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Research Article

Statistical Optimization of Fermentation Process Parameters by Taguchi Orthogonal Array Design for Improved Bioethanol Production

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The statistical optimization of different fermentation process parameters in SSF of mixed MAA and organosolv pretreated 1% (w v^{-1}) wild grass, namely, recombinant *Clostridium thermocellum* hydrolytic enzymes' volume (GH5 cellulase, GH43 hemicellulase), fermentative microbes' inoculum volume (*Saccharomyces cerevisiae*, *Candida shehatae*), pH, and temperature, was accomplished by Taguchi orthogonal array design. The optimized parameters in 100 mL of fermentation medium were (%, v v^{-1}) as follows: 1.0, recombinant GH5 cellulase (5.7 mg^{-1}, 0.45 mg mL^{-1}); 2.0, recombinant GH43 hemicellulase (3.7 U mg^{-1}, 0.32 mg mL^{-1}); 1.5, *S. cerevisiae* (3.9×10^8 cells mL^{-1}); 0.25, *C. shehatae* (2.7×10^7 cells mL^{-1}); pH, 4.3; and temperature, 35°C. pH with p-value 0.001 was found to be the most significant factor affecting SSF. The ethanol titre obtained in Taguchi optimized shake flask SSF was 2.0 g L^{-1} implying a 1.3-fold increase as compared to ethanol titre of 1.5 g L^{-1} in unoptimized shake flask SSF. A 1.5-fold gain in ethanol titre (3.1 g L^{-1}) was obtained with the same substrate concentration in lab scale bioreactor on scaling up the shake flask SSF with Taguchi optimized process parameters.

1. Introduction

Cost-effective fermentation of lignocellulosic hydrolysate to a value-added product, bioethanol, necessitates the conspicuous enhancement in the activities of various hydrolytic enzymes along with efficient mixed sugar utilization by various fermentative microbes [1]. Simultaneous saccharification and fermentation (SSF) is a single step combination of enzymatic hydrolysis of complex polysaccharides with concurrent fermentation of derived monosaccharides to ethanol [2]. The northeast part of India has a wide abundance of lignocellulosic substrate, namely, wild grass (*Achnatherum hymenoides*), rich in cellulose and hemicellulose [3]. As compared to the commercially employed hydrolytic enzyme of the corresponding *Trichoderma* system, the cellulosome of the anaerobic thermophilic bacterium, *Clostridium thermocellum,* exhibits a 50-fold higher specific activity against crystalline cellulose [4]. The advancement in molecular biology has familiarized new area of enzyme production in transformed cells with overexpression and their subsequent use for the breakdown of structural carbohydrates, namely, cellulose and hemicellulose, into simple sugars [3, 5].

According to CAZy database, glycoside hydrolase family 5 (GH5) exhibits activities of chitosanase (EC 3.2.1.132), cellulase (EC 3.2.1.4), glucan 1, 3-β-glucosidase (EC 3.2.1.58), and licheninase (EC 3.2.1.73) whereas glycoside hydrolase family 43 displays β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), and xylanase (EC 3.2.1.8) activities. A number of available pretreatment techniques are used for liberating the cellulosic and hemicellulosic components from the lignin moieties and in turn rendering the accessibility to a better hydrolysis step [6]. *Saccharomyces cerevisiae* has the inherent ability to utilize hexose sugars from the breakdown of cellulose, considerable product tolerance, and resistance to metabolic inhibitions in ethanol production [7]. Xylitol dehydrogenase and xylose reductase are the prime enzymes of *Candida shehatae* that enable it to utilize pentose sugars from hemicellulose degradation for ethanol production [8].

Temperature, pH, hydrolytic enzyme volume, and fermentative microbe's inoculum volume are the process parameters that play a vital role in lignocellulosic ethanol production [9]. The performance of multiple experiments by analyzing one variable at a time (OVAT) approach is time consuming and laborious for identifying various independent variables with their effects [10]. Statistically based experimental designs, namely, Plackett-Burman design, Box-Behnken design, and Taguchi orthogonal array design, summarize the collection and sorting of variables to be taken for consideration, determine the variable amount, and analyze the variable at different parameters and, finally, the effect of variable error. Better quality at low cost is the main aim for generation of Taguchi design of experiments (DOE) approaches to maximize robustness of products and processes [11]. Taguchi experimental design is a fast and considerable way of optimization conferring remarkable outcome in simultaneous study of many factors, making its mark in quality products supplemented with better process performance, and rendering high yield and better stability [12, 13]. The basic principle involved is the encompassment of large experimental data as orthogonal (unbiased) array in determining the effect of various factors which govern the reaction happening, ensuing in experimental error reduction with improved producibility (efficiency) of experimental outcome. Taguchi design established the importance of statistically aligned experiments in speculating the settings of product (and/or processes) on various parameters [14, 15].

The current study emphasizes the Taguchi optimization of different fermentation process parameters such as mixed recombinant enzymes' volume (GH5 cellulase, GH43 hemicellulase), mixed cultures' inoculum volume (*S. cerevisiae, C. shehatae*), pH, and temperature on bioethanol production from mixed pretreated wild grass with subsequent validation of the model at shake flask level and scale-up in a bioreactor.

2. Materials and Methods

2.1. Reagents, Chemicals, and Substrate. Carboxy methyl cellulose (CMC) and rye arabinoxylan were purchased from Sigma Aldrich (St. Louis, USA). The analytical grade reagents and chemicals, namely, LB medium, ampicillin, kanamycin, sodium acetate, glucose, yeast extract, peptone, potassium dichromate ($K_2Cr_2O_7$), sodium carbonate, sodium bicarbonate, sodium potassium tartrate, sodium sulphate, copper sulphate, ammonium molybdate, sodium arsenate, phosphoric acid, and ethanol, were purchased from Merck and Himedia Pvt. Ltd., India. Coomassie brilliant blue G-250 was purchased from Amresco LLC, USA. Lignocellulosic substrate wild grass (*Achnatherum hymenoides*) was collected from the campus of Indian Institute of Technology Guwahati, India. The substrate was washed with water thrice for the removal of adhered dust particles, dried, and finally grinded to 1 mm mesh size.

