). Springer Science and Business Media LLC. https://doi.org/10.1007/s11783-012-0479-7

29. Tan, M., Ma, L., Rehman, M. S. U., Ahmed, M. A., Sajid, M., Xu, X., Sun, Y., Cui, P., & Xu, J. (2019). Screening of acidic and alkaline pretreatments for walnut shell and corn stover biorefining using two way heterogeneity evaluation. In Renewable Energy (Vol. 132, pp. 950–958). Elsevier BV. https://doi.org/10.1016/j.renene.2018.07.131

30. Chen, M., Xia, L., & Xue, P. (2007). Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. In International Biodeterioration & Biodegradation (Vol. 59, Issue 2, pp. 85–89). Elsevier BV. https://doi.org/10.1016/j.ibiod.2006.07.011

31. Blainski, A., Lopes, G., & de Mello, J. (2013). Application and Analysis of the Folin Ciocalteu Method for the Determination of the Total Phenolic Content from Limonium Brasiliense L. In Molecules (Vol. 18, Issue 6, pp. 6852–6865). MDPI AG. https://doi.org/10.3390/molecules18066852

32. Mushimiyimana, I., & Tallapragada, P. (2017). Bioethanol production from agro wastes by acid hydrolysis and fermentation process. In Journal of Scientific & Industrial Research (Vol. 75, pp. 383–388). CSIR-NIScPR.

33. Evcan, E., & Tari, C. (2015). Production of bioethanol from apple pomace by using cocultures: Conversion of agro-industrial waste to value added product. In Energy (Vol. 88, pp. 775–782). Elsevier BV. https://doi.org/10.1016/j.energy.2015.05.090

34. Sun, S., Sun, S., Cao, X., & Sun, R. (2016). The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. In Bioresource Technology (Vol. 199, pp. 49–58). Elsevier BV. https://doi.org/10.1016/j.biortech.2015.08.061

35. Raud, M., Tutt, M., Olt, J., & Kikas, T. (2016). Dependence of the hydrolysis efficiency on the lignin content in lignocellulosic material. In International Journal of Hydrogen Energy (Vol. 41, Issue 37, pp. 16338–16343). Elsevier BV. https://doi.org/10.1016/j.ijhydene.2016.03.190

36. Sarkar, N., Ghosh, S. K., Bannerjee, S., & Aikat, K. (2012). Bioethanol production from agricultural wastes: An overview. In Renewable Energy (Vol. 37, Issue 1, pp. 19–27). Elsevier BV. https://doi.org/10.1016/j.renene.2011.06.045

37. Baruah, J., Nath, B. K., Sharma, R., Kumar, S., Deka, R. C., Baruah, D. C., & Kalita, E. (2018). Recent Trends in the Pretreatment of Lignocellulosic Biomass for Value-Added Products. In Frontiers in Energy Research (Vol. 6). Frontiers Media SA. https://doi.org/10.3389/fenrg.2018.00141

38. Yu, G., Yano, S., Inoue, H., Inoue, S., Endo, T., & Sawayama, S. (2009). Pretreatment of Rice Straw by a Hot-Compressed Water Process for Enzymatic Hydrolysis. In Applied Biochemistry and Biotechnology (Vol. 160, Issue 2, pp. 539–551). Springer Science and Business Media LLC. https://doi.org/10.1007/s12010-008-8420-z

39. de la Torre, I., Ravelo, M., Segarra, S., Tortajada, M., Santos, V. E., & Ladero, M. (2017). Study on the effects of several operational variables on the enzymatic batch saccharification of orange solid waste. In Bioresource Technology (Vol. 245, pp. 906–915). Elsevier BV. https://doi.org/10.1016/j.biortech.2017.08.094

