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Optimization of enzymatic saccharification and fermentation process parameters for production of bioethanol from *Populus nigra* using recombinant enzymes from *Clostridium thermocellum*

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Abstract

The optimization of various parameters such as dosage volume of recombinant hydrolytic enzymes from Clostridium thermocellum, inoculum volume of Candida shehatae, pH and temperature was carried out for improved bioethanol production in simultaneous saccharification and fermentation (SSF) process using Taguchi Orthogonal Array design. The initial SSF trials were performed in 100 ml medium at shake flask level using 1% (w/v) ammonia fibre expansion (AFEX) pretreated Populus nigra leafy biomass. The optimized parameters for SSF process were, 2.0 ml recombinant xylanase (CtXyn30A), 2.0 ml recombinant Acetylxylan esterase (Axe2, 4.4 U/mg, 0.37 mg/ml), 4.6 U/mg, 0.31 mg/ml), 2.0 ml C. shehatae (~4.3 x 10^6 cells/ml), pH 6.5 and temperature 33°C. On the basis of p-value ($p < 0.05$), the three most significant factors were, the inoculum (C. shehatae) volume, temperature and pH. The optimized SSF conditions with 1% (w/v) pretreated biomass at flask level gave an ethanol titre of 1.06 g/l. The monosaccharide analysis of SSF exhibited the release of xylose from hydrolysed biomass. The increased biomass 5% (w/v), under optimized parameters gave an ethanol titre and yield of 6.10 g/l, 0.317 (g of ethanol/g of pretreated biomass) at flask level and its scale-up to 3l bioreactor level contributed ethanol titre of 7.10 g/l and yield 0.369 (g/g), respectively.

Keywords: SSF, Taguchi Orthogonal Array design, HPAEC-PAD, Bioreactor, Bioethanol.

Introduction

The world currently depends significantly upon non-renewable sources such as petroleum along with its derivatives to meet the energy requirements which is depleting day by day[1]. Burning of fossil fuels with increased emission of greenhouse gases has led to an upsurge in atmospheric temperature at an alarming extent. The quest for replacement of fossil fuels has given rise to realizing biofuels as a promising alternative. Lignocellulosic residues contain an abundance of fermentable sugars in the form of hexose and pentose polymers and have a huge potential as a substitute, among renewable energy resource[1,2]. For the cost effective production of bioethanol from lignocellulosic saccharification and fermentation, there is a necessity for the improved activity of hydrolytic enzymes followed by efficient utilization of sugar by fermentative microbes[2]. The leafy and residual agricultural, lignocellulosic biomass is an encouraging raw substrate because of their easy accessibility, economical and categorized under non-competitive food crops[3].

A major requirement in cost effective lignocellulosic biomass processing that involves the employment of effective reactor systems with the optimized process parameters such as pH, hydrolytic enzymes' dosage volume, temperature, inoculum volume of fermentative microbes[4]. The multiple experimental analyses for exploring the effect of various independent variables by the approach of one variable at a time (OVAT) are tough and time taking[5]. Statistically centered various experimental designs like Taguchi orthogonal array, Box-Behnken and Placket-Burman, represents the combination and organizing of various variables along with their quantity analysis at new parameters to be accounted. The main approach of Taguchi design of experiments (DOE) is to abate the time and cost by producing extensive understanding with lesser experimental assessments, without conceding the product quality[6]. Successful implication of the Taguchi design for the optimization of various reaction variables have been involved for the production of biodiesel and activated carbon[7, 8].

In India the abundance of polar (*Populus nigra*), is primarily in the temperate/ sub-temperate regions of north-western states[9]. There is a necessity for exploring the efficient pretreatment required for the delignification, with the subsequent discharge of cellulose and hemicelluloses for effective saccharification of the pretreated lignocellulosic substrate. The AFEX pretreatments of poplar leafy biomass is reported for the enhanced loosening of hemicellulosic component and removal of inhibitors *viz.,* derivatives of furan and phenolic compounds[10], which in turn results in increased accessibility for enzymes involved in saccharification[11].

Clostridium thermocellum, thermophilic anaerobic bacterium encompasses a group of glycoside hydrolases, assembled together to form a high molecular weight complex,

cellulosome[12]. It shows 50-fold higher specific activity against crystalline cellulose as compared to its analogous system of *Trichoderma reesei*, the most studied cellulase producing fungal strain[13,14]. Glycoside hydrolases are a group of enzymes with varying substrate specificity, which also includes cellulases and hemicellulases. The glycoside hydrolase family 30 (GH30) displays the activities of β-xylosidase (EC 3.2.1.37); β-glucosidase (3.2.1.21); β-1, 6-glucanase (EC 3.2.1.75); glucosylceramidase (EC 3.2.1.45); endo-β-1, 6-galactanase (EC: 3.2.1.164), as described by the CAZy database[15]. Acetyl xylan esterase (Axe2) is an accessory enzyme that hydrolyses the xylose moieties present at the side chains of xylan backbone and displays substantial activity with birchwood xylan[16]. These enzyme systems cloned and expressed in *Escherichia coli* for deriving monomeric sugar out of lignocellulosic biomass. This is primarily owing to the cost intensive involvement of high temperature and anaerobic culturing conditions for production of enzymes from *C. thermocellum*.

Candida shehatae, a fungal organism possessing xylitol dehydrogenase and xylose reductase, to uptake C-5 sugars for ethanol production from the hydrolyzed hemicellulosic component of lignocellulosic biomass[17]. In the simultaneous saccharification and fermentation (SSF) process, the phases for saccharification and fermentation are essentially the same, as per separate hydrolysis and fermentation stages, except that both are carried out, simultaneously in the same bioreactor. It thereby, decreases the processing time phase as well as reducing the end-product inhibition of enzyme and thus leading to the improved production of ethanol[18].

The aim of the present study was to optimize and validate the various factors by Taguchi statistical design, for improved bioethanol production from AFEX pretreated poplar leafy biomass. Temperature, pH, dosage volume (ml) of recombinant hydrolytic enzymes from *Clostridium thermocellum* and inoculum volume of *Candida shehatae* are the important parameters considered for bioethanol production. The model was subsequently validated with 1% (w/v) AFEX-treated biomass at shake flask level. Later, the AFEX-treated substrate was increased up to 5% (w/v) at flask level and was subsequently scaled-up to 3liter bioreactor level.