2.2. Microorganisms and Culturing Conditions. The recombinant *E. coli* BL21 (DE3) cells harbouring family 5 glycoside hydrolase (GH5) gene from *Clostridium thermocellum* were cloned in an expression vector pET-21a(+) and expressed earlier [16, 17]. The recombinant GH5 cellulase is available commercially at NZY Tech, Lda, Lisbon, Portugal. The recombinant *E. coli* BL21 (DE3) pLysS cells transformed by family 43 glycoside hydrolase (GH43) gene from *Clostridium thermocellum* and cloned in pET-28a(+) expression vector were expressed earlier [3]. These cells were cast off as a source of recombinant GH43 hemicellulase. These *E. coli* BL21 cells were preserved in LB medium as glycerol stock at −80°C in our laboratory at IIT Guwahati.

The fermentative microbes, *Saccharomyces cerevisiae* (NCIM no. 3215), and *Candida shehatae* (NCIM no. 3500), procured from National Chemical Laboratory, Pune, India, were maintained independently at 4°C on 5 mL of Malt extract glucose yeast extract peptone (MGYP) slants containing malt extract (0.3 g 100 mL^{-1}), glucose (1 g 100 mL^{-1}), yeast extract (0.3 g 100 mL^{-1}), and peptone (0.5 g 100 mL^{-1}) [18]. One loopful from these slant cultures was further introduced into 50 mL of glucose yeast extract (GYE) medium in two separate 100 mL Erlenmeyer flasks containing glucose (1 g 100 mL^{-1}) and yeast extract (0.1 g 100 mL^{-1}) with supplementation of KH_2PO_4 (0.1 g 100 mL^{-1}), $(NH_4)_2SO_4$ (0.5 g 100 mL^{-1}), and $MgSO_4 \cdot 7H_2O$ (0.05 g 100 mL^{-1}). They were incubated at 30°C and 120 rpm for 48 h prior to inoculation into fermentation media. The aliquots measuring 1 mL from each of the actively growing cultures of *S. cerevisiae* (3.9×10^8 cells mL^{-1}) and *C. shehatae* (2.7×10^7 cells mL^{-1}) were aseptically inoculated to 100 mL fermentation medium. The cell count of the actively growing *S. cerevisiae* and *C. shehatae* was measured using a haemocytometer.

2.3. Production of Recombinant Cellulase (GH5) and Hemicellulase (GH43). The recombinant GH5 cellulase production was initiated by inoculating 50 μL of the *E. coli* BL21 (DE3) culture from glycerol stocks into 5 mL of LB medium containing 100 μg mL^{-1} ampicillin with incubation at 37°C and 180 rpm for 16 h. One percent (v v^{-1}) of the culture inoculum was transferred aseptically to 200 mL of LB medium in 500 mL flask containing 100 μg mL^{-1} ampicillin and was incubated at 37°C and 180 rpm till the culture reached the midexponential phase ($A_{600\,nm}$ 0.6). This midexponential phase culture was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) followed by further 8 h incubation for overexpression of recombinant protein [16]. 50 μg mL^{-1} kanamycin was used as a selective marker for *E. coli* BL21 (DE3) plysS cells containing GH43 hemicellulase [3]. Similar production process was employed for GH43 hemicellulase as followed for GH5 cellulase, except that the incubation period was 24°C, 180 rpm, and 24 h for overexpression of protein after IPTG (1 mM final concentration) was added. The overexpressed *E. coli* cells (GH5 or GH43) collected by centrifugation (4°C, 8,510 g, and 30 min) were resuspended in 50 mM sodium phosphate buffer (pH 7.0). Each of the resuspended cell pellets was subjected to sonication (SONICS, Vibra Cell, Newtown, CT, USA) in an ice-bath separately for 15 min with further centrifugation (4°C, 19,650 g, and 30 min). The

Table 1: Factor (parameter) and levels in Taguchi experimental design for shake flask SSF process employing mixed pretreated 1% (w v^{-1}) wild grass at 120 rpm.

Factor/parameter	Levels				
Recombinant GH5 cellulase* (5.7 U mg^{-1}, 0.45 mg mL^{-1})	0.25	0.5	1.0	1.5	2.0
Recombinant GH43 hemicellulase* (3.7 U mg^{-1}, 0.32 mg mL^{-1})	0.25	0.5	1.0	1.5	2.0
*S. cerevisiae** (3.6×10^8 cells mL^{-1})	0.25	0.5	1.0	1.5	2.0
*C. shehatae** (2.1×10^8 cells mL^{-1})	0.25	0.5	1.0	1.5	2.0
pH	3	4.3	5.0	5.5	6
Temperature (°C)	26	28	30	33	35

*The values of levels in (%, v v^{-1}).

two recombinant enzymes, GH5 cellulase and GH43 hemicellulase, were expressed as soluble proteins. The cell free supernatant obtained was employed as the enzyme source for SSF experiments [16].

2.4. Mixed Pretreatment Strategy. Microwave-assisted alkali (MAA) pretreatment loosens the compact structure of cellulose and aids in its hydrolysis to glucose [19]. Organosolv pretreatment with the assistance of different organic acids benefits in relaxing the complex hemicellulose for its efficient hydrolysis to xylose [20]. Owing to the substantial quantities of cellulose and hemicellulose in wild grass, the lignocellulosic substrate was subjected to mixed MAA and organosolv pretreatment strategy.

2.5. Microwave-Assisted Alkali (MAA) Pretreatment. One gram (dry powder) of wild grass (*A. hymenoides*) was suspended in 8 mL of 1% (v v^{-1}) sodium hydroxide aqueous solution in a 100 mL beaker. The beaker was positioned at the centre of a rotating circular glass plate in a domestic microwave oven at 900 W for 25 min [19]. The substrate filtered through muslin cloth was further subjected to organosolv pretreatment.

2.6. Organosolv Pretreatment. The microwave-assisted alkali (MAA) pretreated and filtered wild grass was further subjected to 20 mL of (70 : 30 v v^{-1}) ethanol : water mixture containing 1% (v v^{-1}) of sulphuric acid, hydrochloric acid, acetic acid, and phosphoric acid (1 mL each) at 70°C for 1 h [20]. The substrate was then washed with two ethanolic extracts: 95% (v v^{-1}) ethanol at 60°C for 4 h and 70% (v v^{-1}) ethanol at 30°C for 1 h. The substrate residue was further treated with 4% (v v^{-1}) hydrogen peroxide at 45°C for 16 h. The final washing was done with 70% ethanol at 30°C for 1 h [20]. The mixed pretreated substrate was subsequently subjected to enzymatic hydrolysis.

