COMPARATIVE ASSESSMENT OF SEPARATE *VS*. SIMULTANEOUS SACCHARIFICATION AND FERMENTATION USING *CANDIDA TROPICALIS–SACCHAROMYCES CEREVISIAE* CO-CULTURE FOR 2G BIOETHANOL PRODUCTION FROM MUNICIPAL SOLID WASTE

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This study investigates and compares two bioconversion strategies, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), employing a novel co-culture system of *Saccharomyces cerevisiae* and thermotolerant *Candida tropicalis* for efficient fermentation of dilute acid-pretreated municipal solid waste. In the SHF process, enzymatic hydrolysis was performed at 50 °C using an optimized enzyme cocktail comprising β-glucosidase, endoglucanase, and total cellulase. Subsequent fermentation resulted in a peak ethanol concentration of 29 g/L at 72 h. In contrast, the SSF process where enzymatic saccharification and microbial fermentation occurred simultaneously at 35 °C achieved an ethanol concentration of 32 g/L under similar conditions, demonstrating enhanced process synergy. Optimization of the SSF parameters using Box–Behnken Design (BBD) further improved ethanol titer to 35.134 g/L at 12% substrate concentration, 35.24 °C, and 72 h, with a desirability score of 0.954. The statistical model exhibited strong predictive capability ($R^2 = 0.984$, $p < 0.0001$), confirming the reliability of the optimized conditions.

Keywords: Box Behnken Design, bioethanol, enzyme cocktail, microbes, response surface methodology, waste-to-energy

INTRODUCTION

Fossil fuels currently supply 80% of the globe's energy needs, raising significant concerns about energy security and environment impact.[1] In response, researchers are pioneering innovative renewable energy sources to address these critical issues.[2] Bioethanol production from lignocellulosic biomass is a promising renewable energy source and the estimated natural output of this biomass is about 200 billion tons annually.[3] Lignocellulosic biomass, consisting primarily of lignin, cellulose, and hemicelluloses,[4,5] requires pretreatments to disrupt the strong bonds within their structures for effective utilization.[6] Due to their recalcitrant nature, their chemical structures are not easily biodegradable by microbes, necessitating such pretreatment to make the biomass more accessible for microbial breakdown and application.

Numerous pretreatment methods, including physical,[7] physicochemical,[8] chemical,[9] and

biological,[10] have been reported to achieve pretreatment.[11] After pretreatment, enzymatic saccharification is a critical step that converts the liberated cellulose and hemicelluloses into fermentable simple sugars.[12] This process requires the synergistic action of specific enzymes like cellulases and hemicellulases.[13] Subsequently, a variety of microorganisms can ferment the resulting hydrolysates to produce bioethanol.[14,15] Fermentation can be executed using several approaches such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).[16] SHF allows each process to occur in the separate reactors under optimized conditions.[17] On the other hand, SSF integrates both processes within a single reactor,[18] offering significant advantages over other methods by producing higher concentrations and yields of bioethanol in shorter fermentation durations. SSF has gained significant popularity owing to its cost-effectiveness and high product yield.[19,20]

Besides these benefits, the application of SSF is hampered by the limitation that temperature, which is optimal for the enzymes involved in the hydrolysis process (40-50 °C), does not match the growth range of institutional yeasts such as *Saccharomyces cerevisiae* (optimal 30-35 °C, tolerance up to 40 °C in some strains).[19,21] Thermotolerant strains of *S. cerevisiae* have also been developed and are commercially available, but those can give mixed results due to strain-specific differences, and may not perform as well under commercial scaling.[22] The best solution to this problem would be to introduce the idea of microbial co-culture systems by using yeasts with complementary physiological and metabolic characteristics. The temperature-tolerant yeast, *Candida tropicalis*, has demonstrated good growth at 35-40 °C as well as tolerance to various substrate fermentation inhibitors and has emerged as a promising bioethanol production candidate in SSF processes.[23-25]

Co-cultivation of *S. cerevisiae* and *C. tropicalis* has been suggested as a synergistic approach to enhance ethanol production, expand the type of substances that can be utilized, and alleviate inhibition.[26] In a study, eight robust microorganisms (*Escherichia coli*, *Clostridium saccharoperbutylacetonicum*, *Rhodococcus opacus*, *Geobacillus thermoglucosidasius*, *Pseudomonas putida*, *Zymomonas mobilis*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) were used to produce bioethanol from organic municipal solid waste (MSW).[27] Sovorawet *et al.* compared the SHF and SSF processes for bioethanol production using co-culture of *Candida tropicalis* and *Saccharomyces cerevisiae* by using cassava stalks as a substrate.[28] The study concluded that the fermentation time of SSH was (≈56 h) longer than that of the SSF (24–32 h), but no significant difference was observed in the bioethanol produced. The effectiveness of such co-cultures has been shown in hydrolysates fermentation of agricultural residues, such as rice husk and cassava stalks. The major limitation is that there is still a sizeable knowledge gap in the implementation of co-culture systems on defining more diverse and underutilized sources of biomass, *i.e.* municipal solid waste (MSW).