Material and Methods

Chemicals and substrate: The analytical grade chemicals and reagents *viz.,* yeast extract, sodium potassium tartrate, sodium chloride, glucose, ampicillin, peptone, tryptone, sodium carbonate, copper sulphate, potassium dichromate, magnesium sulphate heptahydrate, sodium sulphate, ammonium molybdate, potassium dihydrogen phosphate, sodium arsenate and agar were procured from Himedia Pvt. Ltd., India. Ethanol and phosphoric acid were obtained from Merck Pvt. Ltd. India., Qualigens India Pvt. Ltd., respectively. Kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) were procured from Sigma Aldrich (St. Louis, USA). Liquid NH_3 was obtained from Merck India Pvt. Ltd. Leafy biomass of poplar (*Populus nigra*) were collected, washed (thrice with H_2O), dehydrated, grinded and consequently passed by a sieve of mesh size, 1 mm.

Microorganisms and culturing conditions: In earlier studies a family 30 glycoside hydrolase (GH30), the glucuronoxylan xylanohydrolase (*Ct*Xyn30A)[15] and a family 2 carbohydrate esterase (CE2), the acetylxylan esterase (Axe2)[16], both from *C. thermocellum* were separately cloned in pET-28a(+) and pET-21a(+) respectively, and hyper-expressed in *E. coli* BL21 (DE3). The cells harbouring the recombinant plasmids were cultured and maintained as glycerol stock at -80°C in the Carbohydrate Enzymes and Biotechnology Laboratory at Department of Biosciences and Bioengineering, IIT Guwahati, India. The recombinant acetylxylan esterase (Axe2) showed activity on xylans whereas recombinant glucuronoarabinoxylan 4-β-D-xylanohydrolase (*Ct*Xyn30A) displayed activity predominantly on xylan polysaccharides substituted with 4-O-methylglucuronic acid (or glucuronic acid) such as beechwood-, birchwood- and glucurono-xylan moreover, it is also capable of hydrolysing xylan polysaccharides decorated with arabinose moiety like rye- wheat- and oat spelt-xylan with one-third less activity as compared to glucuronic acid substituted xylans (unpublished data).

The fermentative microbe, *Candida shehatae* (NCIM no: 3500) primarily pentose sugar utilizing, was acquired from National Chemical Laboratory (NCL), Pune. The culture was grown in MGYP medium containing (g/100 ml) Malt extract (0.3), Glucose (1.0), Yeast extract (0.3) and Peptone (0.5) and 2% agar in 5 ml slants and maintained at 4°C. A loopful from this slant culture (*C. shehatae*) was inoculated into 50 ml GYE (Glucose Yeast extract) medium. The 100 ml GYE medium contained glucose (1.0 g) and yeast extract (0.1g), $MgSO_4.7H_2O$ (0.05g), KH_2PO_4 (0.1g) and $(NH_4)_2SO_4$ (0.5g) at pH 5.0. The culture medium was incubated at shaking of 120 rpm at 30°C for 48 h, prior to its inoculation for fermentation process. One ml culture of actively growing *C. shehatae* (~4.3 x 10^6 cells/ml) was inoculated to fermentation medium (100 ml). The cell count was performed using a haemocytometer.

Production of recombinant xylanase (*Ct*Xyn30A) and acetylxylan esterase (Axe2): 50 µl of the glycerol stocks of *E. coli* cultures containing recombinant *Ct*Xyn30A and Axe2 were separately inoculated into 5 ml Luria Bertani (LB) medium containing kanamycin (50 µg/ml) and ampicillin (100 µg/ml), respectively. The cultures were incubated at 180 rpm for 16 h at 37°C. One percent (v/v) of each of grown culture was inoculated separately to 200 ml of LB medium containing respective antibiotics. The inoculated LB medium was incubated at 37°C and 180 rpm till absorbance at 600 nm (A_{600}) of the cells reached 0.6. After that 1 mM final concentration of Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each culture and further incubated at 24°C, 180 rpm for 16 h, for the protein expression. The *E. coli* cells were harvested by centrifugation at 7426Xg and 4°C for 30 min. The cell pellets

were resuspended in 15 ml, 20 mM sodium phosphate buffer, pH 6.0 and sonicated by keeping on ice-bath for 15 min (5 s on/10 s off pulse, 30% amplitude) using a sonicator (Vibra Cell, Sonics, Newtown, CT, USA). The sonicated cells were centrifuged at 19,650Xg and 4°C for 30 min. The pellets containing cell debris were discarded and the cell free supernatants (CFE), containing the recombinant enzymes viz., xylanase *(CtXyn30A)* and acetylxylan esterase (Axe2) were used for SSF experiments.

Ammonia fibre explosion (AFEX) pretreatment strategy: Due to considerably greater fraction of hemicellulosic content in poplar leafy biomass, it was subjected to AFEX pretreatment as reported earlier[19]. Prior to its usage in saccharification and fermentation, the carbohydrate structural composition of both untreated poplar and AFEX pretreated poplar were analyzed by standardized methods adopted from National Renewable Energy Laboratory (NREL), USA[20].

SSF of pretreated biomass involving xylanase (*Ct*Xyn30A) along with acetylxylan esterase (Axe2) at initial shake flask: The simultaneous enzymatic hydrolysis and fermentation experiments were performed with 1g of AFEX-treated poplar biomass in 100 ml fermentation medium in 250 ml flasks, containing 0.02 M sodium phosphate buffer (pH 7.0), yeast extract (0.2%) and peptone (0.2%) as fermentation medium in 250 ml flasks. 0.5ml, inoculum dosage of each *Ct*Xyn30A (0.31 mg/ml, 4.6 U/mg) and Axe2 (0.37 mg/ml, 4.4 U/mg) were added to the above medium. Simultaneously, 1 ml inoculum volume of fermentative microbe i.e., *C. shehatae* (~4.3 x 10^6 cells/ml) was inoculated to the flasks. The flasks were incubated at 35°C and 120 rpm for 72 h. The 2 ml sample were collected periodically at an interval of 6 h for the estimation of absorbance at 600 nm (A_{600}), ethanol concentration (g/l), specific activity (U/mg) and reducing sugar (g/l).

Statistical optimization of SSF process of AFEX pretreated poplar involving mixed enzyme-single culture system by Taguchi method in shake flask: Optimization using Taguchi statistical orthogonal array design: Experimental design matrix for Taguchi, comprises standard orthogonal array L_{25} (5^5) was used to determine five factors *viz.,* volume (ml) recombinant *Ct*Xyn30A (4.6 U/mg, 0.31 mg/ml), recombinant Acetylxylan esterase Axe2 (4.4 U/mg, 0.37 mg/ml), inoculum volume (ml) of fermentative microbe *viz., C. shehatae* (~4.3 x 10^6 cells/ml), pH and temperature (°C) at five levels, *viz.,* Level 1 to Level 5 (table-1a) in SSF experiments involving 1% (w/v) AFEX-treated biomass at initial shake flask. Levels of all these five factors were considered and the matrix outline of L_{25} Taguchi's orthogonal array is represented in Tables 1a and 1b. All the SSF experiments were performed in fermentation medium of working volume 100 ml at 120 rpm for 3 days at varying temperatures and pH according to (table-1a) with sample collection at an interval of 6 h.