2.7. Simultaneous Saccharification and Fermentation (SSF) Process of Mixed Pretreated 1% (w v^{-1}) Wild Grass at Shake Flask Level. One percent (w v^{-1}) of the mixed microwave-assisted alkali (MAA) and organosolv pretreated wild grass (*A. hymenoides*) was autoclaved in 250 mL Erlenmeyer flask encompassing 100 mL working volume of 20 mM sodium acetate buffer (pH 5.0) supplemented with (0.1%, w v^{-1}) each of the yeast extract and peptone. Then, 0.5 mL of each crude recombinant cellulase (GH5) (5.7 U mg^{-1}, 0.45 mg mL^{-1}) and recombinant hemicellulase (GH43) (3.7 U mg^{-1}, 0.32 mg mL^{-1}) was added as the mixed enzymatic consortium for hydrolysis. At the same time, 0.5 mL of each *S. cerevisiae* (3.9×10^8 cells mL^{-1}) and *C. shehatae* (2.7×10^7 cells mL^{-1}) inoculum was added for fermentation. The flasks were kept at 30°C and 120 rpm for 72 h and the sample was collected at every 6 h interval. The monitoring of SSF dynamic profile was done with the measurement of the cell OD ($A_{600\,nm}$), reducing sugar (g L^{-1}), ethanol concentration (g L^{-1}), and specific activity (U mg^{-1}).

2.8. Optimization of Process Parameters of Simultaneous Saccharification and Fermentation (SSF) Involving Mixed Pretreated Wild Grass at Shake Flask Level by Taguchi Method

2.8.1. Statistical Optimization Using Taguchi Orthogonal Array Design. Taguchi experimental design matrix, a standard orthogonal array L_{25} (6_5), was used to examine six factors, namely, recombinant GH5 cellulase (5.7 U mg^{-1}, 0.45 mg mL^{-1}) volume (mL), recombinant GH43 hemicellulase (3.7 U mg^{-1}, 0.32 mg mL^{-1}) volume (mL), *S. cerevisiae* (3.9×10^8 cells mL^{-1}) inoculum volume (mL), *C. shehatae* (2.7×10^7 cells mL^{-1}) inoculum volume (mL), pH, and temperature (°C) in five levels, namely, Level 1 to Level 5 (Table 1), in SSF experiments involving mixed pretreated 1% (w v^{-1}) wild grass at shake flask level. The lower and upper levels of optimized factors were selected on the basis of the suitable conditions for the active functioning of the recombinant hydrolytic enzymes and the desired growth of the fermentative microbes for efficient bioethanol production. The L and the subscript (25) represent the Latin square and the number of experimental runs, respectively. The levels of the factors studied and the layout of the L_{25} Taguchi's orthogonal array are represented in Tables 1 and 2. Each of the twenty-five simultaneous saccharification and fermentation (SSF) experiments denoted by "runs" was carried out as per the defined values of six different parameters in five levels (Table 2). All the SSF experiments were carried out in 100 mL of fermentation media at 120 rpm for 72 h at varying

Table 2: Matrix layout of the L_{25} Taguchi orthogonal array design.

Run/expt. no.	Recombinant GH5 cellulase*	Recombinant GH43 hemicellulase*	*S. cerevisiae**	*C. shehatae**	pH	Temperature
1	0.25	1	1	1	5	30
2	0.5	1.5	2	0.25	4.3	30
3	1	2	0.5	1.5	3	30
4	1.5	0.25	1.5	0.5	6	30
5	2	0.5	0.25	2	5.5	30
6	0.25	0.25	0.25	0.25	3	26
7	0.5	0.5	1	1.5	6	26
8	1	1	2	0.5	5.5	26
9	1.5	1.5	0.5	2	5	26
10	2	2	1.5	1	4.3	26
11	2	0.25	2	1.5	5	28
12	1.5	2	1	0.25	5.5	28
13	1	1.5	0.25	1	6	28
14	0.5	1	1.5	2	3	28
15	0.25	0.5	0.5	0.5	4.3	28
16	0.25	1.5	1.5	1.5	5.5	33
17	0.5	2	0.25	0.5	5	33
18	1	0.25	1	2	4.3	33
19	1.5	0.5	2	1	3	33
20	2	1	0.5	0.25	6	33
21	0.25	2	2	2	6	35
22	0.5	0.25	0.5	1	5.5	35
23	1	0.5	1.5	0.25	5	35
24	1.5	1	0.25	1.5	4.3	35
25	2	1.5	1	0.5	3	35

*The values of levels in (%, v v^{-1}).

temperatures (Table 1) with sample collection at every 6 h interval.

2.8.2. Analysis of the Taguchi Orthogonal Array Experiments (Runs). The MINITAB statistical software package (Design Expert, version 8.0) was used to determine the outcomes of the fermentation runs. The signal-to-noise ratio (S/N), which is the logarithmic function of desired output, served as objective function for optimization.

For each run, S/N ratio corresponding to larger-the-better objective function was computed using relation in

$$\frac{S}{N} = -10 \log_{10} \frac{1}{n} \sum_{i=1}^{n} \frac{1}{y_i^2}, \quad (1)$$

where "y_i" is the signal and "n" is the number of repetitions in each experiment.

The response values in terms of ethanol titre (%, v v^{-1}) and S/N ratios of Taguchi experimental design in 25 runs were analysed to extract independently the main effects of the factors; the analysis of variance technique was then applied to determine which factors were statistically significant. The controlling factors were identified, with the magnitude of the effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. The analysis of variance (ANOVA) for the responses of ethanol production was carried out according to the factors' contribution by the Taguchi method. The factors in the experimental design considered to be statistically significant at 95% confidence limit were used to determine the ratio (F) and the p-value ($p < 0.05$).

