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An insight - A statistical investigation of consolidated bioprocessing of *Allium ascalonicum* leaves to ethanol using *Hangateiclostridium thermocellum* KSMK1203 and synthetic consortium

S. Kavitha [a, *], T. Gajendran [b], K. Saranya [a], P. Selvakumar [c], V. Manivasagan [a], S. Jeevitha [d]

[a] Department of Biotechnology, Adhiyamaan College of Engineering, Hosur, Tamil Nadu, India
[b] Department of Biotechnology, BIT Campus, Anna University, Tiruchirappalli, Tamil Nadu, India
[c] Department of Chemical Engineering, School of Mechanical, Chemical and Materials Engineering, Adama Science and Technology University, Adama, 1888, Ethiopia
[d] Department of Biotechnology,Vivekanandha College of Engineering for Women, Tiruchengode, Tamil Nadu, India

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ABSTRACT

Bioethanol can be produced by cellulolytic and ethanogenic bacterial species could efficiently degrade lignocelluloses and have potential for biofuels production through consolidated bioprocessing (CBP). In this study, on-site cellulase production followed by saccharification and fermentation of pre-treated *Allium ascalonicum* leaves to bioethanol in a single reactor using *Hangateiclostridium thermocellum* KSMK1203 and consortium of *Cellulomonas fimi* MTCC 24 and *Zymomonas mobilis* MTCC 92. *A. ascalonicum* leaf pre-treatment with different alkalis and different pre-treatment conditions such as alkali dosage and time for proficient removal of hemicellulose and cellulose recovery was optimized using RSM method with the maximum hemicellulose removal of 85.25%. The essential medium components were screened through PBD and further optimized using RSM method for *H. thermocellum* KSMK1203 and the consortium for maximized bioethanol yield. Hence, this study proposed that wild-type *H. thermocellum* KSMK1203 strain and consortium could be used for cellulase secretion and simultaneous bioethanol conversion.

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1. Introduction

The importance of developing a sustainable alternative energy source is highlighted by rising energy demands and the rapid use of non-renewable fossil fuels [1,2]. Compared to traditional fuel production technologies, biological fuel production from renewable resources has gotten a lot of attention [3]. Bioethanol is a colorless, low-toxicity, and easily biodegradable alternative fuel to fossil fuels. The exasperated problems caused by greenhouse emissions can be tackled by using bioethanol near carbon-neutral fuel, thus reducing CO_2 emissions [4]. It has a wide variety of pharmaceuticals, cosmetics, foods, and medical applications, making it a possible supply for chemical companies. Agricultural waste biomass and forestry residues such as corn cobs, sugar cane bagasse, wheat straw, and wood chips have been used to make bioethanol. Lignocellulosic biomass consists of cellulose, hemicellulose, lignin, extractives, and ash, plentiful and readily available. Lignocellulosic materials are the most cost-effective alternatives for producing organic fuels. Bioethanol produced from converting lignocellulosic biomass to bio bioethanol is referred to as second-generation biofuel [4,5].

The lignocellulosic biomaterial is generally composite and, because of its recalcitrance, highly resistant to enzymatic hydrolysis and pre-treatments needed to enhance the hydrolysis rates [6,7]. Pre-treatment of lignocellulosic biomass is highly crucial in the bioethanol production process prior to the fermentation stage as it exposes the cellulose that is to be converted into bioethanol [8]. It is engaged in destroying the rigid structure of the raw material and subsequently separate the various components present in it, thus aiding in removing lignin content present in the lignocellulosic biomass [6]. Various pre-treatment methods are used, which could be either physical or chemical. Alkali pre-treatment is preferred here due to its high efficiency [9].

* Corresponding author. Department of Biotechnology, Adhiyamaan College of Engineering, Hosur, Tamil Nadu, 635130, India.
E-mail address: kavitha.biotechnology@gmail.com (S. Kavitha).