Exploration of various experiments (runs) from the Taguchi orthogonal array: To determine the outcome of the fermentation runs, the software MINITAB®, Design Expert, version 8.0 was used. Taguchi experimental design for 25 runs comprising the response values, determined in term of ethanol titre (%, v/v) and S/N ratios were analysed to find out the main effects of the factors independently; thereafter determining the factors that were statistically significant, by analysis of variance technique (ANOVA). The controlling factors were explored, along with the degree of effects and later the statistically significant effects were determined. Accordingly, the ideal conditions were analyzed by combining the levels of factors that had the uppermost main effect value. For the outcome of ethanol production values, the ANOVA was performed to confer each factor contribution by Taguchi approach. For individual run, the S/N ratio conforming to larger-the-better primarily function was calculated using the following relation described in Eq. 1

$$\frac{S}{N} = -10 \log_{10} \frac{1}{X} \sum_{z=1}^{X} \frac{1}{k_z^2} \quad (1)$$

Where, 'k_z' is signal and 'X' is repetitions number during each experiment.

To ascertain the ratio (F) and the p value ($p< 0.05$), the parameters (factors) in the experimental design at 95% confidence limit were accounted to be statistically significant.

Validation of Taguchi model optimized parameters: The validation of the model was accomplished by performing the SSF trials involving the parameters optimized through Taguchi method using 1% (w/v) pretreated poplar biomass in 100 ml of fermentation medium contained in 250 ml flasks. The optimised parameters for fermentation process were 2.0 ml of each recombinant xylanase, *Ct*Xyn30A (4.6 U/mg, 0.31 mg/ml) and recombinant Acetylxylan esterase (Axe2) (4.4 U/mg, 0.37 mg/ml) along with 2.0 ml of *C. shehatae* (~4.3 x 10^6 cells/ml), pH 6.5 and temperature 33°C. These optimised parameters were used for validation of the Taguchi model by determining the value of ethanol titre (%, v/v). The enzymatic saccharification and fermentation was carried out for 72 h at 120 rpm and the samples (2 ml) were collected at every 6 h.

SSF of 5% (w/v) AFEX pretreated leafy biomass at shake flask level: Five grams of AFEX-treated poplar biomass was utilized for SSF trials in 100 ml of medium containing 20 mM sodium phosphate buffer (pH 6.5) along with yeast extract (0.2%) and peptone (0.2%), in 250 ml flasks. Mixed enzymatic consortium of inoculum dosage, 10 ml each of *Ct*Xyn30A (4.6 U/mg, 0.31 mg/ml) and Axe2 (4.4 U/mg, 0.37 mg/ml) were added in 250 ml flasks. Simultaneously, the inoculum volume of 10 ml of *C. shehatae* (~4.3 x 10^6 cells/ml) was added to the flasks for the reduced sugar utilization. The SSF experiment was carried out at 33°C and 120 rpm for three days. The samples (2 ml) were collected at every 6 h for estimation of

cell growth (A_{600}), specific activities (U/mg), ethanol concentration (g/l) and reducing sugar concentration (g/l). All the saccharification and fermentation experimental trails were carried out in triplicates.

SSF of 5% (w/v) AFEX pretreated leafy biomass at bioreactor level: AFEX-treated poplar leafy biomass (50 g) was added to 1 liter of 20 mM sodium phosphate (pH 6.5) buffer along with yeast extract (0.2%) and peptone (0.2%), in applikon bioreactor (3 liter) (Bio Console ADI 1025, Netherlands). Mixed dosage of recombinant enzymes, containing 100 ml each of *Ct*Xyn30A (4.6 U/mg, 0.31 mg/ml) and Axe2 (4.4 U/mg, 0.37 mg/ml) were employed together for saccharification along with 100 ml of *C. shehatae* (~4.3 x 10^6 cells/ml) for fermentation. The SSF trials were performed at 33°C and 120 rpm for 3 days in batch mode. 40% dissolved oxygen (DO) level, was maintained by keeping an aeration rate of 1 vvm, through mass flow controller for the proficient growth of *C. shehatae*. 2 ml sample was collected from bioreactor after every 6 h for assessment of cell growth (A_{600}), reducing sugar concentration (g/l), ethanol concentration (g/l) and specific activity of Axe2 (U/mg) and *Ct*Xyn30A (U/mg). During the experiment, a constant pH of 6.5 was sustained constantly by addition of 1N NaOH and1N HCl.

Analytical methods: Monosaccharide analysis by HPAEC: The monosaccharides released by the enzymatic saccharification of AFEX pretreated poplar biomass by *Ct*Xyn30A and Axe2 during the bioreactor run was analyzed by High Pressure Anion Exchange Chromatography (HPAEC) (Dionex, ICS-3000). 100 µl samples from 5% (w/v) SSF process running in the bioreactor were taken periodically at 0, 18, 36, 54 and 72h. Each sample was diluted by two volumes of ethanol. The samples were prepared for monosaccharide analysis as described elsewhere[21]. The sample volume (diluted supernatant) of 25 µl was run at 30°C using CARBOPACK^{TM} PA-20 column (15 x 0.3 cm, Dionex), connected to guard column of CarboPac^{TM} PA20 (3 x 0.3 cm, Dionex). In column, the flow rate of 500 µl/min, was maintained throughout the analysis. The monosaccharide was eluted with 300 mM NaOH and analyzed by PAD (pulsed amperometric detector) attached with Ion chromatography system (Dionex, ICS-3000). The monosaccharide, D-xylose (10 µg/ml) was used as the standard.

Enzyme assay and determination of protein content: The activity of *Ct*Xyn30A was determined by using 1% (w/v) beechwood xylan as substrate in 50 mM sodium phosphate buffer, pH 6.0 in 100 µl reaction mixture incubated at 70°C for 10 min (unpublished results). The reaction mixture was analysed for reducing sugar as described earlier by Nelson and Somogy[22,23]. The activity of Axe2 was carried out by incubating enzyme (10 µl) with 1% (w/v) birchwood xylan in 20 mM sodium phosphate buffer (pH 6.0) in 100 µl reaction mixture incubated at 55°C for 10 min[19]. 1 unit (U) of enzyme activity is the amount of enzyme required to liberate 1 µmole of xylose per minute.