MSW is commonly misunderstood as non-lignocellulosic material because it is collected domestically, commercially and institutionally. Nevertheless, most MSW, especially the organic and biodegradable component, consists of lignocellulosic materials that include paper, cardboard, yard waste, food waste, textiles, and wood waste fragments.[29] The organic part of MSW (OFMSW) has been depicted as a probable feedstock with the capacity to satisfy the generation of 2G bioethanol, particularly in those regions with considerable waste management issues and energy insecurity. Developed countries and developing ones generate 1.3 billion tons of MSW per year, and it will grow significantly because of further urbanization and the increase of the population rate. In Pakistan especially, MSW generation was about 49.6 million tons in 2024 and there is poor infrastructure to separate and recycle waste and valorize it.[30,31] The existing MSW disposal activities like open dumping and incineration are extremely detrimental to community health, causing air, water and soil pollution.[32-34]

Thus, the process of bioethanol production out of MSW has a two-fold advantage: waste reduction and the possibility to produce renewable energy. To date, an increasing number of researchers have adopted the SHF or SSF approach in bioconversion of MSW or its

fractions.[35-38] Ebrahimian *et al.*[37] proved the production of bioethanol from the organic part of MSW using SHF, whereas Nwobi *et al*. evaluated SSF on mild heat pretreated domestic waste.[39] There is limited information on comparing the SHF and SSF processes using co-cultures of thermotolerant yeasts to work on MSW-derived substrates. In addition, the importance of process optimization in improving SSF performance has not been sufficiently examined by previous literature using statistical modeling tools like the Response Surface Methodology (RSM) and Box-Behnken Design (BBD).[40]

To fulfill these research gaps, the current study will conduct a comparative analysis of SHF and SSF in the production of bioethanol on acid-pretreated MSW using a combination of *Saccharomyces cerevisiae* and *Candida tropicalis*, and to optimize key SSF parameters, including temperature, substrate concentration and fermentation time using BBD to maximize bioethanol yield. This work, to the best of our knowledge, presents the first effort to integrate microbial co-culture with statistically optimized SSF to utilize lignocellulosic-rich MSW. Besides offering a sustainable solution to the urban organic waste issue, the proposed approach will also address the development of scalable, cost-effective, and environmentally friendly 2G biofuel technologies.

EXPERIMENTAL

Raw material and reagents

The raw material (MSW) was collected from the Lakhodair landfill site, Lahore, Pakistan (74°23'08.57" East and 31°36'39.90" North). The sample was prepared by separating the biodegradable fraction[41] (vegetable and fruit waste, spoiled food, textile waste, yard waste, wood chips, cardboard and paper waste) from the non-biodegradable fraction[42] (polythene bags, plastic, glass pieces, and metal) manually, then ground, followed by drying as described earlier.[43] Table 1 presents the characteristics of MSW before and after drying. Yeast extract, peptone, dextrose, xylose, ammonium sulfate, potassium dihydrogen phosphate, and calcium chloride used in this study were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO, USA).

Enzyme cocktail

In this research, the same wheat bran (WB) enzyme cocktail was used for both SHF and SSF processes. The enzyme cocktail contained endoglucanase, β-glucosidase, and total cellulase activities as described earlier.[44] For both processes, enzyme loading rates were maintained at 6.43 U of endoglucanase, 22.76 U of β-glucosidase, and 3.01 FPU of total cellulase per gram of substrate, ensuring consistency across the processes.

Yeast inoculum

In this study, *Saccharomyces cerevisiae* (baker's yeast) was used for the fermentation of hydrolysate for bioethanol production, and it was acquired from a local market. It was activated in yeast extract peptone dextrose (YPD) medium, which was prepared in an Erlenmeyer flask containing peptone (20.0 g/L), yeast extract (10.0 g/L), and dextrose (20.0 g/L), and autoclaved for sterilization at 121 °C, 15 psi for 15 minutes. The activated yeast was then transferred to a Petri dish containing the sterilized YPD medium and incubated at 30 °C for 24 hours in a laboratory incubator (Wise cube WON-105). After incubation, colonies of *Saccharomyces cerevisiae* were sampled by scraping the surface with an inoculation loop and transferring it to 10 mL of phosphate buffer solution. Then it used as inoculum in the fermentation process.[45]

Candida tropicalis was acquired from the University of Punjab fungal bank, Lahore Pakistan, and used for the fermentation process as a thermotolerant yeast. It was grown on a sterilized medium containing peptone (20 g/L), yeast extract (10 g/L), and xylose (20 g/L) in Petri dishes. Briefly, the microorganism was inoculated in a Petri dish containing 50 mL sterilized medium and then incubated for 24 h at 30 °C.[46] The freshly grown culture was used by scraping the top of five cultured Petri dishes using an inoculation loop and inoculated in a fermentation medium for bioethanol production.

Pretreatment

The sample was chemically pretreated under optimized conditions (3 days and 5% acid concentration) using a toilet cleaner, acidic in nature (A_{TC}) (30% HCl, 10% $C_{11}H_{24}$ (LABSA-96%), 5% $C_6H_8O_7$, and 55% water) as described earlier.[43] Briefly, 5 g of sample was taken into a 100 mL Erlenmeyer flask with 15 mL of 5% (vol/vol) A_{TC} with a 1:3 solid-to-liquid ratio. The flask was then allowed to stand at room temperature for three days. After the pretreatment processes, the sample was filtered using Whatman-42 filter paper, and analyzed for the concentration and composition of reducing sugar (RS) by the dinitro-salicylic acid (DNS) method[47] and high-performance liquid chromatography (HPLC) analysis respectively.[48]

Separate hydrolysis and fermentation

In the SHF process, enzymatic hydrolysis and fermentation were conducted separately. The first step of SHF involved the enzymatic hydrolysis of

the substrate to release RS previously performed[44] and the hydrolysate was used in the fermentation stage of the current study. The hydrolysate, containing primarily RS, was then fermented using a co-culture of *Saccharomyces cerevisiae* and *Candida tropicalis*.