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Nomenclature

ºC	Degree celsius
h	Hour
v/v	Volume per volume
g	Gram
mL	Milli litter
%	Percentage
mM	Milli mole
min	Minutes
cm	Centimeter
g/L	Gram per liter
mmoles L^{-1}	Milli moles per liter
PBD	PlackettBurman design
ANOVA	Analysis of variance
RSM	Response surface methodology
CCRD	Central Composite Rotatable Design
Y_{EC}	Ethanol yield for *H. thermocellum* KSMK1203
Y_{ECC}	Ethanol yield for consortium
$Y_{p/s}$	Bioethanol yield
rs	Rate of cellulose consumption
rp	Yield formation rate
rx	Biomass formation rate

Allium ascalonicum, also known as shallots or red onions, is a type of *Allium* that varies from the standard variety *A. cepa* since it produces smaller bulbs that resemble big garlic cloves and has a milder flavour [10]. It is an herbaceous plant that belongs to the *Liliaceae* family and has been used as a spice since ancient times [11]. It is an integral part of many people's diets, and it is widely grown in West Africa due to its climate suitability [10]. *Allium ascalonicum* leaves are commonly considered waste and discarded. As a result, the leaves (Lignocellulosic biomass) can be effectively used as a raw material for bioethanol production.

Cellulolytic and high product yield microorganisms with toxicity resistance are needed to build up the cost-effective fermentation of lignocellulosic biomaterial into bioethanol by combining the saccharification and fermentation processes [12–14]. Consolidated bioprocessing (CBP) through the cellulolytic and ethanogenic bacterium is thought to be a low-cost processing scheme for lignocellulosic bioethanol production [15]. CBP combines enzyme production, cellulose hydrolysis, and impartial utilization of the full spectrum of liberated sugars, and robust bioethanol fermentation in a single bioreactor [16]. CBP can be attained through a single microorganism that possesses both cellulolytic and ethanogenic activity [9,17] and/or a consortium of microorganisms [6]. In order to enhance the maximized yield, various statistical methods such as Plackett Burman design (PBD) and response surface methodology (RSM) have been employed for the optimization process [9,18]. Only the most important factors with positive significance are chosen in the Plackett Burman design (PBD), which significantly reduces the number of experiments and provides knowledge for evaluating the target factors as much as possible for further optimization [4,9,19]. The response surface methodology (RSM) is a collection of mathematical and statistical techniques that are used in designing experiments, building models, evaluating the impact of numerous factors and obtaining optimum condition of factors for desirable responses, which provides the relationship between one or more measured dependent responses and several input factors [20].

The present study was aimed to optimize the pre-treatment of *A. ascalonicum* leaf biomass with different alkalis (NaOH, KOH, Urea, NaOH + Urea and KOH + Urea) and different pre-treatment conditions such as alkali dosage and time for proficient removal of hemicellulose and improved cellulose recovery. Central Composite Rotatable Design (CCRD) was used to optimize pre-treatment conditions and essential medium components using RSM for bioethanol production from *A. ascalonicum* leaf biomass using *Hangateiclostridium thermocellum* KSMK1203 and the consortium of *Cellulomonas fimi* MTCC 24 and *Zymomonas mobilis* MTCC 92. *H. thermocellum* KSMK1203 is a consolidated bioprocessing candidate that produces cellulases for cellulose hydrolysis and produces bioethanol from hydrolysate in a single reactor. The strains chosen for the consortium, are used for direct bioconversion of cellulose into bioethanol. *C. fimi* MTCC 24 strain has the capability to secrete cellulase to hydrolyze cellulose and *Z. mobilis* MTCC 92 utilizes simple sugars in the hydrolysate to convert into bioethanol. This method of fermentation results the effective fermentation of lignocellulosic biomass into bioethanol through the consolidated bioprocessing. The selected medium components from PBD, namely pre-treated *A. ascalonicum* leaf, malt extract, sodium chloride and potassium dihydrogen phosphate on cellulase and bioethanol production using *H. thermocellum* KSMK1203 strain and the consortium (*C. fimi* MTCC 24 and *Z. mobilis* MTCC 92) were investigated.