40. Li, W.-C., Li, X., Zhu, J.-Q., Qin, L., Li, B.-Z., & Yuan, Y.-J. (2018). Improving xylose utilization and ethanol production from dry dilute acid pretreated corn stover by two-step and fed-batch fermentation. In Energy (Vol. 157, pp. 877–885). Elsevier BV. https://doi.org/10.1016/j.energy.2018.06.002

41. Xu, H., Zhao, N., Yao, H., Qin, H., Zeng, J., Ran, Y., Yang, Y., Qiao, D., & Cao, Y. (2019). Lipid production from corn stover by a cost-efficient system featuring ammonium carbonate-steam explosion and recirculating enzymatic hydrolysis. In Biomass and Bioenergy (Vol. 120, pp. 387–395). Elsevier BV. https://doi.org/10.1016/j.biombioe.2018.11.020

42. Kazemi Shariat Panahi, H., Dehhaghi, M., Aghbashlo, M., Karimi, K., & Tabatabaei, M. (2020). Conversion of residues from agro-food industry into bioethanol in Iran: An under-valued biofuel additive to phase out MTBE in gasoline. In Renewable Energy (Vol. 145, pp. 699–710). Elsevier BV. https://doi.org/10.1016/j.renene.2019.06.081

43. Wakai, S., Nakashima, N., Ogino, C., Tsutsumi, H., Hata, Y., & Kondo, A. (2019). Modified expression of multi-cellulases in a filamentous fungus Aspergillus oryzae. In Bioresource Technology (Vol. 276, pp. 146–153). Elsevier BV. https://doi.org/10.1016/j.biortech.2018.12.117

44. Abd Razak, D. L., Abd Rashid, N. Y., Jamaluddin, A., Sharifudin, S. A., Abd Kahar, A., & Long, K. (2017). Cosmeceutical potentials and bioactive compounds of rice bran fermented with single and mix culture of Aspergillus oryzae and Rhizopus oryzae. In Journal of the Saudi Society of Agricultural Sciences (Vol. 16, Issue 2, pp. 127–134). Elsevier BV. https://doi.org/10.1016/j.jssas.2015.04.001

45. Londoño-Hernández, L., Ramírez-Toro, C., Ruiz, H. A., Ascacio-Valdés, J. A., Aguilar-Gonzalez, M. A., Rodríguez-Herrera, R., & Aguilar, C. N. (2017). Rhizopus oryzae – Ancient microbial resource with importance in modern food industry. In International Journal of Food Microbiology (Vol. 257, pp. 110–127). Elsevier BV. https://doi.org/10.1016/j.ijfoodmicro.2017.06.012

46. Naga Padma, P., Anuradha, K., & Reddy, G. (2011). Pectinolytic yeast isolates for cold-active polygalacturonase production. In Innovative Food Science & Emerging Technologies (Vol. 12, Issue 2, pp. 178–181). Elsevier BV. https://doi.org/10.1016/j.ifset.2011.02.001

47. Almeida D., Soares Da Silva R.D.C., Brasileiro P., Luna J., Silva M.D.G., Rufino R., Costa A., Dos Santos V. A., & Sarubbo L. (2018). Application of a biosurfactant from candida tropicalis ucp 0996 produced in low-cost substrates for hydrophobic contaminants removal. In Chemical Engineering Transactions (Vol. 64, pp. 541–546). AIDIC-Italian Association of Chemical Engineering. https://doi.org/10.3303/CET1864091

48. El Baz, A. F. (2011). Kinetic behavior of Candida tropicalis during xylitol production using semi-synthetic and hydrolysate based media. In African Journal of Biotechnology (Vol. 10, Issue 73). Academic Journals. https://doi.org/10.5897/ajb11.1766

49. Spohner, S. C., Schaum, V., Quitmann, H., & Czermak, P. (2016). Kluyveromyces lactis: An emerging tool in biotechnology. In Journal of Biotechnology (Vol. 222, pp. 104–116). Elsevier BV. https://doi.org/10.1016/j.jbiotec.2016.02.023