2.8.3. Validation of the Experimental Model. The model was validated by performing the SSF trial employing Taguchi optimized fermentation process parameters on mixed pretreated 1% (w v^{-1}) wild grass in 100 mL of fermentation medium. The best fermentation process parameters comprised 1.0 mL of recombinant GH5 cellulase (5.7 U mg^{-1}, 0.45 mg mL^{-1}), 2.0 mL of recombinant GH43 hemicellulase (3.7 U mg^{-1}, 0.32 mg mL^{-1}), 1.5 mL of *S. cerevisiae* (3.9×10^8 cells mL^{-1}), 0.25 mL of *C. shehatae* (2.7×10^7 cells mL^{-1}), pH of 4.3, and temperature of 35°C. The fermentation was carried out at 120 rpm for 72 h with 6 h sample collection interval. The validation of the experimental model was executed by determining the ethanol titre (%, v v^{-1}).

2.8.4. Scale-Up of Taguchi Optimized Simultaneous Saccharification and Fermentation (SSF) Process Parameters Involving Mixed Pretreated 1% (w v^{-1}) Wild Grass at Bioreactor Level. The Taguchi optimized SSF process parameters involving mixed pretreated 1% (w v^{-1}) wild grass were scaled up to 1 L in a 2 L lab scale fermentor (Applikon, model Bio Console ADI 1025, Holland). 10 g L^{-1} of mixed MAA and organosolv pretreated wild grass (*A. hymenoides*) was used as substrate for bioreactor SSF experiments. 10 mL of isolated crude recombinant GH5 cellulase (5.7 U mg^{-1}, 0.45 mg mL^{-1}) along with 20 mL of recombinant GH43 hemicellulase (3.7 U mg^{-1}, 0.32 mg mL^{-1}) was employed for saccharification. 15 mL of *S. cerevisiae* (3.9×10^8 cells mL^{-1}) and 2.5 mL of *C. shehatae* (2.7×10^7 cells mL^{-1}) were engaged for bioethanol production. The SSF was carried out at 35°C, pH 4.3, and agitation of 120 rpm. For the efficient growth of fermentative microbes, an aeration rate of 1 vvm was controlled by a mass flow controller to maintain the dissolved oxygen (DO) level of minimum 40%. Growth was observed at 600 nm using UV-visible spectrophotometer (Varian Cary50, Australia). The online process parameters, namely, temperature (°C), pH, and stirring rate (rpm), were noted for every 1 min. The different parameters of cell OD ($A_{600\,nm}$), reducing sugar (g L^{-1}), ethanol concentration (g L^{-1}), and specific activity (U mg^{-1}) were surveyed at 6 h fixed interval. The addition of 1 N HCl and 1 N NaOH to maintain the pH at 4.3 prohibited the pH excursions of the organism below the set point.

2.9. Analytical Methods

2.9.1. Estimation of Structural Carbohydrate. The structural carbohydrates like cellulose, hemicellulose, and lignin of untreated and pretreated wild grass were estimated by the standardized methods of NREL, USA [21]. 0.3 g of dry powdered substrate (untreated or pretreated) was mixed with 3 mL of H_2SO_4 (27 N) and kept at 30°C for 1 h. Then 84 mL of distilled water was added to lower down the concentration of H_2SO_4 to 1.5 N. Then, the sample was autoclaved at 121°C for 1 h. The substrate was cooled to room temperature and the biomass (untreated or pretreated) was filtered using a vacuum filtration unit. The residue weighed was lignin (acid insoluble lignin). The pH of the collected filtrate was neutralized by addition of $CaCO_3$ (1 M). Finally, the filtrate was assessed for reducing sugar (glucose) and in turn cellulose was calculated (1 g cellulose = 1.1 g of glucose). The remaining content was hemicellulose.

2.9.2. Recombinant GH5 Cellulase, GH43 Hemicellulase Assay, and Protein Content Determination. The recombinant GH5 cellulase assay was performed by incubating the enzyme (10 μL) in a 100 μL reaction mixture with 1% (w v^{-1}) final concentration of CMC in 20 mM sodium acetate buffer (pH 4.3) at 50°C and 10 min. The mixture was assessed for the released reducing sugar [22, 23]. The released reducing sugar was used to determine the enzyme activity. The GH43 hemicellulase activity was tested by incubating 10 μL of the recombinant enzyme in a 100 μL reaction mixture with 1% (w v^{-1}) final concentration of rye arabinoxylan in 100 mM sodium acetate buffer (pH 5.4) at 50°C for 10 min. The absorbance was measured using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) at 500 nm against a blank with D-glucose or L-arabinose as standard. One unit (U) of cellulase activity is defined as the amount of enzyme that liberates 1 μmole of reducing sugar (glucose) per min under the above assay conditions. On the other hand, one unit (U) of hemicellulase activity is defined as the amount of enzyme that releases 1 μmole of reducing sugar (arabinose) per min under the above assay conditions. The concentration of protein was detected by mixing the enzyme (10 μL) with distilled water (90 μL) in a total reaction volume (100 μL) with final addition of 1 mL Bradford reagent [24]. The reaction mixture was upheld at 25°C for 20 min and OD was determined using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) at 595 nm. A BSA standard curve was used to determine the protein concentration.

2.9.3. Ethanol Content Determination by Gas Chromatography and Dichromate Method. The ethanol fraction in fermentation broth was determined by gas chromatography furnished with flame ionization detector (GC-FID, Varian 450) and Porapak (Hayesep) Q packed column (3.0 m × 2.0 mm i.d., 80–100 mesh, Varian). A constant flow rate (55 cm^3 min^{-1}) of nitrogen was used as the carrier gas with the oven temperature kept constant at 150°C for 20 min as per Bandaru et al., 2006 [25]. Both the injector and detector temperatures were maintained at 170°C. The injection volume used for ethanol analysis was 1 μL.

The dichromate method was also employed to detect the ethanol content by its conversion to acid following dichromatic reaction [26]. The cell free supernatant of fermentation broth (1 mL) was mixed with 0.115 M $K_2Cr_2O_7$ (2 mL) with final addition of 9 mL distilled water. The 12 mL reaction mixture was kept for 10 min in a boiling water bath. Finally, the absorbance of the cooled sample was measured against a blank of potassium dichromate ($K_2Cr_2O_7$) as standard using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) at 600 nm.

The ethanol yield (g of ethanol g of substrate^{-1}) was obtained by dividing the maximum ethanol concentration (g L^{-1}) attained in SSF experiments with initial cellulose and hemicellulose concentration (g L^{-1}) of the pretreated wild grass (lignin was not taken into account). When these ethanol yields are compared with the theoretical 0.51 g ethanol/g of sugar (glucose or xylose) yield, since the residual cellulose and hemicellulose contents after fermentation were not determined in our SSF studies, the amount of cellulose and hemicellulose consumed could not be calculated. Similar method for calculation of ethanol yield has been reported earlier [27].