2. Materials and methods

2.1. Materials

Sodium chloride, carboxymethyl cellulose, dinitro salicylic acid, ammonium chloride, citric acid, disodium hydrogen phosphate, cellulase, and glucose in high purity were procured from Sigma–Aldrich (Bommasandra, Bengaluru, India) used for proximate biochemical analysis. Sodium chlorite, sodium hydroxide, potassium hydroxide and ureawere used to pre-treat *A. ascalonicum* leaves and purchased from Merck Chemicals Ltd., Mumbai, India. Additional chemicals and solvents used in this research study were purchased from Sigma Aldrich or Merck.

2.2. Sample collection and handling

A. ascalonicum leaves were collected from the agricultural fields of Erode District, Tamil Nadu, India. The *A. ascalonicum* leaves were dried in a hot air oven at 60 °C for 3 h and ground into powder [6]. The powdered leaves were then stored at 4 °C for further use. The proximate biochemical analysis of the *A. ascalonicum* leaves biomass was performed for the determination of moisture [21], lipid [21], cellulose [22], hemicellulose [23] and lignin [6] using the standard procedure.

2.3. Optimization of thermo-chemical pre-treatment of *A. ascalonicum leaf*

A. ascalonicum leaves were defatted with toluene: ethanol (v/v) for 24 h using magnetic stirring and the residue after filtration was used for further proceedings. The defatted residues were treated to obtain $NaClO_2$-delignified residue through Teramoto et al. [23] procedure, where about 1.5 g of defatted *A. ascalonicum* leaf biomass was heated with 100 mL water containing 0.2 mL acetic acid and 2.0 g $NaClO_2$ for 1 h at 75 °C and 0.2 mL acetic acid and 1.0 g $NaClO_2$ were added into the system for every hour until a total

Table 1
Experimental range and levels of independent variables in the Plackett-Burman experiment.

$$Y_i = \alpha_0 + \sum \alpha_i |X_i$$

Code	Variables	Range (g/L)	
		High	Low
A	Pre-treated sample	5	3
B	Yeast extract	5	3
C	Malt extract	5	3
D	Ammonium chloride	3	1
E	Dipotassium hydrogen phosphate	3	1
F	Sodium chloride	3	1
G	Potassium dihydrogen phosphate	3	1
H	Ammonium nitrate	1	0.5
I	Magnesium chloride	1	0.5
J	Copper sulphate	1	0.5
K	Zinc sulphate	1	0.5

The major effect was calculated and based on a first-order model, the variables were screened.

of 0.8 mL acetic acid and 5.0 g $NaClO_2$ have been added. The delignified holocellulose residue constitutes cellulose and hemicellulose, was washed with distilled water, dried at 100 °C for 2 h in a hot air oven and used for further studies. $NaClO_2$-delignified residue was separately treated with mild alkali of NaOH, KOH, Urea, NaOH + Urea and KOH + Urea were utilized for the treatment of *A. ascalonicum* leaf biomass and optimized for efficient hemicellulose removal, was determined as xylose extrusion [24]. About 100 mL of 0.5%, 1%, 1.5%, 2% and 2.5% NaOH, KOH, NaOH + Urea and KOH + Urea were used to hydrolyze 1.0 g of $NaClO_2$-delignified biomass and subjected to heat at 100 °C for 15, 30, 60, 90 and 120 min. The hydrolyzed $NaClO_2$-delignified *A. ascalonicum* leaf biomass was filtered using muslin cloth and residue was dried in a hot air oven at 100 °C. The effective alkali was selected based on the experimental conditions and further optimized using response surface methodology with the coded values. Statistical analysis was performed by response surface methodology based on central composite rotatable design (CCRD). The experimental values were fitted in the quadratic model. The coefficients of variables were evaluated by analysis of variance (ANOVA). The model was validated by comparing experimental and predicted values for dilute alkali pre-treatment of biomass and performing six sets of experiments at optimum conditions [25].