50. Shariq, M., & Sohail, M. (2019). Application of Candida tropicalis MK-160 for the production of xylanase and ethanol. In Journal of King Saud University - Science (Vol. 31, Issue 4, pp. 1189–1194). Elsevier BV. https://doi.org/10.1016/j.jksus.2018.04.009

51. Nguyen, N. H., Suh, S.-O., Marshall, C. J., & Blackwell, M. (2006). Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, Spathaspora passalidarum gen. sp. nov. and Candida jeffriesii sp. nov. In Mycological Research (Vol. 110, Issue 10, pp. 1232–1241). Elsevier BV. https://doi.org/10.1016/j.mycres.2006.07.002

52. Zuza-Alves, D. L., Silva-Rocha, W. P., & Chaves, G. M. (2017). An Update on Candida tropicalis Based on Basic and Clinical Approaches. In Frontiers in Microbiology (Vol. 8). Frontiers Media SA. https://doi.org/10.3389/fmicb.2017.01927

53. Akyilmaz, E., Guvenc, C., & Koylu, H. (2020). A novel mıcrobıal bıosensor system based on C. tropicalis yeast cells for selectıve determınatıon of L-Ascorbıc acid. In Bioelectrochemistry (Vol. 132, p. 107420). Elsevier BV. https://doi.org/10.1016/j.bioelechem.2019.107420

54. Wu, C., & Tu, X. (2016). Biological and fermentative conversion of syngas. In Handbook of Biofuels Production (pp. 335–357). Elsevier. https://doi.org/10.1016/b978-0-08-100455-5.00012-6

55. Alriksson, B., Cavka, A., & Jönsson, L. J. (2011). Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical in-situ detoxification with reducing agents. In Bioresource Technology (Vol. 102, Issue 2, pp. 1254–1263). Elsevier BV. https://doi.org/10.1016/j.biortech.2010.08.037

56. Hossain, T., Miah, A. B., Mahmud, S. A., & Mahin, A.-A.-. (2018). Enhanced Bioethanol Production from Potato Peel Waste Via Consolidated Bioprocessing with Statistically Optimized Medium. In Applied Biochemistry and Biotechnology (Vol. 186, Issue 2, pp. 425–442). Springer Science and Business Media LLC. https://doi.org/10.1007/s12010-018-2747-x

57. Izmirlioglu, G., & Demirci, A. (2015). Enhanced Bio-Ethanol Production from Industrial Potato Waste by Statistical Medium Optimization. In International Journal of Molecular Sciences (Vol. 16, Issue 10, pp. 24490–24505). MDPI AG. https://doi.org/10.3390/ijms161024490

58. Yu, X., Guo, N., Chi, Z., Gong, F., Sheng, J., & Chi, Z. (2009). Inulinase overproduction by a mutant of the marine yeast Pichia guilliermondii using surface response methodology and inulin hydrolysis. In Biochemical Engineering Journal (Vol. 43, Issue 3, pp. 266–271). Elsevier BV. https://doi.org/10.1016/j.bej.2008.10.018

59. Zabed, H., Faruq, G., Sahu, J. N., Azirun, M. S., Hashim, R., & Nasrulhaq Boyce, A. (2014). Bioethanol Production from Fermentable Sugar Juice. In The Scientific World Journal (Vol. 2014, pp. 1–11). Hindawi Limited. https://doi.org/10.1155/2014/957102

60. Raja Sathendra, E., Baskar, G., Praveenkumar, R., & Gnansounou, E. (2019). Bioethanol production from palm wood using Trichoderma reesei and Kluveromyces marxianus. In Bioresource Technology (Vol. 271, pp. 345–352). Elsevier BV. https://doi.org/10.1016/j.biortech.2018.09.134

61. Ezhumalai, S., & Thangavelu, V. (2010). Kinetic and optimization studies on the bioconversion of lignocellulosic material into ethanol. In BioResources (Vol. 5, Issue 3, pp. 1879–1894). BioResources. https://doi.org/10.15376/biores.5.3.1879-1894