The concentration of protein was detected by mixing the enzyme (10 µl) with distilled water (90 µl) and adding 1 ml Bradford reagent. The mixture was incubated at 25°C for 20 min and the absorbance at 595nm (A_{595}) was measured. The Bovine serum albumin (BSA) standard curve was prepared by varying the concentration between 0.01-0.2 mg/ml to determine the protein concentration. The specific activity (U/mg) of the cell free supernatant was calculated by dividing enzyme activity (U/ml) and protein concentration (mg/ml).

Ethanol estimation by gas chromatography (GC): The analysis of ethanol formed during the process of fermentation was estimated by gas chromatography equipped with flame ionization detector (Varian 450) and Porapaq column (3.0 m x 0.2 cm i.d., 80-100 mesh, Varian). 1 µl sample was analysed and the temperature at injector and detector was maintained at 170°C. N_2 gas as carrier with constant flow rate of 55 cm^3/ min was utilized and the isothermal oven temperature was maintained at 150°C for 20 min.

Results and Discussion

Unoptimized SSF trails of AFEX pretreated 1% (w/v) poplar involving *Ct*Xyn30A along acetylxylan esterase (Axe2) with at flask level: The enzymatic hydrolysis of 1% (w/v) pretreated poplar leafy biomass by mixed (*Ct*Xyn30A and Axe2) are shown as reducing sugar and the ethanol titre profiles in Figure-1. In the initial phase of SSF, the sugar concentration increased to 1.16 g/l at 6 h and later it slightly declined to 0.95 g/l at 18 h and subsequently increased to 1.42 g/l at 36 h (figure-1). The ethanol titre increased exponentially during 36 h-48 h reaching at 0.94 g/l at 60 h (table-2, figure-1). The maximum cell absorbance (A_{600}) of 0.74 reached at 66 h and thereafter a decline in the cell OD and ethanol titre marked the end of the SSF.

Optimization of process parameters in SSF by Taguchi method involving AFEX pretreated 1% (w/v) poplar: In SSF trials, involving 1% (w/v) AFEX pretreated poplar leaves, the influence of 5 factors was analyzed in 25 runs by Taguchi experimental design (table-1a and 1b). For all 5 parameters *i.e.,* recombinant *Ct*Xyn30A, recombinant Acetylxylan esterase (Axe2) dosage volume (ml), inoculum volume (ml) of *C. shehatae* (~4.3 x 10^6 cells/ml), temperature (°C) and pH, giving 25 different combinations of experiments showed an ethanol concentration ranging from 0.095 – 0.130 v/v (table-3). The response data and S/N ratio of 1% (w/v) AFEX pretreated poplar shown leaves are shown in the table-3. Taguchi optimized values for the various fermentation parameters are represented in figure-2, with the prime objective "larger the better" S/N ratio.

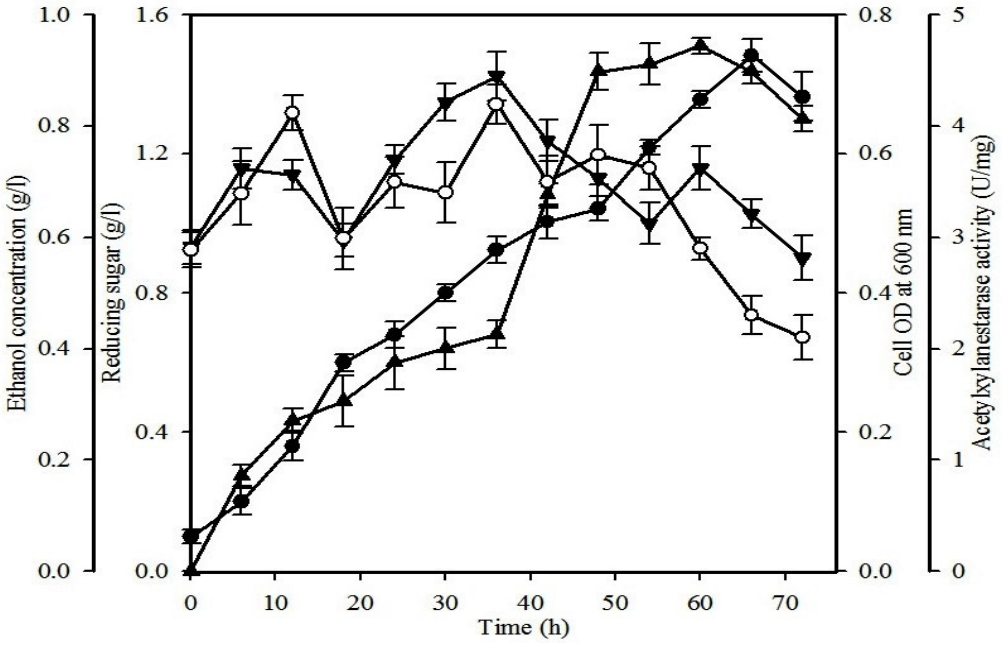

Figure-1

SSF profile of 1% (w/v) *Ct*Xyn30A+Axe2 with AFEX pretreated poplar and *C. shehatae* in shake flask, using unoptimized fermentation process parameters. (▲) Ethanol concentration (g/l), (▼) reducing sugar concentration (g/l), (●) cell OD taken at 600 nm and (○) specific activity of Acetylxylan esterase (Axe2) (U/mg) with time (h). SSF was performed in fermentation medium (100 ml) contained in shake flask (250 ml); initial pH 7.0; agitation rate of 120 rpm and temperature 35°C. Values are mean ± SE (n=3)