3. Results and Discussion

The improved saccharification of cellulosic and hemicellulosic components of lignocellulosic biomass by competent hydrolytic enzymes with simultaneous consumption of

Table 3: Comparison of unoptimized and Taguchi optimized SSF combinations with mixed pretreated wild grass.

SSF combination	Substrate concentration (%, w v^{-1}) and mode of SSF	Reducing sugar* (g L^{-1})	Ethanol yield (g of ethanol g of pretreated substrate^{-1})	Ethanol titre* (g L^{-1})
GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (unoptimized)	1% shake flask	1.70 ± 0.09	0.228	1.50 ± 0.06
GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (Taguchi optimized)	1% shake flask	2.31 ± 0.05	0.304	2.0 ± 0.04
GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (Taguchi optimized)	1% bioreactor	4.02 ± 0.03	0.472	3.10 ± 0.07

*The values correspond to the maximum reducing sugar and maximum ethanol at a particular time; values are mean ± SE ($n = 3$).

monomeric sugars by fermentative microbes is the technoeconomic viability of an efficient SSF process. The structural carbohydrates determination of wild grass (*A. hymenoides*) revealed greater amount of cellulose (50.09 ± 0.32%, w w^{-1}) followed by hemicellulose (29.9 ± 0.67% w w^{-1}), suggesting wild grass as the suitable candidate for SSF based bioethanol production. The microwave-assisted alkali (MAA) pretreatment is reported to increase cellulose hydrolysis [19] and the organosolv pretreatment is more effective for hemicellulosic content breakdown of agroresidues [20]. The carbohydrate composition of wild grass after mixed pretreatment revealed cellulose (43.32 ± 0.51%, w w^{-1}) and hemicellulose (22.35 ± 0.48% w w^{-1}). In the current study, the desired volume of recombinant *C. thermocellum* mixed enzymes for the production of simple sugars and the inoculum volume of mixed fermentative microbes along with other process parameters for bioethanol production from mixed MAA and organosolv pretreated wild grass were optimized by Taguchi statistical design in shake flask and scaled up in bioreactor.

3.1. Unoptimized Simultaneous Saccharification and Fermentation (SSF) Process of Mixed Pretreated 1% (w v^{-1}) Wild Grass at Shake Flask Level. The dynamic profile of SSF involving unoptimized process parameters for ethanol production from mixed pretreated 1% (w v^{-1}) wild grass at shake flask level is represented in Figure 1. The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited negligible lag phase in their growth with steady increase till 66 h with slight decrease thereafter (Figure 1). The growth-associated ethanol formation began from 12 h of SSF with a gradual increase till 36 h after which a sharp rise was observed till 54 h (Figure 1). The maximum ethanol titre achieved was 1.50 g L^{-1} (Table 3, Figure 1) with a yield of 0.228 (g of ethanol g of substrate^{-1}). Thereafter, a decrease in ethanol production was witnessed. The initial phase of the SSF represented an accumulation of available sugars till 18 h with a gradual decline. The maximum reducing sugar concentration was 1.70 g L^{-1} (Figure 1). The activities of both the recombinant enzymes decreased with the progress in fermentation. The dynamic profile of only recombinant GH5 cellulase has been shown in Figure 1 as wild grass contains more cellulose. Interestingly, the microbial growth and ethanol production shared an inverse relationship with enzyme activities and, in turn, the reducing sugars released

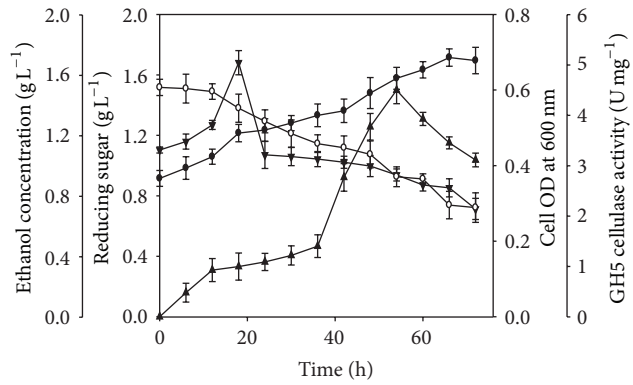

Figure 1: SSF profile of 1% (w v^{-1}) mixed MAA and organosolv pretreated wild grass (*Achnatherum hymenoides*) using unoptimized fermentation process parameters, namely, recombinant cellulase (GH5), recombinant hemicellulase (GH43) along with *S. cerevisiae*, *C. shehatae,* pH, and temperature at shake flask level showing variation of (●) cell OD measured at 600 nm, (▲) ethanol concentration (g L^{-1}), (▼) reducing sugar (g L^{-1}), and (○) specific activity (U mg^{-1}) of GH5 cellulase with time (h). Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown).

clearly demonstrating the fact of sugar utilization by the organisms for growth and ethanol formation (Figure 1).

3.2. Optimization of Process Parameters of Simultaneous Saccharification and Fermentation (SSF) Involving Mixed Pretreated Wild Grass by Taguchi Method. Taguchi experimental design is a good positive option for the optimization of biotechnological processes. The fermentation process parameters, namely, temperature, pH, hydrolytic enzyme volume, and fermentative microbe's inoculum volume, play an important role in lignocellulosic ethanol production [9]. In this case, the influence of 6 factors on the SSF process was tested by Taguchi experimental design in 25 runs (Tables 1 and 2). The response values in terms of ethanol titre (%, v v^{-1}) and S/N ratios of Taguchi experimental design in 25 runs, for the six factors, that is, recombinant GH5 cellulase volume, recombinant GH43 hemicellulase volume, *S. cerevisiae* inoculum volume, *C. shehatae* inoculum volume, pH, and temperature (°C), chosen for optimization of ethanol production by SSF process (Table 4), show the efficiency of

Table 4: Response values and S/N ratio of L_{25} Taguchi orthogonal array design.