Enzymatic hydrolysis

The pretreated substrate was enzymatically hydrolyzed in a 100 mL Erlenmeyer flask using a WB enzyme cocktail (β-glucosidase, cellulase, and endoglucanase) under optimized conditions such as 10% substrate loading, 36 h time, and 50 °C temperature.[44] Afterward, the sample was immediately centrifuged using a centrifuge (80-2 Electronic centrifuge, Jin Yi, China) for 10 min at 10,000 rpm to get the clear supernatant. Finally, the hydrolysis process was stopped by autoclaving. RS concentration of the hydrolysate was measured using a T80+ UV Spectrophotometer (PG Instruments, UK) and 3,5-dinitro-salicylic acid by Miller's method.[47]

Fermentation

The fermentation process was performed in a 250 mL Erlenmeyer flask with a working volume of 100 mL containing 10 mL of fermentation medium of $MgSO_4$ (1 g/L), $(NH_4)_2SO_4$ (1 g/L), yeast extract (5 g/L), and KH_2PO_4 (2 g/L) mixed with the 90 mL hydrolysate of 12% substrate concentration. Then, 10% (v/v) yeast inoculum (co-culture of *Candida tropicalis* and *Saccharomyces cerevisiae* with 1:1 ratio) was seeded in the fermentation flasks. The pH of the fermentation medium was adjusted to 5 using toilet cleaner (A_{TC}). After the inoculation, the flasks were covered and incubated in a laboratory incubator (BJPX-H80, BIOBASE, Shandong, China) at 35 °C for 96 h. The samples were withdrawn after every 12 h and centrifuged at 10,000 rpm for 10 min using centrifuge (80-2 Electronic centrifuge, Jin Yi, China) to get a clear supernatant. Later, the concentration of bioethanol was measured using HPLC. All fermentation process experiments were run in triplicate, and average results are reported with standard deviations.

Simultaneous saccharification and fermentation (SSF)

In contrast to SHF, the SSF process combined the hydrolysis and fermentation processes into a single process. Therefore, pretreated MSW was subjected to simultaneous enzymatic hydrolysis and fermentation by adding the enzyme cocktail and co-culture of *Saccharomyces cerevisiae* and *Candida tropicalis*. This approach allows for more efficient utilization of the substrate, as both hydrolysis and fermentation occur simultaneously, thereby reducing process time and enhancing overall biofuel production efficiency

The SSF process was performed in a 250 mL Erlenmeyer flask, with a working volume of 100 mL. 12% w/v acid-pretreated sample (90 mL) was mixed with 10 mL of fermentation medium containing (g/L): K_2HPO_4, 3.5; yeast extract, 5; $MgSO_4 \cdot 7H_2O$, 0.75; $(NH_4)_2SO_4$, 7.5; $CaCl_2 \cdot 2H_2O$, 1. The pH of the medium was adjusted to 5.5 using A_{TC}. After that, the solution was sterilized in an autoclave at a temperature of 121 °C for 15 min. For enzymatic hydrolysis, the enzyme cocktail with an enzyme loading of 22.76 U β-glucosidase, 6.43 U endoglucanase, and 3.01 FPU total cellulase per gram of substrate was added in the medium. While for the fermentation process, a (10% (v/v)) co-culture of *Saccharomyces cerevisiae* and *Candida tropicalis* with 1:1 ratio was added. Afterwards, the Erlenmeyer flask was covered with cotton plugs and placed in an incubator for 96 h at 35 °C. Then the concentration of bioethanol was measured by HPLC. After this pre-test, SSF process parameters were optimized using BBD. After every specified time, the concentration of bioethanol was measured. The schematic diagram of the process is shown in Figure 1.

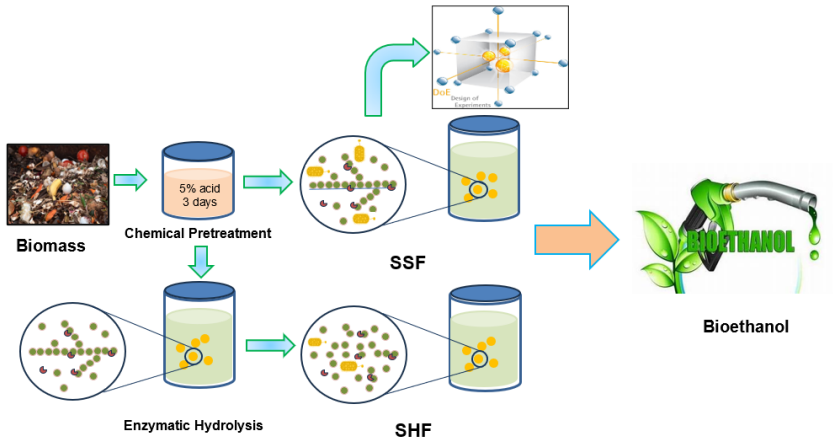

Figure 1: Schematic diagram of the bioethanol production process

Box Behnken design and statistical analysis

SSF process parameters were optimized and analyzed using BBD, and RSM in Design Expert software (version 12.0.1.0). The ranges of SSF process parameters were substrate concentration (8–12%, w/v), temperature (30-40 °C), and time (12-72 h). Seventeen runs of BBD were created with three variables and bioethanol as a dependent variable. All the experiments were performed three times and mean data values were reported.