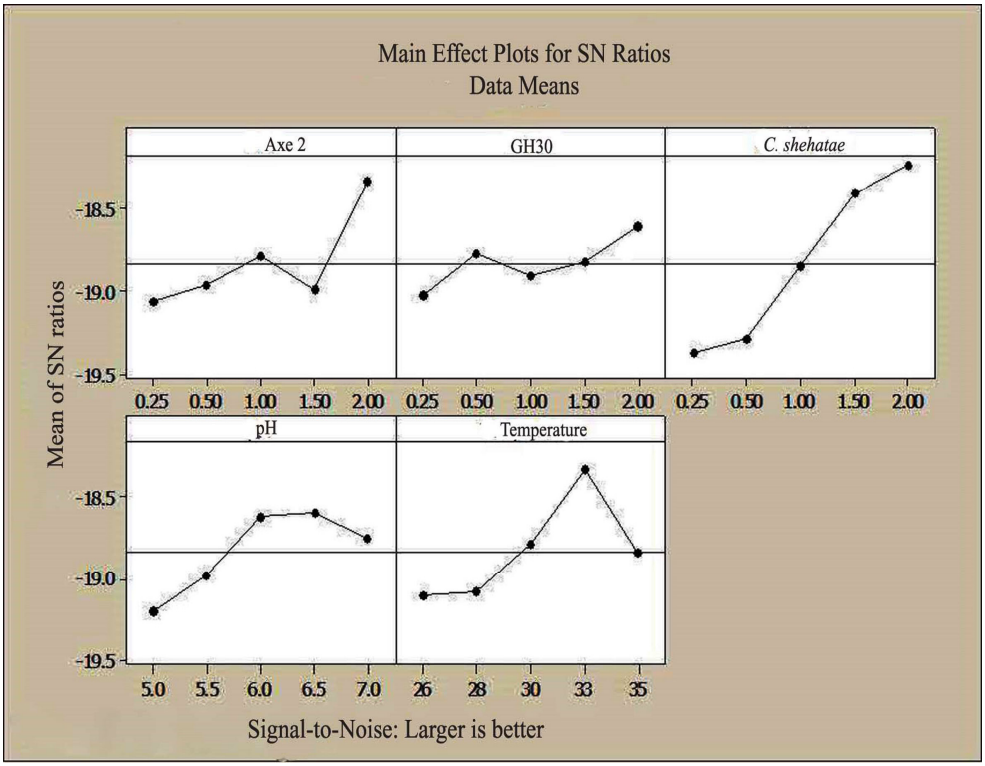

Figure-2

Main effect plots for S/N ratios with the better objective function of Taguchi optimized fermentation process parameters for AFEX pretreated poplar

In the first experimental runs a combination of recombinant *Ct*Xyn30A (0.25 ml), recombinant acetylxylan esterase (Axe2) (0.25 ml), *C. shehatae* inoculum volume (0.25 ml), pH (5) and temperature (26°C), resulted in lowest S/N ratio of -20.45, with an ethanol concentration of 0.095 (%, v/v) (table-3). While in 25th experimental run, maximum ethanol titre of 0.130 (%, v/v) with the greatest response and maximum S/N ratio (-17.72) (table-3) was observed. The best process parameters in 100 ml of fermentation medium obtained were a combination of recombinant *Ct*Xyn30A volume (2 ml), acetylxylan esterase (Axe2) volume (2 ml), *C. shehatae* inoculum volume (1.5 ml), pH (6) and temperature (28°C). The analysis of variance (ANOVA) was performed as each factor contribution gave different values for ethanol by the Taguchi process (table-4a). It can be determined from the calculated ratios (F), that for the experimental design, the factors considered are statistically significant at a confidence limit of 95%. The contributions of selected factors are shown in table-4b. On the basis of *p*-value ($p< 0.05$), it can be observed that *C. shehatae* inoculum volume with rank 1 is the most significant of all other factors and shows highest positive impact on the ethanol production (p=0.001). Later, temperature is considered as the 2nd most significant factor (p=0.008), and then the volume of recombinant acetylxylan esterase as 3rd most significant factor (p=0.010) and then pH played a major role (p=0.017). Earlier reports showed that *C. shehate* gave the maximum yield and productivity among the 200 species of yeast examined for the production of ethanol from xylose[16]. It has also been reported earlier that the pH and temperature play important role in the fermentation for the metabolism and the enzymatic activity of fermentative microbes[4,24]. Therefore, the statistical data obtained in the present study, confirmed the importance of inoculum volume of *C. shehatae*, along with other process parameters such as temperature, pH, hydrolytic enzyme volume play a vital role in lignocellulosic ethanol production[25].

Experimental validation of Taguchi model for SSF of 1% (w/v) AFEX pretreated leafy biomass of poplar at shake flask level: The experimental validation of Taguchi model for SSF is provided in table-5. It was observed that the Taguchi optimum values for response (ethanol %, v/v) (0.134) and S/N ratio (-17.40) was only slightly larger than the experimental optimum data for the ethanol concentration (0.130%, v/v) and S/N ratio (-17.72) thereby, validating the SSF process parameters optimized by Taguchi orthogonal design for bioethanol production (Table 5). The Taguchi SSF trials gave the necessary outcome for improving the efficiency of ethanol production. Taguchi optimized SSF conditions with 1% (w/v) AFEX-treated leafy biomass at initial flask level gave an ethanol titre of 1.06 g/l along with reducing sugar concentration of 1.55g/l and yield, 0.285 (g of ethanol/g of pretreated biomass) (table-2). Thus, a~1.3-fold rise in ethanol concentration, observed with Taguchi optimized SSF process parameters as compared to unoptimized parameters.

SSF experiments with 5% (w/v) AFEX pretreated poplar using Taguchi optimized parameters at shake flask level: "In 5% (w/v) SSF, a consistent increment was observed in cell-biomass, reaching a maximum A_{600} of 1.44 at 66 h (figure-3). The reducing sugar concentration 6.82 g/l was observed at 36 h and that later declined to 3.9 g/l at 66 h. A maximum ethanol concentration of 6.10 g/l was obtained at 66 h of fermentation with a yield, 0.317 g of ethanol/g of pretreated biomass (table-2, figure-3). Escalating the concentration of AFEX pretreated biomass from 1% (w/v) to 5% (w/v) at flask level, resulted a~6-fold increase in ethanol titre (table-2). It has also been reported that on increasing the concentration of substrate an escalation in ethanol concentration is observed[26].

Scale up of Taguchi optimized SSF process with 5% (w/v) AFEX pretreated poplar leaves at bioreactor level: The SSF profile using bioreactor involving 5% (w/v) poplar with mixed enzymes (*Ct*Xyn30A+Axe2) and *C. shehatae* is shown in figure-4. The fermentative organism showed an initial lag phase (A_{600} of 0.7) for first~6 h (figure-4). Increased cell biomass with a maximum cell growth with A_{600} of 5.23 was obtained during the log phase (~66 h) followed by a decline in the growth (figure-4). An initial increase of 2.90 g/l ethanol formation was observed at 24 h, followed by further upsurge of 6.80 g/l at 54 h (table-2, figure-4). The maximum ethanol titre of 7.10 g/l and yield, 0.369 (g of ethanol/g of pretreated biomass) was obtained at 66 h (table-2, figure-4). The maximum reducing sugar concentration released from the enzymatic saccharification of poplar leafy biomass using *Ct*Xyn30A+Axe2 was 10.2 g/l at 36 h. For Axe2, the maximum specific enzyme activity of 4.2 U/mg (6 h) and a minimum of 2.4 U/mg (30 h) were observed (figure-4). The precise pH (6.5) with aeration rate (1 vvm) and volumetric oxygen transfer coefficient, K_{La}= 0.206 per min were maintained throughout the SSF experiment in bioreactor. The ethanol titre (7.1 g/l) with an yield, 0.369 (g of ethanol/g of pretreated biomass) with 5% (w/v) AFEX-treated biomass in SSF carried out in bioreactor resulted in a ~1.20-fold increment in both ethanol titre and yield over SSF at shake flask level (6.10 g/l, 0.317 g of ethanol/g of pretreated biomass) at same biomass concentration (table-2).