Run/expt. no.	Response in terms of ethanol titre (%, v v^{-1})*	S/N ratio
1	0.1527 ± 0.05	−16.32
2	0.1892 ± 0.04	−14.46
3	0.1427 ± 0.09	−16.92
4	0.1888 ± 0.02	−14.48
5	0.1828 ± 0.07	−14.76
6	0.1203 ± 0.08	−18.39
7	0.1874 ± 0.06	−14.55
8	0.2102 ± 0.01	−13.55
9	0.1837 ± 0.03	−14.72
10	0.2252 ± 0.06	−12.95
11	0.1910 ± 0.05	−14.38
12	0.1938 ± 0.07	−14.26
13	0.2093 ± 0.08	−13.59
14	0.1226 ± 0.08	−18.23
15	0.2170 ± 0.04	−13.27
16	0.2024 ± 0.05	−13.87
17	0.2006 ± 0.03	−13.95
18	0.2056 ± 0.02	−13.74
19	0.1245 ± 0.01	−18.09
20	0.2198 ± 0.08	−13.16
21	0.2170 ± 0.09	−13.27
22	0.2179 ± 0.07	−13.24
23	0.2512 ± 0.03	−11.99
24	0.2225 ± 0.02	−13.05
25	0.1267 ± 0.05	−17.94

*The values correspond to the maximum ethanol at a particular time; values are mean ± SE ($n = 3$).

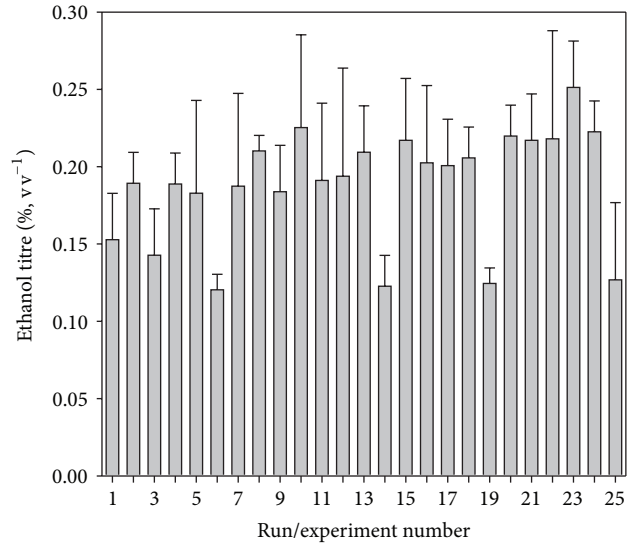

Figure 2: Comparative results of response in terms of ethanol titre (%, v v^{-1}) of Taguchi L_{25} orthogonal array of experiments.

ethanol production ranging from 0.120 (%, v v^{-1}) to 0.251 (%, v v^{-1}) corresponding to the combined effect of the six factors in their specific ranges. The experimental results suggest that these factors at optimum level strongly support the production of ethanol. In run (expt. 6), with a combination of recombinant GH5 cellulase volume (0.25 mL), recombinant GH43 hemicellulase volume (0.25 mL), *S. cerevisiae* inoculum volume (0.25 mL), *C. shehatae* inoculum volume (0.25 mL), pH (3), and temperature (26°C), an ethanol concentration of 0.120 (%, v v^{-1}) was observed (Table 4, Figure 2). A maximum ethanol titre of 0.251 (%, v v^{-1}) ethanol was observed in run (expt. 23) with a combination of recombinant GH5 cellulase volume (1.0 mL), recombinant GH43 hemicellulase volume (0.5 mL), *S. cerevisiae* inoculum volume (1.50 mL), *C. shehatae* inoculum volume (0.25 mL), pH (5), and temperature (30°C) with the best response and maximum S/N ratio (−11.99) (Table 4, Figure 2).

The Taguchi optimized fermentation process parameters are shown in Figure 3. The best process parameters in 100 mL of fermentation medium comprised 1.0 mL of recombinant GH5 cellulase (5.7 U mg^{-1}, 0.45 mg mL^{-1}), 2.0 mL of recombinant GH43 hemicellulase (3.7 U mg^{-1}, 0.32 mg mL^{-1}), 1.5 mL of *S. cerevisiae* (3.9×10^8 cells mL^{-1}), 0.25 mL of *C. shehatae* (2.7×10^7 cells mL^{-1}), pH of 4.3, and temperature of 35°C.

The analysis of variance (ANOVA) for the responses of ethanol production was carried out according to the factors' contribution by the Taguchi method (Table 5). From the calculated ratios (F), it can be inferred that the factors considered in the experimental design are statistically significant at 95% confidence limit. Table 6 represented the contribution of the selected factors to bioethanol production. It can be observed that, on the basis of p-value ($p < 0.05$), pH with rank 1 is the most significant of all other factors and shows the highest positive impact on the ethanol production. *C. shehatae* inoculum volume showed the least impact on ethanol production among the factors studied with the assigned variance of values. Several scientists have reported that the transport of chemical products and enzymes across the cell membrane is affected by the pH of the fermentation medium, influencing many enzymatic reactions [28]. The statistical outcomes in our research also confirmed fermentation medium pH to be an important factor affecting SSF. Similar findings have been reported in the literature [9]. *C. shehatae* inoculum volume showed the least impact among the factors studied with the assigned variance of values.

3.3. Validation of Taguchi Experimental Model. The validation of Taguchi experimental model is represented in Table 7. It was observed that the response (ethanol %, v v^{-1}) (0.254) as well as S/N ratio (−10.95) for Taguchi optimum values was more than the experimental optimum values for ethanol production (0.2512%, v v^{-1}) and S/N ratio (−11.99) (Table 7). This validated the Taguchi optimized SSF process parameters. Thus, there was a 1.3-fold increase in ethanol titre with Taguchi optimized SSF process parameters as compared