The second-order polynomial model is shown in Equation (1):

$$X = a_0 + \sum_{i=1}^{3}(a_1 Y_i) + \sum_{n=1}^{\infty}(a_2 Y_i^2) + \sum_{\cdot}\sum_{\cdot}(a_3 Y_i Y_j) \quad (1)$$

where X is bioethanol (response); a_o, a_1, a_2, and a_3 are constant, linear, quadratic, and interaction coefficients, respectively, while Y_i and Y_j are variables. In the model, experimental data values were used to find out the values of the regression coefficient. The model efficiency and validation are checked by F-test, coefficient of determination (R^2), and *p*-value of lack of fit attained from ANOVA. The desirability of DOE was used for the process optimization of the variables and response. Regression analysis, contour plots, and 3D response surface plots were generated to analyze the individual and interactive effects of the variables on the response. Kumar *et al.* used RSM for the optimization of enzymatic saccharification and bioethanol fermentation using waste potato.[49]

Analytical method

HPLC was used to determine bioethanol concentrations at specified times during the fermentation process. To analyze, an aliquot of fermentation broth (5 mL) was centrifuged in the centrifuge at 5000 rpm to clear out suspended particulates at a UV setting of 4 °C within 5-10 minutes. The supernatant obtained was clarified and filtered using 0.45-0.1 µm syringe-driven membrane filters and introduced into HPLC vials to be analyzed. The HPLC system was Agilent 1260 Infinity, and the C18 reversed-phase analytical column was used. Separation was performed with an isocratic elution scheme where a mixture of bioethanol and deionized water of the 30:70 (v/v) ratio was used as the mobile phase. The temperature of the column was kept constant and steady at 25 °C and the flow rate was kept constant at 0.6 mL min^{-1}. Bioethanol detection was done at the wavelength of 280 nm. The retention time of bioethanol was determined by injecting standard bioethanol solutions in the mobile phase under same chromatographic conditions.[50]

RESULTS AND DISCUSSION

Separate hydrolysis and fermentation process

The hydrolysability and fermentability of MSW was measured in a SHF process with a co-culture of *Saccharomyces cerevisiae* and *Candida tropicalis.* The results of enzymatic hydrolysis have been discussed in detail in our previous study.[44] Briefly, the results showed that the maximum RS yields, *i.e.*, 87% and 83%, were achieved after enzymatic hydrolysis of the acid pretreated sample at 50 °C temperature, 10% substrate concentration, and 36 h of hydrolysis time by WB and MSW enzyme cocktail, respectively. Subsequently, the product was hydrolyzed and the hydrolysate, which contained high quantities of glucose, was used in the present study as a substrate for bioethanol production.

The MSW applied in this experiment mainly consisted of cellulose and hemicelluloses, which are structural polysaccharides and are readily digestible and fermentable.[51] MSW, composed of high levels of cellulose (Table 1), qualifies as a second-generation feedstock for bioethanol that is in line with worldwide biofuel development toward the use of biomass.[52] The conversion of cellulose and hemicelluloses into fermentable sugars remains key towards the attainment of industrially relevant bioethanol titers, commonly at >40 g/L in order to defray downstream distillation cost.[53]

Figure 2 contains time-course data of the bioethanol production and glucose consumption in the SHF procedure. The hydrolysate contained an initial glucose concentration of 73.48 g/L. It was noted that during the initial 12 h of fermentation, glucose was consumed rapidly, leaving residual glucose at 60 g/L and bioethanol production at 17 g/L. This lagged phase relates to the exponential growth phase of *C. tropicalis* and *S. cerevisiae* where the two yeasts have high level of metabolic activities and rapid rates of substrate uptakes. The concentration of glucose decreased drastically between 12 and 24 h to 15 g/L, and during the same period, the concentration of bioethanol increased to 21 g/L. At 36 h, glucose levels were 6 g/L and bioethanol concentration was 24 g/L. At 48 h, glucose was completely depleted, a fact that proves complete substrate conversion. It was

then associated with a gradual rise in bioethanol concentration, which reached the highest level at 29 g/L at 72 h. Bioethanol concentration stagnated after 72 h, with small declines seen at 84 h (28 g/L) and 96 h (27 g/L). The tendency can be described as a re-consumption or (microbial) degradation of bioethanol at long incubation periods. It has been found previously that long-term exposure to bioethanol concentrations induces stress in the yeast organism, which results in a decline in the efficiency of the fermentation and the potential metabolization of bioethanol as a second carbon source.[54] The rapid cleavage both in glucose (within 48 h) and bioethanol production that lasted up to 72 h demonstrates the effectiveness of the co-culture system. This result complies with the literature that has reported the highest bioethanol yields reported of 85.0 g/L using lignocellulosic substrates under controlled SHF conditions.[55] In addition, the fact that pretreated MSW substrate is a high carbohydrate-containing substrate further enabled effective saccharification and fermentation process, thereby credibly supporting its competence as a second-generation bioethanol feedstock.