The hydrolyzed product released from AFEX pretreated of 5% (w/v) pretreated biomass with mixed (*Ct*Xyn30A + Axe2) enzymatic saccharification carried out in bioreactor was analyzed by HPAEC-PAD at the different time interval (figure-5 a,b,c,d,e and f). The retention time for xylose (standard) was 4.98 min. The sample analysis at 0 h showed less xylose content, which might have been released from the initial AFEX pretreatment. The substantial increase in xylose content was observed at 18 h and its maximum content reached at 36 h of SSF process (figure-5d). The decreased concentration of xylose after 36 h was observed that was due to its consumption as carbon source by *C. shehatae*. Further, the decrease in xylose peak was observed at 72 h, showing its utilization by *C. shehatae* for its growth and subsequent ethanol production (figure-5-f). The ethanol titre and xylose concentration obtained

in present saccharification and fermentation studies are comparable to the other reports. 60% of the theoretical yield (0.34 g of ethanol/g of substrate) and 41% of xylose recovery was obtained in SSF process involving steam exploded woody poplar biomass[27]. SSF experiments using 6% (w/v) sawdust of pretreated poplar gave a yield of 55% with use of fermentative microbe *Saccharomyces cerevisiae* was reported[28].

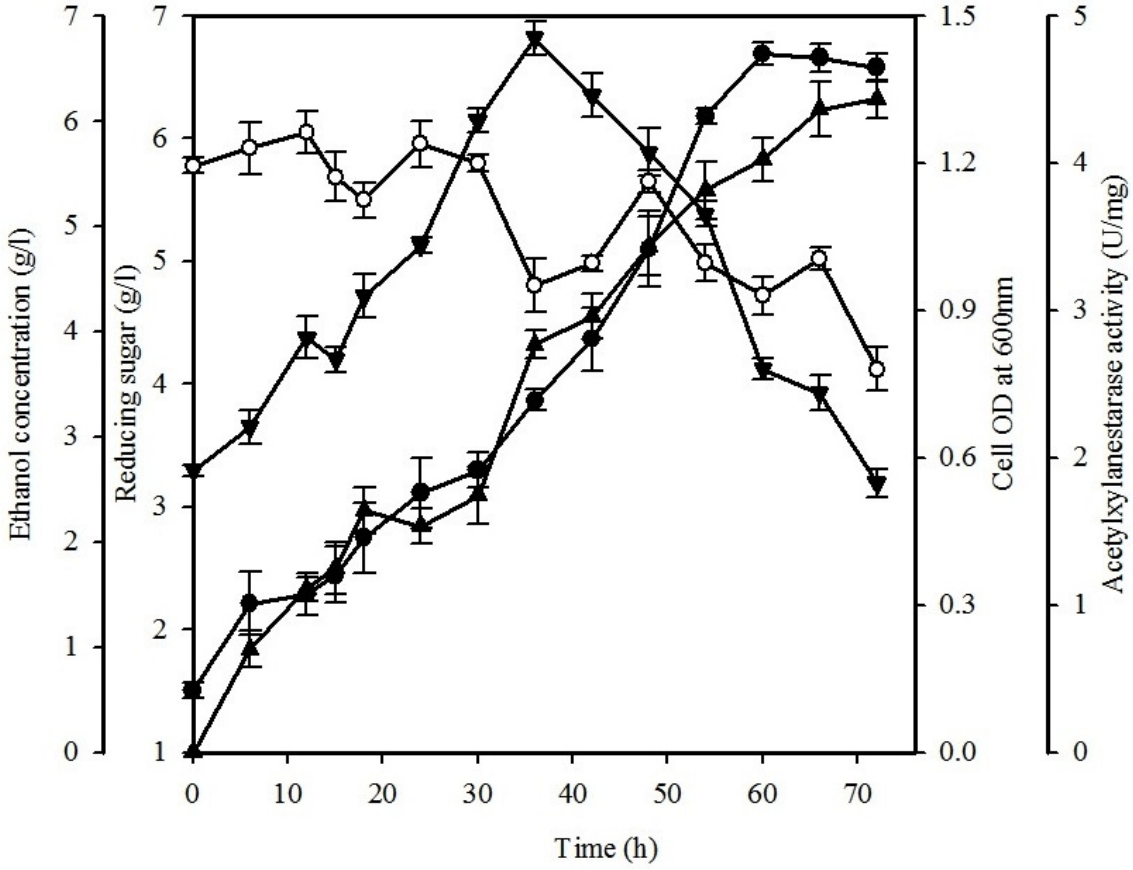

Figure-3

SSF experiments of 5% (w/v) AFEX pretreated poplar using Taguchi optimized parameters in shake flask. (▲) Ethanol concentration (g/l), (▼) reducing sugar concentration (g/l), (●) cell OD taken at 600 nm and (○) specific activity of Acetylxylan esterase (Axe2) (U/mg) with time (h). SSF was performed in fermentation medium (100 ml) contained in shake flask (250 ml); initial pH 6.5; shaking 120 rpm and temperature 33°C. Similar specific enzyme activity profiles were acquired for recombinant enzyme *Ct*Xyn30A (data not provided). Values are mean ± SE (n=3)

Table-1(a)

Various Parameter (Factor) and levels in Taguchi Experimental Design for simultaneous saccharification and fermentation (SSF) experiments at shake flask employing AFEX pretreated 1% (w/v) poplar at 120 rpm

Parameter/Factor	Levels				
Recombinant Axe2* (0.37 mg/ml, 4.4 U/mg)	0.25	0.5	1.0	1.5	2.0
Recombinant *Ct*Xyn30A* (0.31 mg/ml, 4.6 U/mg)	0.25	0.5	1.0	1.5	2.0
*C. shehatae** (~4.3 x 10^6 cells/ml)	0.25	0.5	1.0	1.5	2.0
pH	5.0	5.5	6.0	6.5	7.0
Temperature (°C)	26.0	28.0	30.0	33.0	35.0

* indicates the values of levels in (%, v/v)

Table-1(b)
Matrix outline of the L_{25} Taguchi orthogonal array design for 1% (w/v) AFEX pretreated poplar at 120 rpm