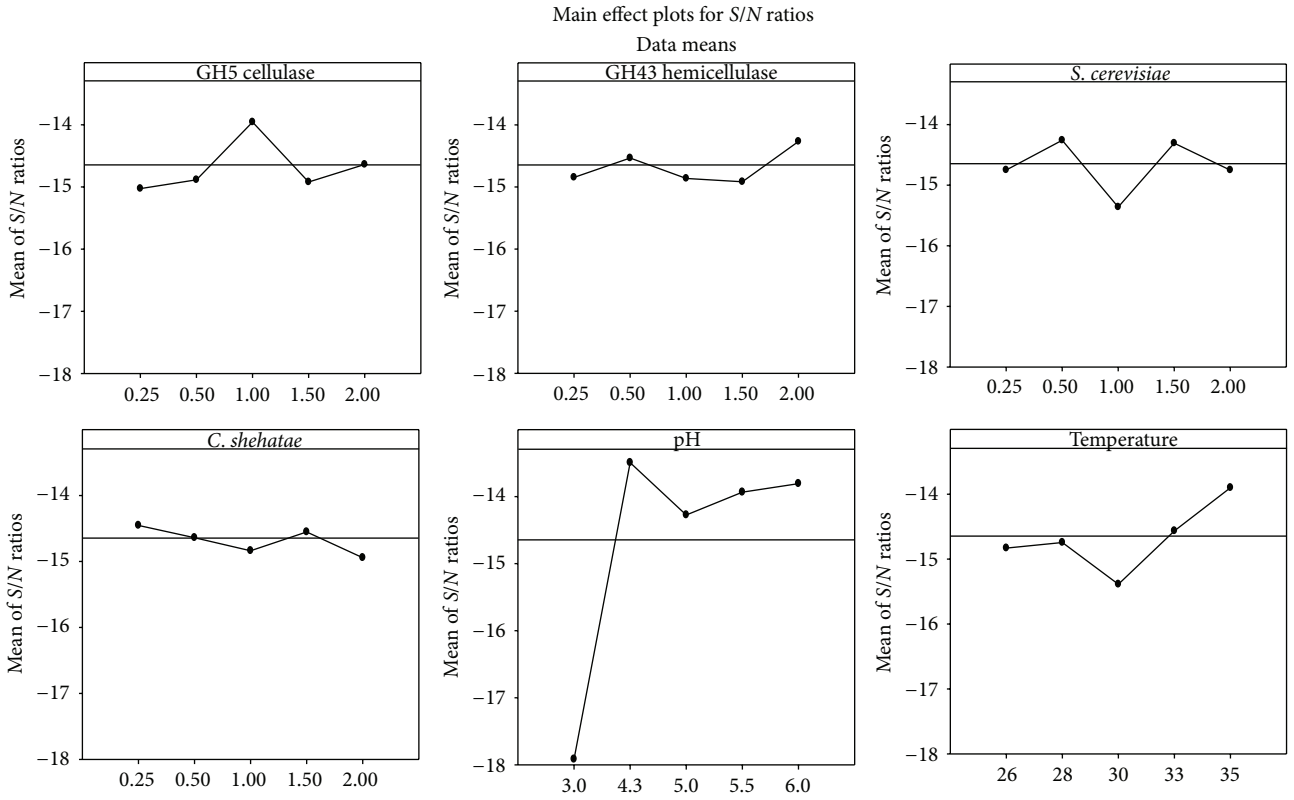

Figure 3: Main effect plots for S/N ratios with larger-the-better objective function of Taguchi optimized fermentation process parameters.

Table 5: Analysis of variance for the responses of ethanol production.

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Recombinant GH5 cellulase	1	0.0000660	0.0000660	0.0000660	0.08	0.783
Recombinant GH43 hemicellulase	1	0.0000637	0.0000637	0.0000637	0.08	0.786
S. cerevisiae	1	0.0000000	0.0000000	0.0000000	0.00	0.996
C. shehatae	1	0.0002732	0.0002732	0.0002732	0.32	0.576
pH	1	0.0150152	0.0150152	0.0150152	17.85	0.001
Temp	1	0.0012587	0.0012587	0.0012587	1.50	0.237
Error	18	0.0151394	0.0151394	0.0008411		
Total	24	0.0318162				

DF: degrees of freedom, SS: sum of squares, and MS: mean of squares.

to unoptimized parameters (Table 3). These experiments supported the analysis of the main effect of each constituent of the medium. The Taguchi SSF experiments provided basic information for the improvement of the ethanol production efficiency. Finally, using the Taguchi optimized fermentation process parameters (Table 7), the SSF process was scaled up at bioreactor level.

3.4. Scale-Up of Taguchi Optimized Simultaneous Saccharification and Fermentation (SSF) Process Parameters Involving Mixed Pretreated 1% ($w\,v^{-1}$) Wild Grass at Bioreactor Level. It is a well-established fact that the fermentation dynamics and, in turn, the final ethanol titre are significantly affected by the parameters, namely, pH and aeration [29]. The SSF process involving statistically designed Taguchi optimized fermentation process parameters and mixed pretreated 1% ($w\,v^{-1}$) wild grass was finally scaled up in an automated bioreactor enabling the stringent monitoring of important process parameters (Figure 4).

S. cerevisiae and *C. shehatae* remained in a very short lag phase of initial 6 h and displayed an exponential growth profile (Figure 4). Until the 66 h, the biomass concentration increased considerably as the organisms entered the log phase reaching a maximum cell OD ($A_{600\,nm}$) of 1.4, and, finally, a decline phase was observed thereafter. A biphasic

Table 6: Rank and significance of various factors.

Factor/parameter	Rank	p-value
Recombinant GH5 cellulase* ($5.7\,U\,mg^{-1}$, $0.45\,mg\,mL^{-1}$)	4	0.783
Recombinant GH43 hemicellulase* ($3.7\,U\,mg^{-1}$, $0.32\,mg\,mL^{-1}$)	5	0.786
*S. cerevisiae** (3.6×10^8 cells mL^{-1})	3	0.996
*C. shehatae** (2.1×10^8 cells mL^{-1})	6	0.576
pH	1	0.001
Temperature	2	0.237

$p < 0.05$.

Table 7: Validation of Taguchi experimental data values.