2.4. Bacterial strains, culture conditions, and inoculum preparation

Hangateiclostridium thermocellum KSMK1203 strain (Gene Bank Accession No. MT117842) was isolated from compost soil & identified using 16s rRNA sequencing and has been submitted to National Centre for Biotechnology Information (NCBI) [9]. *Cellulomonas fimi* MTCC 24 and *Zymomonas mobilis* MTCC 92 were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Bacterial strains were maintained on nutrient agar slants at 4 °C and sub-cultured twice a month. The inoculum of the bacterial strains was prepared with 1 mL of stock culture that was inoculated into 100 mL of nutrient broth maintained under aerobic conditions at 30 °C in a reciprocal shaker at 100 rpm [6].

2.5. Medium formulation and optimization

2.5.1. Plackett-Burman design

Plackett-Burman design [19], an efficient way to identify the significant factors among a large number of variables, was used in the present study to screen the important variables that significantly influenced cellulase and bioethanol production using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 strains from pre-treated *A. ascalonicum* leaf biomass. In this study, a 12-run Plackett-Burman statistical design was used to evaluate eleven medium components were examined at two levels: −1 for the low level and +1 for the high level that targets significant variables forthe production by screening "n" variables in "n+1" experiments [4]. In this investigation, eight assigned variables together with three dummy variables were tested in 12 experiments at two levels based on PBD. Table 1 illustrates the variables and their corresponding levels used in the experimental design and the values of two levels were set according to our previous preliminary experimental results [5,9]. PBD and the response value of cellulase and bioethanol production and analysis of variance (ANOVA) are shown in Table 3.where Y_i was the response (bioethanol concentration), α_0 was the model intercept and α_i was the variable estimates. Design Expert 10.0.3 software was employed to screen the variables in this present investigation.

2.5.2. Response surface methodology - central composite rotatable design

In this study, selected medium components (pre-treated biomass, malt extract, sodium chloride and KH_2PO_4) and pre-treatment conditions (NaOH + urea concentration, solid loading and time) were examined through RSM-CCRD method. The ranges of the independent variables were selected based on preliminary experiments and assessed at three levels [9]. To evade redundancy of experiments, a central composite rotatable design (CCRD) was applied to produce 30 & 20 experimental conditions respectively for medium component and pre-treatment condition optimization were used to study the effects of the chosen factors. Six centre points were used to evaluate the lack of fit and pure error in the suggested model. The quality of the fitted model was evaluated by significance test and analysis of variance (ANOVA) that denoted in second-order polynomial equations Eqs. (2) and (3) for medium components and pre-treatment condition optimization, respectively.

$$Y_{\text{bioethanol yield}} = \alpha_0 + \alpha_1 * X_1 + \alpha_2 * X_2 + \alpha_3 * X_3 + \alpha_4 * X_4 + \alpha_{12} * X_1X_2 + \alpha_{13} * X_1X_3 + \alpha_{14} * X_1X_4 + \alpha_{23} * X_2X_3 + \alpha_{24} * X_2X_4 + \alpha_{34} * X_3X_4 + \alpha_{11} * X_1^2 + \alpha_{22} * X_2^2 + \alpha_{33} * X_3^2 + \alpha_{44} * X_4^2 \quad (2)$$

$$Y_{\text{xylose yield}} = \alpha_0 + \alpha_1 * X_1 + \alpha_2 * X_2 + \alpha_3 * X_3 + \alpha_{12} * X_1X_2 + \alpha_{13} * X_1X_3 + + \alpha_{23} * X_2X_3 + \alpha_{11} * X_1^2 + \alpha_{22} * X_2^2 + \alpha_{33} * X_3^2 \quad (3)$$

where α_0 was the intercept value, α_1, α_2, α_3, and α_4 were the first order coefficients, α_{12}, α_{13}, α_{14}, α_{23}, α_{24}, and α_{34} were the interaction coefficients, α_{11}, α_{22}, α_{33} and α_{44} represents the quadratic coefficients and X_1, X_2, X_3, and X_4 represent the independent variables.