Table 1
Characterization of raw material before and after drying

Parameters	Content	
	Before drying	After drying
pH	4.75	4.73
Total solids (%)	28.05 ± 1.32	86.3 ± 2.68
Volatile solids (%)	26.47 ± 1.12	81.01 ± 2.38
Ash (%)	1.44 ± 0.69	16.56 ± 0.64
Moisture (%)	70.6 ± 2.65	11.02 ± 0.47
Cellulose (%)	31.7 ± 1.58	32.9 ± 1.43
Hemicelluloses (%)	27.5 ± 1.38	16.75 ± 1.21
Lignin (%)	35.5 ± 1.83	33.8 ± 1.71

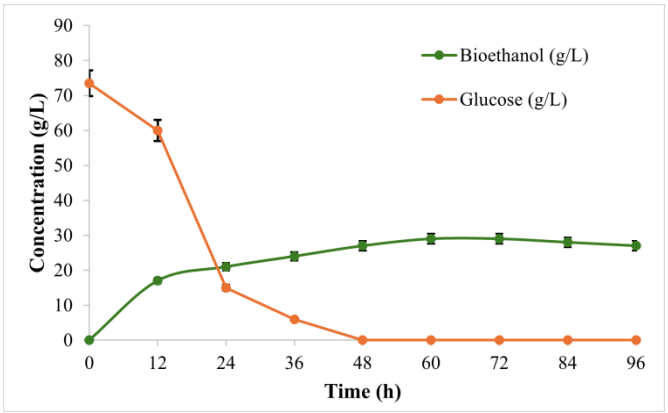

Figure 2: Time course of fermentation during separate hydrolysis and fermentation of pretreated MSW

The co-culture approach succeeds in improving fermentation outcomes given that both strains of *S. cerevisiae* and *C. tropicalis* could utilize glucose through Embden-Meyerhof-Parnas (EMP) pathway under anaerobic circumstances.[56] Experiments with analogous growth kinetics have been described previously: exponential yeast growth takes place over 12-24 hours, after which it enters a stationary phase, caused by bioethanol buildup or nutrient exhaustion.[57] The combination of MSW and co-culture is seen to produce higher bioethanol concentration than in this study because of the high cellulose content of MSW and the co-culture synergistic effect, which allows the complete conversion of sugars even at a high substrate loading level of 12% (w/w) where limitation is often caused by enzyme inhibition and mixing. An all-inclusive conclusion proven by these findings is that enzyme-treated MSW is an attractive substrate, which can be utilized in the production of

biomass bioethanol through SHF with high performance. The fact that sugar was consumed quickly and that long-term bioethanol production was possible confirmed the feasibility of co-culture-based fermentation platforms and provided a scalable and convenient path towards the valorization of MSW.

Simultaneous saccharification and fermentation process

In this research, SSF would be utilized as an alternative to SHF to maximize bioethanol production in MSW utilizing thermotolerant yeast *C. tropicalis* along with the more routinely used *S. cerevisiae*. SSF has a clear advantage over SHF that enzymatic hydrolysis and fermentation could be combined into one step, end-product inhibition could be minimized, and process simplification could be achieved. The performance of this co-culture in SSF conditions was characterized at 35 °C and 12% (w/w) of the substrate loading. The monitoring of the SSF process was done on a 96 h interval, especially on the depletion of glucose and the subsequent accumulation of bioethanol (Fig. 3). The effective time course of saccharification of MSW before fermentation is proved by the initial glucose concentration of 61 g/L at 0 h. It should be noted that an unexplained over-shoot in glucose level was observed at 12 h (79 g/L) maybe because of involuntary prolonged enzymatic hydrolysis during the inoculation or could be due to the slow uptake of sugar by the yeast consortium. Nevertheless, the growth of bioethanol started early, with a 20 g/L production recorded at 12 h, which is indicative of high levels of fermentation at the initial phases of growth. After 12-24 h, the concentration of glucose decreased to 12 g/L hence producing an increased amount of bioethanol of 23 g/L. The high rate of this decline signifies that there is a high rate of metabolism and conversion by co-culture. At the time point of 36 h, almost all available glucose was depleted (5 g/L), and bioethanol level reached 26 g/L. The time point of 48 h was the maximal time when the substrates were completely consumed, and bioethanol production tended towards constant. High bioethanol concentration (32 g/L) was achieved after 72 h, proving strong fermentative capacity of the yeast consortium, especially at the high solids loading applied (12% w/w). After maxima, the bioethanol slightly decreased (32-31 g/L at 84-96 h), which may be described by cellular reassimilation, evaporative loss, or microbial stress response, as reported in other SSF studies.[58]

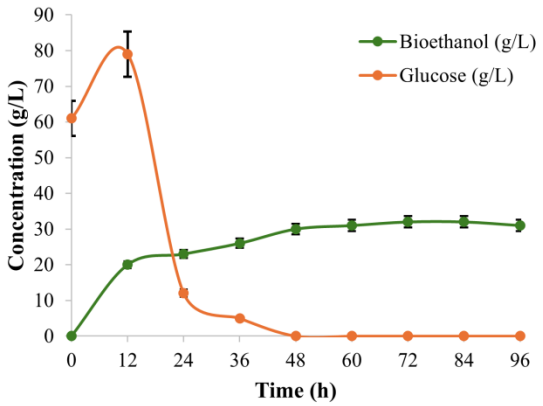

Figure 3: Time course of fermentation during simultaneous saccharification and fermentation of pretreated MSW

The pattern of the fermentation observed closely corresponds to classical yeast growth dynamics. After an initial lag period, *S. cerevisiae* will normally make the growth transition into the exponential phase during the first 12-24 h, where the bioethanol production is followed by a stationary state.[59] Correspondingly, *C. tropicalis* grows best at 30 to 37 °C during the first 24 h of growth before metabolism decelerates.[60] The high final concentration of bioethanol (32 g/L) is also commercially relevant (>30 g/L), thus

supporting the use of MSW as a feedstock of second-generation bioethanol. The SSF showed a higher performance compared to SHF regarding the glucose conversion, as well as bioethanol yield. The maximum level of bioethanol was achieved in SSF (32 g/L) compared to that in SHF (29 g/L) under comparable conditions of substrate loading, which justified the operative superiority of the combined strategy. Bioethanol productivities measured at 72 h also emphasized the increased efficiency of SSF with ~6% greater conversion of glucose to bioethanol compared to SHF. This enhancement is consistent with earlier study results,[59] highlighting the advantage of inhibiting enzyme-related feedback and sustaining the constant fermentable sugary flow inside SSF systems. The fact that the levels of glucose remained at early levels after 48 h also implies a concurrent equilibrium between the rate of saccharification and fermentation, an attribute that will minimize the chances of osmotic or catabolite repression among yeast cells. Compared to SHF, in which bioethanol buildup was offset by successive processing, SSF produced a high rate and consistent bioethanol production, with no noticeable microbial inhibition during the presentation. This implies the compatibility of the yeast strains chosen and the strength of the process setup.