Run/ Expt. No.	Recombinant acetylxylan esterase (Axe2)*	Recombinant *Ct*Xyn30A*	*C. shehatae**	pH	Temper-ature
1	0.25	0.25	0.25	5	26
2	0.25	0.5	0.5	5.5	28
3	0.25	1	1	6	30
4	0.25	1.5	1.5	6.5	33
5	0.25	2	2	7	35
6	0.5	0.25	0.5	6	33
7	0.5	0.5	1	6.5	35
8	0.5	1	1.5	7	26
9	0.5	1.5	2	5	28
10	0.5	2	0.25	5.5	30
11	1	0.25	1	7	28
12	1	0.5	1.5	5	30
13	1	1	2	5.5	33
14	1	1.5	0.25	6	35
15	1	2	0.5	6.5	26
16	1.5	0.25	1.5	5.5	35
17	1.5	0.5	2	6	26
18	1.5	1	0.25	6.5	28
19	1.5	1.5	0.5	7	30
20	1.5	2	1	5	33
21	2	0.25	2	6.5	30
22	2	0.5	0.25	7	33
23	2	1	0.5	5	35
24	2	1.5	1	5.5	26
25	2	2	1.5	6.0	28

* *indicates the values of levels in (%, v/v)*

Table-2
SSF using *Ct*Xyn30A, Axe2 and *C. shehatae* with AFEX pretreated poplar leafy biomass under unoptimized and Taguchi optimized parameters

SSF trails and Biomass concentration	SSF mode and Volume	Reducing sugar (g/L)*	Ethanol titre (g/L)*	Ethanol yield (g of ethanol/g of pretreated biomass)
Unoptimized, 1% (w/v)	Shake flask (100 ml)	1.42 ± 0.09	0.94 ± 0.06	0.244
Taguchi optimized, 1% (w/v)	Shake flask (100 ml)	1.55 ± 0.05	1.06 ± 0.07	0.285
Taguchi optimized, 5% (w/v)	Shake flask (100 ml)	6.82 ± 0.06	6.10 ± 0.07	0.317
Taguchi optimized 5% (w/v)	Bioreactor (1000 ml)	10.20 ± 0.04	7.10 ± 0.08	0.369

*Values correspond to the maximum reducing sugar and maximum ethanol concentration at a particular time and the values are mean ± SE (n=3)

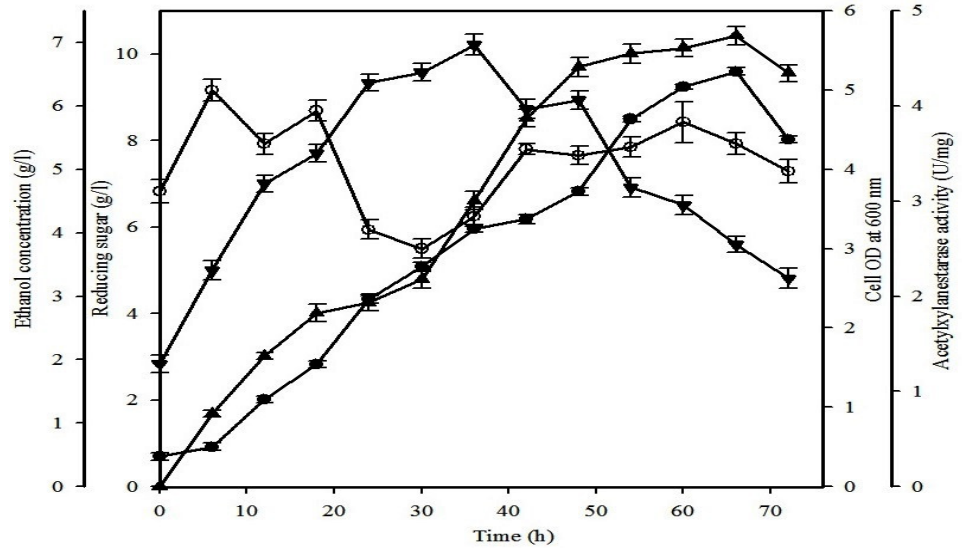

Figure-4

SSF of 5% (w/v) AFEX pretreated poplar using *Ct*Xyn30A+Axe2 and fermentative microbes, *C. shehatae* in reactor level, (▲) Ethanol concentration (g/l), (▼) reducing sugar concentration (g/l), (●) cell OD taken at 600 nm and (○) specific activity of Acetylxylan esterase (Axe2) (U/mg) with time (h). Saccharification and fermentation was carried out in 1 liter medium contained in bioreactor (3 liter); initial pH 6.5; temperature 33°C, agitation 120 rpm and aeration 1 vvm. Similar specific enzyme activity profiles were acquired for recombinant enzyme *Ct*Xyn30A (data not provided). Values are mean ± SE (n=3)

Table-3

Obtained responsesand S/N ratio of Taguchi orthogonal array design (L_{25}) for SSF of 1% (w/v) AFEX pretreated poplar at 120 rpm

Run/ Expt. No.	Response (Ethanol titre, %, v/v)*	S/N ratio			
1	0.095 ± 0.05	-20.45	13	0.127 ± 0.08	-17.93
2	0.100 ± 0.04	-20.00	14	0.109 ± 0.04	-19.25
3	0.114 ± 0.09	-18.86	15	0.112 ± 0.08	-19.02
4	0.127 ± 0.02	-17.93	16	0.114 ± 0.05	-18.86
5	0.124 ± 0.07	-18.13	17	0.120 ± 0.03	-18.42
6	0.114 ± 0.08	-18.86	18	0.105 ± 0.02	-19.58
7	0.116 ± 0.06	-18.71	19	0.108 ± 0.01	-19.33
8	0.113 ± 0.01	-18.94	20	0.115 ± 0.08	-18.79
9	0.113 ± 0.03	-18.94	21	0.129 ± 0.09	-17.79
10	0.107 ± 0.06	-19.41	22	0.123 ± 0.07	-18.21
11	0.110 ± 0.05	-19.17	23	0.109 ± 0.03	-19.25
12	0.118 ± 0.07	-18.56	24	0.116 ± 0.02	-18.72
			25	0.130 ± 0.05	-17.72