2.6. Modeling of biomass hydrolysis, fermentation and yield formation

The hydrolysis rate of alkali pre-treated *A. ascalonicum* leaves in bioethanol bioconversion through consolidated bioprocessing using *H. thermocellum* KSMK1203 and consortium were determined to calculate hydrolysis percentage of cellulose and kinetic parameters. For this study, 100 mL of pre-treated and untreated sample medium was sterilized, 5% (v/v) (10^3 initial cells/mL) inoculum was added to the culture medium and incubated under 150 rpm for 24 h and incubated at 34 °C. The parameter of bioethanol yields over cellulose totally consumed cellulose, total cellulose in bioethanol conversion and bioethanol productivity were determined

Table 2
RSM-CCRD comparative result of experimental and predicted values and analysis of variance for pre-treatment condition.

Variables	Code	Levels		
		−1	0	+1
NaOH + urea concentration (%, v/v)	X_1	2	2.5	3
Solid loading (g/100 mL)	X_2	2.5	5	7.5
Time (min)	X_3	45	60	75

RSM-CCRD comparative result

Run	X_1	X_2	X_3	Xylose concentration (g/L) Exp.	RSM
1	0	0	0	8.251	8.790
2	1	1	−1	11.235	11.775
3	−1	−1	−1	3.251	3.791
4	−1	1	−1	8.211	8.752
5	1	−1	−1	10.24	10.783
6	1	0	0	11.654	12.194
7	1	−1	1	10.014	10.554
8	0	0	−1	8.350	8.894
9	0	0	0	8.120	8.665
10	0	1	0	6.982	7.526
11	0	0	0	8.782	9.327
12	1	1	1	12.021	12.568
13	−1	0	0	6.213	6.759
14	0	0	1	6.982	7.521
15	0	−1	0	5.124	5.662
16	0	0	0	8.352	8.893
17	0	0	0	8.541	9.084
18	−1	−1	1	3.981	4.525
19	0	0	0	8.812	9.356
20	−1	1	1	8.914	9.457

Analysis of variance

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Model	97.07	9	10.79	17.31	<0.0001[a]
X_1	60.53	1	60.53	97.14	<0.0001[a]
X_2	21.76	1	21.76	34.92	0.0001[a]
X_3	0.038	1	0.038	0.061	0.8092 [b]
X_1 X_2	5.93	1	5.93	9.52	0.0115[a]
X_1 X_3	0.095	1	0.095	0.15	0.7046 [b]
X_2 X_3	0.12	1	0.12	0.19	0.6697 [b]
$X_1^{\wedge 2}$	5.94	1	5.94	9.53	0.0115[a]
$X_2^{\wedge 2}$	5.48	1	5.48	8.80	0.0141[a]
$X_3^{\wedge 2}$	0.11	1	0.11	0.18	0.6790 [b]
Residual	6.23	10	0.62	14.51	
Lack of Fit	5.83	5	1.17		0.0853[b]
Pure Error	0.40	5	0.080		
Cor Total	103.30	19			
R- Squared		0.9397	Adeq Precision		15.254
Adj. R-Squared		0.8854	Std. Dev.		0.79
Pred. R- Squared		0.6540	Mean		8.20

Exp.: Experimental values, RSM: RSM predicted values; a- significant at $p \geq 0.05$ level, b-Not significant at $p \geq 0.05$ level; X_1: NaOH concentration, X_2: Solid loading, X_3:Time,

Table 3
Plackett-Burman design matrix for 11 independent variables with response and ANOVA.