62. Sharma, N., Kalra, K. L., Oberoi, H. S., & Bansal, S. (2007). Optimization of fermentation parameters for production of ethanol from kinnow waste and banana peels by simultaneous saccharification and fermentation. In Indian Journal of Microbiology (Vol. 47, Issue 4, pp. 310–316). Springer Science and Business Media LLC. https://doi.org/10.1007/s12088-007-0057-z

63. Jambo, S. A., Abdulla, R., Marbawi, H., & Gansau, J. A. (2019). Response surface optimization of bioethanol production from third generation feedstock - Eucheuma cottonii. In Renewable Energy (Vol. 132, pp. 1–10). Elsevier BV. https://doi.org/10.1016/j.renene.2018.07.133

64. Sininart Chongkhong. (2017). Response surface optimization of ethanol productionfrom banana peels by organic acid hydrolysis and fermentation. Songklanakarin Journal of Science and Technology (SJST), 39, 2. https://doi.org/10.14456/SJST-PSU.2017.28

65. Hashem, M., Alamri, S. A., Alrumman, S. A., & Qahtani, M. S. A. A.-. (2015). Enhancement of Bio-Ethanol Production from Date Molasses by Non-Conventional Yeasts. In Research Journal of Microbiology (Vol. 10, Issue 3, pp. 114–125). Science Alert. https://doi.org/10.3923/jm.2015.114.125

66. Lee, S. C., & Park, S. (2016). Removal of furan and phenolic compounds from simulated biomass hydrolysates by batch adsorption and continuous fixed-bed column adsorption methods. In Bioresource Technology (Vol. 216, pp. 661–668). Elsevier BV. https://doi.org/10.1016/j.biortech.2016.06.007

67. Singh, A., Bajar, S., & Bishnoi, N. R. (2014). Enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by Saccharomyces cerevisiae, Scheffersomyces stipitis and their co-culture. In Fuel (Vol. 116, pp. 699–702). Elsevier BV. https://doi.org/10.1016/j.fuel.2013.08.072

68. Zhang, Y., Xia, C., Lu, M., & Tu, M. (2018). Effect of overliming and activated carbon detoxification on inhibitors removal and butanol fermentation of poplar prehydrolysates. In Biotechnology for Biofuels (Vol. 11, Issue 1). Springer Science and Business Media LLC. https://doi.org/10.1186/s13068-018-1182-0

69. Nuwamanya, E., Chiwona-Karltun, L., Kawuki, R. S., & Baguma, Y. (2011). Bio-Ethanol Production from Non-Food Parts of Cassava (Manihot esculenta Crantz). In AMBIO (Vol. 41, Issue 3, pp. 262–270). Springer Science and Business Media LLC. https://doi.org/10.1007/s13280-011-0183-z

70. Patle, S., & Lal, B. (2007). Ethanol production from hydrolysed agricultural wastes using mixed culture of Zymomonas mobilis and Candida tropicalis. In Biotechnology Letters (Vol. 29, Issue 12, pp. 1839–1843). Springer Science and Business Media LLC. https://doi.org/10.1007/s10529-007-9493-4

71. Wu, W.-H., Hung, W.-C., Lo, K.-Y., Chen, Y.-H., Wan, H.-P., & Cheng, K.-C. (2016). Bioethanol production from taro waste using thermo-tolerant yeast Kluyveromyces marxianus K21. In Bioresource Technology (Vol. 201, pp. 27–32). Elsevier BV. https://doi.org/10.1016/j.biortech.2015.11.015

72. Sudhakar, D. V., & Maini, S. B. (2000). Isolation and characterization of mango peel pectins. In Journal of Food Processing and Preservation (Vol. 24, Issue 3, pp. 209–227). Hindawi Limited. https://doi.org/10.1111/j.1745-4549.2000.tb00414.x