However, some limitations were identified. The characteristic lower incremental sugar release in the later steps of SSF may be explained by the fact that enzymatic activity is suboptimal at 35 ℃ below the temperature (= 50 ℃) at which most cellulolytic enzymes are most effectively used. Also, the degradation of the enzymes due to ageing must have led to lower saccharification effectiveness. These constraints imply that additional means of enzyme stabilization, or thermotolerant enzyme compositions could be required to maintain long-term activity during SSF systems. Lastly, it must be noted that MSW is variable in composition. The high bioethanol concentration was attained in the study however, the capacity to replicate the process with MSW is dependent on the inherent heterogeneity of MSW. However, the co-culture *S. cerevisiae* and *C. tropicalis* was characterized by an effective glucose fermentation kinetics, particularly in the initial 24 h, as in the previous studies,[59-61] and proved to be stable throughout the course of the process. Collectively, these results show that SSF is superior to SHF regarding process complexity, glucose conversion, and yield of bioethanol, confirming the industrial interests of SSF application to MSW valorization into second generation bioethanol.

Optimization of SSF using BBD

In this study, SSF process parameters were optimized to achieve the maximum yield of bioethanol.[62] BBD generated seventeen experimental runs, and to achieve a high degree of accuracy, all experiments were conducted in triplicates. The quadratic model of seventeen runs was obtained using experimental results of the SSF process and analyzed by regression and ANOVA analysis. BBD-RSM was conducted to investigate the mutual effect of SSF process parameters on bioethanol. Table 2 shows the BBD design runs and the production of bioethanol, demonstrating that experimental and predicted results are very close. Moreover, errors associated with the concentration of bioethanol are also presented, and the range of bioethanol (17 g/L to 36 g/L) was observed in Table 2. The multinomial quadratic model for bioethanol obtained from the actual results of the SSF process (Table 2) is expressed in Equation (2):

$$X\ (g/L) = -195.778 - (0.390*Y1) + (11.315*Y2) + (4.912*Y3) + (0.008*Y1*Y2) + (0.012*Y1*Y3) - (0.100*Y2*Y3) + (0.002*Y1^2) - (0.152*Y2^2) - (0.075*Y3^2) \quad (2)$$

The coded model compares the relative effect of independent variables with the variable's coefficients. Furthermore, the factors-based regression model can be used to predict the responses for the actual value of each process parameter. In Equation (2), negative and positive signs exhibit process variables' antagonistic and synergistic effects on the SSF process. Y1, Y2, and Y3 correspond to time (h), temperature (℃), and substrate concentration (%), respectively. At the same time, X is bioethanol (g/L).

The statistical significance of the model was checked by the Fisher (F) test. At the same time, regression analysis was performed to check the quadratic model's fitness. The F value of 48.92 with a p-value < 0.05 at 95% confidence, as shown in Table 3, indicates the significance of

the model. Figure 4 shows the comparison of experimental and predicted data values of bioethanol, and it clearly shows that all the concentration values of bioethanol (actual and predicted) are within the vicinity of the regression line. The R^2 value of model was 0.984 and very close to 1, indicating the accuracy of the model.[63-65] Furthermore, the coefficient of determination (R^2 = 0.984) comparable with the adjusted R^2 value (0.964) shows that the optimization operation of the model can be performed effectively. It also presents a significant correlation between the experimental results of bioethanol and predicted responses.[66]

ANOVA analysis for bioethanol produced by the SSF process is presented in Table 3. A statistically significant multiple regression relationship between the process parameters and response is seen. In the ANOVA analysis, the lack of fit test is used to determine the adequacy of the model.[36] For this test, the F value will be significant with a p-value > 0.05, which indicates the fitness of the actual results for the response.[67] For the lack of fit test, the F-value of the model was 1.35, which means that it is not significant relative to the error. Conversely, for the lack of fit test, if the *p*-value is less than 0.05 (significant), then a more complex model would be required to fit the result.[40] In this model, the *p*-value for the lack of fit was 0.376, *i.e.*, not significant, which indicates the good fit of the model.[68] To further visualize and explain the effects of process parameters like time, temperature, and substrate concentration on bioethanol, 2D contour plots were generated. In Figure 5(a-c), the shapes of the curves provide the pictured impact of the process parameters of SSF on the response.[69] In the 2D plots, circular-shaped maps show the insignificant interaction of process parameters, while the oval-shaped maps display significant interaction of process parameters.[67]