Plackett-Burman design matrix														Analysis of Variance						
Run	A	B	C	D	E	F	G	H	I	J	K	EC_C	EC	Variable	df.		Co-efficient		P value	
															EC_C	EC	EC_C	EC	EC_C	EC
1	1	1	−1	1	1	1	−1	−1	−1	1	−1	3.845	1.443	Model	11	11	0.059	0.078	<0.0001[a]	<0.0001[a]
2	1	1	1	−1	−1	−1	1	−1	1	1	−1	6.610	7.708	A	1	1	0.034	0.004	0.00134[a]	0.00254[a]
3	−1	−1	1	−1	1	1	−1	1	1	1	−1	3.830	2.378	B	1	1	−0.042	−0.063	0.494[b]	0.694 [b]
4	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	9.982	8.938	C	1	1	0.057	0.048	0.033[a]	0.043[a]
5	1	−1	1	1	−1	1	1	1	−1	−1	−1	3.868	2.624	D	1	1	−0.065	−0.057	0.997 [b]	1.097 [b]
6	1	−1	−1	−1	1	−1	1	1	−1	1	1	2.202	1.795	E	1	1	−0.065	−0.058	0.564 [b]	0.675 [b]
7	−1	1	1	−1	1	1	1	−1	−1	−1	1	5.724	6.068	F	1	1	0.087	0.061	0.0042[a]	0.0022[a]
8	−1	1	−1	1	1	−1	1	1	1	−1	−1	8.286	7.708	G	1	1	0.024	0.011	<0.0001[a]	<0.0001[a]
9	−1	−1	−1	1	−1	1	1	−1	1	1	1	5.905	4.641	H	1	1	−0.047	−0.052	0.565 [b]	0.678 [b]
10	−1	1	1	1	−1	−1	−1	1	−1	1	1	4.815	5.403	I	1	1	−0.037	−0.054	0.454 [b]	0.654 [b]
11	1	−1	1	1	1	−1	−1	−1	1	−1	1	9.921	7.626	J	1	1	−0.034	−0.021	0.654 [b]	0.587 [b]
12	1	1	−1	−1	−1	1	−1	1	1	−1	1	8.181	6.396	K	1	1	−0.063	−0.051	0.854 [b]	0.941 [b]

EC_C: Bioethanol concentration for consortium (*C. fimi* MTCC24 + *Z. mobilis* MTCC92) (g/L); EC: Bioethanol concentration for *H. thermocellum* KSMK 1203 (g/L); a-significant at $p \geq 0.05$ level; b-not significant at $p \geq 0.05$ level.

according to the procedure of Selvakumar et al. [6]. The growth rate parameters, total cellulose consumption rate, and bioethanol production rate were determined from cell kinetics, bioethanol productivity and total cellulose consumption [6,26]. Also, the primary fermentation end products an (acetate and bioethanol) effect on cellulosic biomass conversion was investigated for *H. thermocellum* KSMK1203 and consortium. It was determined by the addition of sodium acetate in the varying concentrations ranging from 20 to 180 mM and bioethanol ranging from 0 to 12% v/v into the treated medium.

2.7. Analytical and statistical methods

The standard methods were used for the characteristics of alkali pre-treated *A. ascalonicum* leaf biomass such as cellulose, hemicellulose, and lignin [9]. Cellulase enzyme in the fermentation medium was analyzed by the method of Dension and Koehn [27] and concentration of fermentation end products (bioethanol, lactate, formate and acetate) was measured using Gas Chromatography (GC-MS, PerkinElmer, Bengaluru, Karnataka, India) [28]. The cellulose content in the fermentation medium was determined using anthrone reagent [22]. Cell concentration in the production medium was quantified using Neubauer counting and the viable cell concentration was articulated as the logarithm of cells per mL (log cells/mL) [23]. All of the experiments in this study were done in triplicate, and the results were expressed as an average of three values. PBD and RSM-CCRD were used to design the experiments for optimizing experimental parameters of bioethanol production and xylose yield using Design Expert 10.0.3, Stat-Ease Inc. 2020 east Hennipin Ave., suite 480 Minneapolis, MN 55413 [29].