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

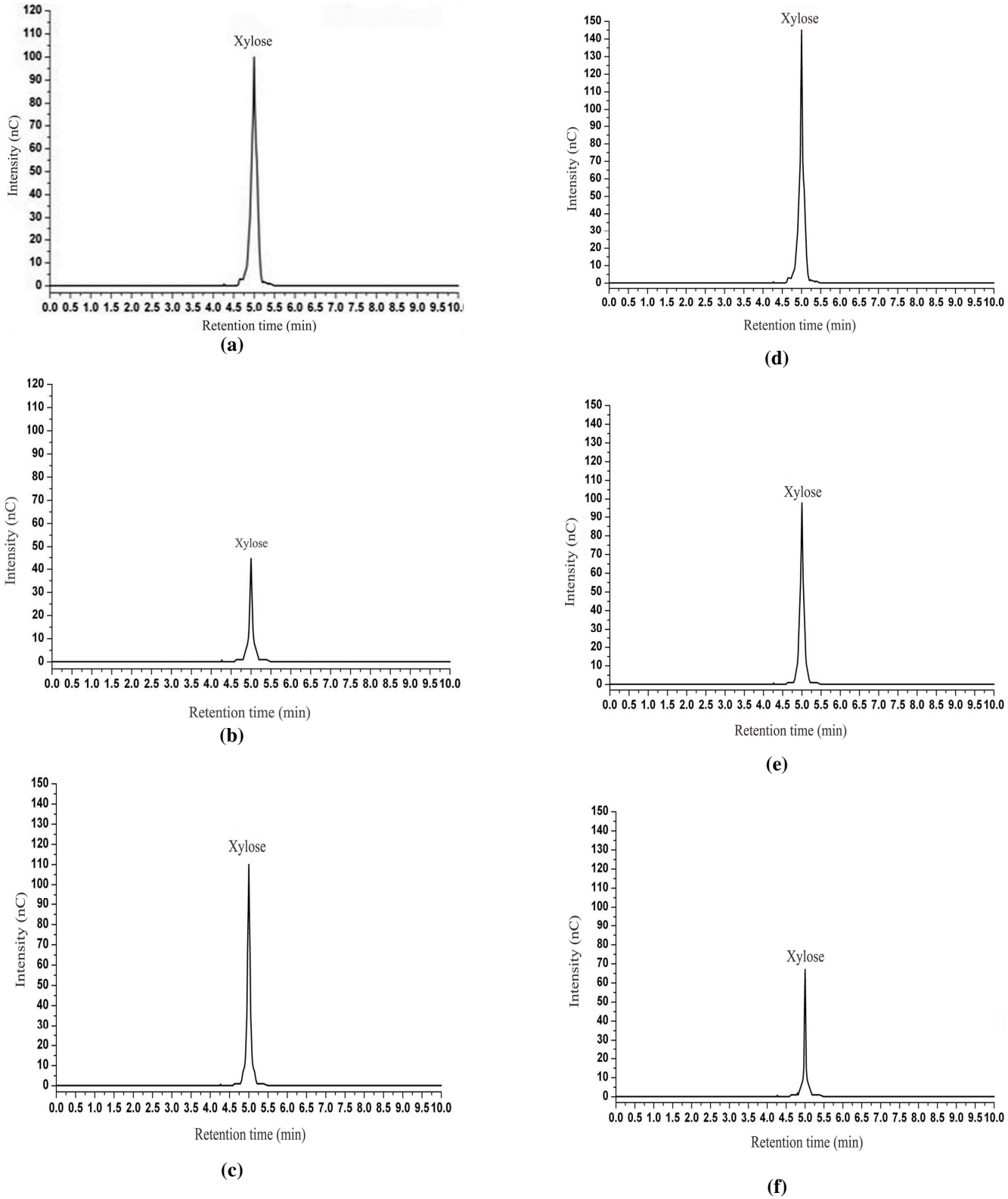

Figure-5

HPAEC profiles of xylose attained from bioreactor saccharification and fermentation experiments of 5% (w/v) AFEX pretreated poplar. The chromatogram enzymatically reduced sugar hydrolysate *viz.,* xylose was observed at periodic time intervals by HPAEC-PAD (a) Standards (b) 0 h (c) 18 h (d) 36 h (e) 54 h (f) 72 h

Table-4(a)
Analysis of Variance for the responses of ethanol production from SSF of AFEX pretreated poplar

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Recombinant Axe2	4	1.7673	1.7673	0.44183	16.21	0.010
Recombinant *Ct*Xyn30A	4	0.4723	0.4723	0.11807	4.33	0.092
C. shehatae	4	5.2281	5.2281	1.30703	47.96	0.001
pH	4	1.2886	1.2886	0.32216	11.82	0.017
Temp	4	1.9064	1.9064	0.47661	17.49	0.008
Error	4	0.1090	0.1090	0.02725		
Total	24	10.7718				

DF- Degrees of freedom, SS-Sum of squares, MS-Mean of Squares

Table-4(b)
Rank and significance of various factors for SSF from poplar

Factor/Parameter	Rank	*p*-value
Recombinant Axe2 (4.4U/mg, 0.37 mg/ml)	3	0.010
Recombinant *Ct*Xyn30A (4.6U/mg, 0.31 mg/ml)	5	0.092
C. shehatae (~4.3 x 10^6cells/ml)	1	0.001
pH	4	0.017
Temperature	2	0.008

$p < 0.05$

Table-5
Validation of Taguchi experimental obtained values for SSF from poplar.

Factor/ Parameter	Taguchi optimum predicted	Experimental optimum
Recombinant Axe2* (4.4U/mg, 0.37mg/ml)	2.0	2.0
Recombinant *Ct*Xyn30A*(4.6U/mg, 0.31mg/ml)	2.0	2.0
*C. shehatae**(~4.3 x 10^6cells/ml) (%, v/v)	2.0	1.5
pH	6.5	6.0
Temperature (°C)	33	28
S/N ratio	-17.40	-17.72
Response predicted Ethanol titre (%, v/v)	0.139	0.134
Response experimental Ethanol titre (%, v/v)	0.135	0.130
Ethanol titre (g/l)	1.065	1.026
Ethanol yield (g of ethanol/g of pretreated biomass)	0.276	0.266

* indicates the values of levels in (%, v/v)

Conclusion

Taguchi optimization of SSF process parameters for bioethanol production from AFEX pretreated leafy biomass of *Populus nigra* was reported for the first time. Shake flask unoptimized SSF trials, gave an ethanol titre of 0.94 g/l using the combination of *Ct*Xyn30A and acetylxylan esterase (Axe2) as saccharifying enzymes and *C. shehatae* as fermentative microbe. Taguchi optimized enzymatic hydrolysis and fermentation conditions at shake flask level with 1% and 5% (w/v) AFEX-treated leafy biomass gave a maximum ethanol titre of 1.06 g/l and 6.10 g/l, respectively. The scale-up of 5% (w/v) feed biomass shake flask SSF at bioreactor level over shake flask experiments gave a ~1.2-fold higher ethanol titre with improved yield of ethanol 7.10 g/l, with an yield of 0.369 (g/g).

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