Table 2
Actual and predicted responses for bioethanol by SSF

Runs	Time (h)	Temperature (°C)	Substrate concentration (%)	Bioethanol (g/L)		
				Actual	Predicted	Error
1	72	35	12	36	33.4	7.7%
2	72	35	8	32	30.2	6.0%
3	12	35	8	20	20.7	3.4%
4	12	40	10	17	16.2	5.0%
5	42	35	10	25	24.8	0.7%
6	12	35	12	21	21.0	0.2%
7	42	35	10	27	24.8	8.8%
8	72	40	10	31	29.6	4.8%
9	42	35	10	25	24.8	0.7%
10	42	30	8	20	18.8	6.4%
11	42	35	10	26	24.8	4.7%
12	12	30	10	19	18.5	2.8%
13	72	30	10	28	27.1	3.5%
14	42	40	12	21	20.7	1.7%
15	42	30	12	23	22.5	2.0%
16	42	40	8	22	20.9	5.2%
17	42	35	10	25	24.8	0.7%

Figure 5 (a-c) presents the contour lines of elliptical shape for bioethanol, which indicates the strong and interactive effect of process variables, *i.e.*, substrate concentration and temperature (Fig. 5a), temperature, and time (Fig. 5b), and substrate concentration and time (Fig. 5c), on bioethanol. The highest bioethanol concentration by SSF process was 36 g/L, achieved at 12% substrate concentration for 72 h at 35 °C temperatures.

Table 3
ANOVA analysis of CV of biofuel produced by SSF

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	405.6676	9	45.07418	48.91772	< 0.0001	Significant
A-Time	312.5	1	312.5	339.1473	< 0.0001	
B-Temperature	0.125	1	0.125	0.135659	0.7235	
C-Substrate concentration	6.125	1	6.125	6.647287	0.0366	
AB	6.25	1	6.25	6.782946	0.0352	
AC	2.25	1	2.25	2.44186	0.1621	
BC	4	1	4	4.341085	0.0757	
A^2	16.01053	1	16.01053	17.37576	0.0042	
B^2	60.8	1	60.8	65.9845	< 0.0001	
C^2	0.378947	1	0.378947	0.411261	0.5417	
Residual	6.45	7	0.921429			
Lack of Fit	3.25	3	1.083333	1.354167	0.3762	Not significant
Pure Error	3.2	4	0.8			
Cor. Total	412.1176	16				

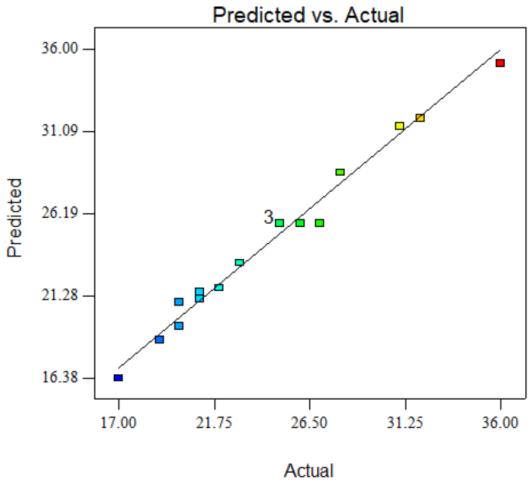

Figure 4: Predicted *vs* actual plot of bioethanol produced by SSF

To visualize the effects of the parameters between other parameters, 3-D response surface plots are effectively used. In this study, 3-D plots were constructed to evaluate the impact of process parameters on the response (bioethanol).[70] Figure 5(d-f) shows the interaction of process parameters like temperature and substrate concentration (Fig. 5d), time and temperature (Fig. 5e), and time and substrate concentration (Fig. 5f) on the response (bioethanol). Temperature is an important process parameter for SSF as enzymes and microorganisms work together. Enzymes hydrolyze the substrate, and then microorganisms converted this hydrolysate into bioethanol. As these two processes co-occur, the temperature must be favorable for both. Figure 5d shows that with the increase in temperature, bioethanol increases, and after 35 °C, it starts decreasing; therefore, 35 °C is the most favorable temperature to produce bioethanol. Similarly, with the increase in substrate concentration, bioethanol increases, and at 12% concentration, maximum bioethanol concentration was achieved. The substrate may inhibit enzymes at very high substrate concentration, and it may also hinder the homogenous mixing of enzymes and substrate.[71] Furthermore, very high substrate concentration may cause diffusion of the end-product and limit the attack of enzymes on the substrate.[72] Enzymes released from *Aspergillus niger* are temperature dependent and work efficiently at specific temperatures.[73] Time for

the SSF process is another critical parameter because microorganisms require a particular time to convert substrate into bioethanol. The effect of time and temperature (Fig. 5e) clearly shows that bioethanol increases with the increase in time, and at the 72 h of SSF with 35 °C temperatures, the concentration of bioethanol was maximum.