73. Plackett, R. L., & Burman, J. P. (1946). The design of optimum multifactorial experiments. In Biometrika (Vol. 33, Issue 4, pp. 305–325). Oxford University Press (OUP). https://doi.org/10.1093/biomet/33.4.305

74. Pan, X., Gilkes, N., & Saddler, J. N. (2006). Effect of acetyl groups on enzymatic hydrolysis of cellulosic substrates. In Holzforschung (Vol. 60, Issue 4, pp. 398–401). Walter de Gruyter GmbH. https://doi.org/10.1515/hf.2006.062

75. El Sayed, W. M. M., & Ibrahim, H. A. H. (2016). Evaluation of Bioethanol Production from Ulva lactuca By Saccharomyces cerevisiae. In Journal of Biotechnology & Biomaterials (Vol. 6, Issue 2). OMICS Publishing Group. https://doi.org/10.4172/2155-952x.1000226

76. Deesuth, O., Laopaiboon, P., Jaisil, P., & Laopaiboon, L. (2012). Optimization of Nitrogen and Metal Ions Supplementation for Very High Gravity Bioethanol Fermentation from Sweet Sorghum Juice Using an Orthogonal Array Design. In Energies (Vol. 5, Issue 9, pp. 3178–3197). MDPI AG. https://doi.org/10.3390/en5093178

77. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. In Analytical Chemistry (Vol. 31, Issue 3, pp. 426–428). American Chemical Society (ACS). https://doi.org/10.1021/ac60147a030

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Contact Address:

Noha Sorour, University of Sadat City, Genetic Engineering and Biotechnology Research Institute, Department of Industrial Biotechnology, Egypt,
Tel.: 01229703235
E-mail: noha.sorour@mail.mcgill.ca
ORCID: https://orcid.org/0000-0002-6117-7980

***Saqer Herzallah**, Mutah University, Faculty of Agriculture, Department of Nutrition and Food Tech. Karak, Jordan,
Tel.: +962-785103354
E-mail: saqmhy@mutah.edu.jo
ORCID: https://orcid.org/0000-0003-1171-4271

Nazieh Alkhalaileh, Mutah University, Faculty of Agriculture, Department of Nutrition and Food Tech. Karak, Jordan,
Tel.: +962-078661531547
E-mail: nazieh@mutah.edu.jo
ORCID: https://orcid.org/0000-0001-6081-0468

Amer Mamkagh, Mutah University, Faculty of Agriculture, Department of Plant Production. Karak, Jordan,
Tel.: +962-79579-6414
E-mail: amer_mam@mutah.edu.jo
ORCID: https://orcid.org/0000-0001-9353-2345

Ashraf El-Baz, University of Sadat City, Genetic Engineering and Biotechnology Research Institute Department of Industrial Biotechnology, Egypt,
Tel.: 01229703235
E-mail: ashrafhaase@gmail.com
ORCID: https://orcid.org/0000-0002-8772-7194

Esraa Shalaby, University of Sadat City, Genetic Engineering and Biotechnology Research Institute Department of Industrial Biotechnology, Egypt,
Tel.: 01229703235
E-mail: esraaosama808@yahoo.com
ORCID: https://orcid.org/0000-0002-3912-3281

Hani Dmoor, Albalqa Applied University, Faculty of Agriculture, Department of Nutrition and Food Processing Alsalt, Jordan,
Tel.: +962-79649-3086
E-mail: Dmour@bau.edu.jo
ORCID: https://orcid.org/0000-0002-8837-9806

Rateb Abbas, University of Sadat City, Genetic Engineering and Biotechnology Research Institute Department of Microbial Biotechnology, Egypt,
Tel.: 01229703235
E-mail: rateb.youssef@gebri.usc.edu.eg
ORCID: https://orcid.org/0000-0002-7182-4803

Corresponding author: *