Factor/parameter	Taguchi optimum	Experiment optimum
Recombinant GH5 cellulase ($5.7\,U\,mg^{-1}$, $0.45\,mg\,mL^{-1}$) (%, $v\,v^{-1}$)	1.0	1.0
Recombinant GH43 hemicellulase ($3.7\,U\,mg^{-1}$, $0.32\,mg\,mL^{-1}$) (%, $v\,v^{-1}$)	2.0	0.5
S. cerevisiae (3.6×10^8 cells mL^{-1}) (%, $v\,v^{-1}$)	1.5	1.5
C. shehatae (2.1×10^8 cells mL^{-1}) (%, $v\,v^{-1}$)	0.25	0.25
pH	4.3	5.0
Temperature (°C)	35	35
S/N ratio	−10.95	−11.99
Response experimental ethanol titre (%, $v\,v^{-1}$)	0.2540	0.2512
Response predicted (%, $v\,v^{-1}$) ethanol titre (%, $v\,v^{-1}$)	0.2705	0.2677
Ethanol titre ($g\,L^{-1}$)	2.00	1.98
Ethanol yield (g of ethanol g of substrate^{-1})	0.200	0.198

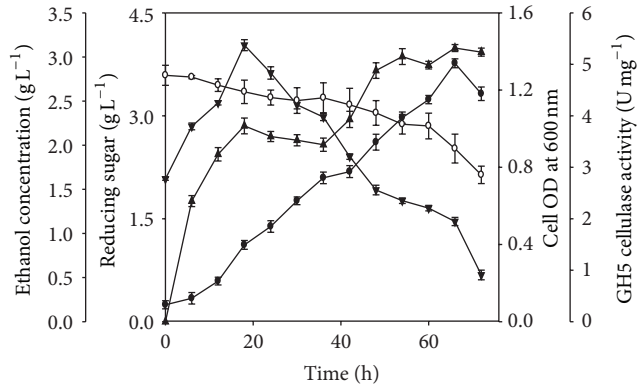

Figure 4: SSF profile of 1% ($w\,v^{-1}$) mixed MAA and organosolv pretreated wild grass (*Achnatherum hymenoides*) using statistically designed Taguchi optimized fermentation process parameters, namely, recombinant cellulase (GH5), recombinant hemicellulase (GH43) along with *S. cerevisiae*, *C. shehatae,* pH, and temperature, at bioreactor level showing variation of (●) cell OD measured at 600 nm, (▲) ethanol concentration ($g\,L^{-1}$), (▼) reducing sugar ($g\,L^{-1}$), and (○) specific activity ($U\,mg^{-1}$) of GH5 cellulase with time (h). Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown).

ethanol formation was recorded. The initial phase of ethanol production documented a titre of $2.25\,g\,L^{-1}$ at 18 h of SSF followed by a slight decrease in ethanol synthesis rate till 36 h. The final phase of ethanol kinetics witnessed a maximum ethanol concentration of $3.10\,g\,L^{-1}$ (Figure 4) with an ethanol yield of 0.472 (g of ethanol g of substrate^{-1}) at 66 h and then a declination in ethanol titre was observed till the end of the fermentation process (Table 3, Figure 4). The reducing sugar concentration peaked during the initial 18 h of fermentation reaching a maximum concentration of $4.02\,g\,L^{-1}$ (Table 3, Figure 4). As *A. hymenoides* have more cellulosic content, the dynamic profile of only recombinant GH5 cellulase has been presented in Figure 4. The activities of mixed enzymatic consortium decreased with the progress of SSF. The drop in reducing sugar concentration after 18 h clearly indicated the sugar uptake by the hexose and pentose utilizing microbes for their growth, maintenance, and ethanol production.

The controlled parameters of pH and aeration rate significantly affected the growth and ethanol concentration. A threshold dissolved oxygen (DO) level of minimum 40% was maintained by 1 vvm aeration rate for the efficient growth of bioethanol producers and, in turn, a good product yield. The ethanol titre obtained in Taguchi optimized shake flask SSF was $2.0\,g\,L^{-1}$ (Table 3) implying a 1.3-fold rise as compared to ethanol titre of $1.5\,g\,L^{-1}$ (Table 3) in unoptimized shake flask SSF. A 1.5-fold upsurge in ethanol titre ($3.1\,g\,L^{-1}$) (Table 3) was obtained in lab scale bioreactor on scaling up the shake flask SSF ($2.0\,g\,L^{-1}$) (Table 3) with Taguchi optimized SSF process parameters. The dynamic profiles of various offline measurements from various SSF batch runs established a complex interplay between the rates of saccharification by the mixed recombinant enzymes, utilization of sugar by bioethanol producers, and finally the formation of ethanol. The reducing sugar profile was inversely proportional to the rate of ethanol formation. The repressed enzyme activities in the later stages of fermentation might be attributed to sugar accumulation in the broth. A depleted reducing sugar concentration was observed without any further upturn in ethanol titre during the late log phase indicating the sugars utilization only for maintenance and endurance of the fermentative microbes.

The ethanol titre values obtained in our research are comparable with the findings reported in the literature. An ethanol titre of $2.1\,g\,L^{-1}$ has been reported from 1% ($w\,v^{-1}$) mango leaves with recombinant GH43 hemicellulase from *C. thermocellum* and *C. shehatae* [3]. The coculture of *C. thermosaccharolyticum* HG8 and *Thermoanaerobacter ethanolicus* ATCC 31937 provided an ethanol concentration ($2.2\,g\,L^{-1}$) from 1% ($w\,v^{-1}$) of banana waste [30]. The recombinant cellulase from *Clostridium thermocellum* offered an ethanol titre of $1.4\,g\,L^{-1}$ from 1% ($w\,v^{-1}$) Jamun (*Syzygium cumini*) leafy biomass [31]. A SSF process from 6% ($w\,w^{-1}$) solka floc employing commercial cellulase and *Kluyveromyces*

marxianus contributed to an ethanol yield of 0.337 ($g\,g^{-1}$) [27]. An ethanol titre of $1\,g\,L^{-1}$ from 1% ($w\,v^{-1}$) wheat straw using crude unprocessed *Trichoderma reesei* cellulase has been reported [32].

4. Conclusions

This study reported for the first time the statistical optimization and validation of different fermentation process parameters for bioethanol production from mixed MAA and organosolv pretreated 1% ($w\,v^{-1}$) wild grass using Taguchi orthogonal array design, namely, mixed recombinant *C. thermocellum* hydrolytic enzymes' volume along with mixed fermentative microbes' inoculum volume, pH, and temperature. The model was successfully validated at shake flask level with pH as the most significant factor. Finally, the optimized process parameters were scaled up at bioreactor level with a gain of significant ethanol titre. In essence, the statistical optimization of fermentation process parameters involving recombinant enzymes can transform the weed, *A. hymenoides,* into the fuel of tomorrow, bioethanol.

Conflict of Interests

The authors clearly state that they do not have any possible conflict of interests with the mentioned commercial identities.

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