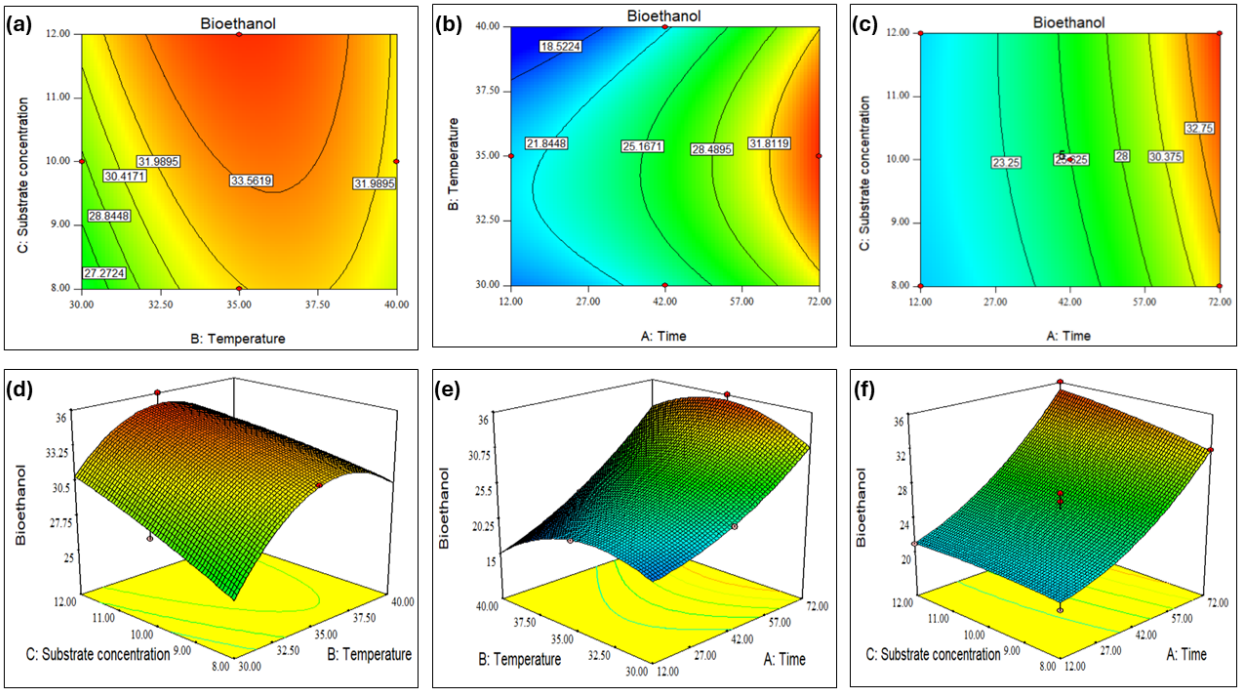

Figure 5: 2D and 3D plots of bioethanol produced by SSF

Figure 5f presents the effect of time and substrate concentration on bioethanol. Maximum concentration of bioethanol was achieved at 72 h and 12% substrate concentration. Figure 5 shows that 35 °C temperature is optimum for both processes (saccharification and fermentation).

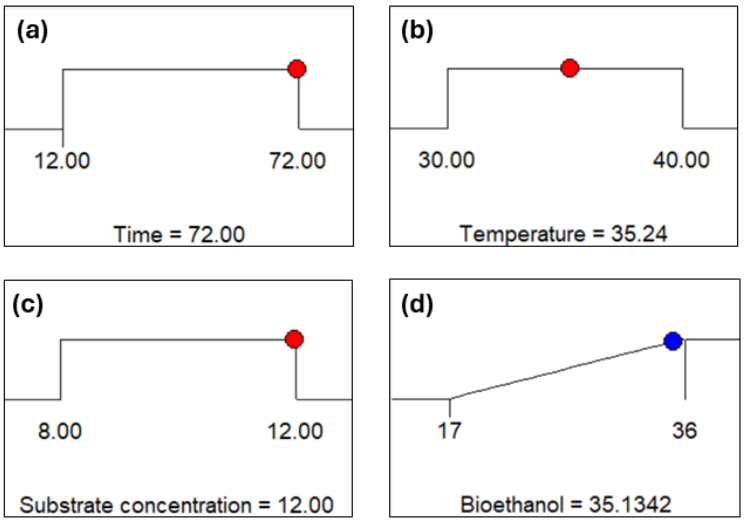

Figure 6: Optimization of SSF variables for biofuel: (a) time, (b) temperature, (c) substrate concentration, and (d) bioethanol (red balls show the optimal process variables, blue balls show the optimal response (bioethanol))

Verification of optimum conditions for SSF

The desirability of DOE (Design Expert, Version 12.0.1.0) predicts the model capability. The response (bioethanol) was set as the maximum goal, while the SSF process variables (time, temperature, and substrate concentration) were set in ranges. Figure 6 presents the optimum results obtained. The maximum bioethanol was 35.134 g/L at time of 72 h, 35.24 °C temperature, and substrate concentration of 12% for SSF. The SSF process was performed under these optimized conditions to verify the maximized bioethanol results. The maximum bioethanol was slightly higher than the achieved experimental value, *i.e*., 36 g/L. However, compared to the experimental value, this optimization is more reliable because it is statistical model based. Thus, the result shows that the model fits well the SSF process

CONCLUSION

The current study has positioned the use of acid-treated MSW as a viable and low-cost source of lignocellulosic material toward second-generation bioethanol production. Comparative analysis showed that SSF process is better than SHF because it offers higher yields of bioethanol and shorter process time. BBD optimization led to an increase in the process efficiency further where the fermentation time was reduced to 72 h and a high concentration of ethanol was obtained – of 36 g/L. These results indicate that the co-culture of *Saccharomyces cerevisiae* and *Candida tropicalis* in an optimized SSF is promising as it allows to produce higher amounts of ethanol and leads to environmental friendliness by turning organic waste into a useful biofuel. The problems of inefficiency and compatibility with the temperature are also overcome by this dual yeast system. In future research, pilot-scale development of this process should be undertaken to determine robustness and cost-effectiveness on the industrial scale. The economic feasibility of the process could be improved by investigations into genetically engineered thermotolerant yeast strains, enzyme recycling and continuous fermentation systems. Techno-economic and life cycle assessments are necessary to assess the overall economic and environmental implications as well. In the end, the research creates a solid basis of including MSW-based production of bioethanol as a part of new urban waste management approaches that would lead to the creation of a circular bioeconomy, a sustainable alternative to the use of fossil fuels.

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