3. Results and discussion

3.1. Effect of thermo-chemical pre-treatment on A. ascalonicum leaf biomass

Lignocellulosic materials such as cellulose, hemicelluloses, and lignin are still being investigated as a source of fermentable sugars for biofuel (bioethanol) production [6]. It has been appeared to be an appealing biomass feedstock for biorefineries because of its availability, low cost and high bioethanol productivities [30]. In the present investigation, the *A. ascalonicum* leaf biomass was primarily characterized for bioethanol production with the results: total cellulose, 21.86 ± 0.03%; hemicellulose, 11.82 ± 0.04%; lignin, 0.92 ± 0.05%; ash, 1.8 ± 0.01% and moisture 75 ± 0.03%. This shows that *A. ascalonicum* leaf biomass is rich in lignocellulose which makes it suitable for bioethanol bioconversion. *A. ascalonicum* leaf biomass was treated with $NaClO_2$ and CH_3COOH to obtain delignified residue, which constitutes cellulose and hemicellulose. The $NaClO_2$-delignified biomass required further pre-treatment in order to remove hemicellulose through physico-chemical factors (temperature, acid/alkaline concentration and time) that influences efficient hemicellulose hydrolysis [9]. The thermo-chemical treatment of *A. ascalonicum* leaf biomass was accomplished with different alkalis (NaOH, KOH, NaOH + Urea and KOH + Urea) and varying concentrations at 100 °C for up to 120 min. The maximum hemicellulose removal was assessed repeatedly for selecting low and high levels for each variable and further optimized with response surface methodology.

3.1.1. Influence of physico-chemical factors on hemicellulose removal

The pre-treatment of $NaClO_2$-delignified *A. ascalonicum* leaf biomass using dilute alkali with a shorter time and high temperature is a suitable condition to remove hemicellulose. Dilute alkali pre-treatment was performed with 0.5%, 1%, 1.5%, 2% and 2.5% alkalis, 2 atm pressure for 15, 30, 60, 90 and 120 min at 100 °C. This physico-chemical circumstance solubilizes hemicelluloses in the biomass and partially hydrolysis the cellulose. Alkaline pre-treatment completely removes the hemicellulose fraction and limits the formation of inhibitors, reduces energy requirements and cost [31]. Because of the hemicellulose solubilization, this process enhances the disintegration of the complex carbohydrate structure that could facilitate microbial utilization of cellulose. During the pre-treatment of $NaClO_2$-delignified biomass with NaOH, KOH, NaOH + Urea and KOH + Urea, the hydrolysis of hemicellulose had been increased with increasing time. The enhanced hemicellulose solubilization was attained at 60 min and efficiency had decreased beyond that. The maximum hemicellulose removal was found to be 71.5%, 72.9%, 73.6%, 74.1% and 74.9% at 15, 30, 60, 90 and 120 min, respectively, for the treatment with NaOH (Fig. 1a). Likewise, for KOH pre-treatment, the maximum hemicellulose removal was observed to be 67.2%, 69.6%, 72.6%, 72.9% and 73.3% at 15, 30, 60, 90 and 120 min (Fig. 1b). Also, for NaOH + Urea and KOH + Urea as shown in Fig. 1c and d, maximized hemicellulose solubilization attained were found to be 82.5%, 89.6%, 92.4%, 92.9% and 93.4%, and 63.8%, 69.2%, 72.5%, 72.9% and 73.2% at 15, 30, 60, 90 and 120 min respectively. During the conduct of the pre-treatment at 15–120 min, an insignificant hemicellulose removal was observed at 15–30 and 90–120 min. Hence the conduction of the study at

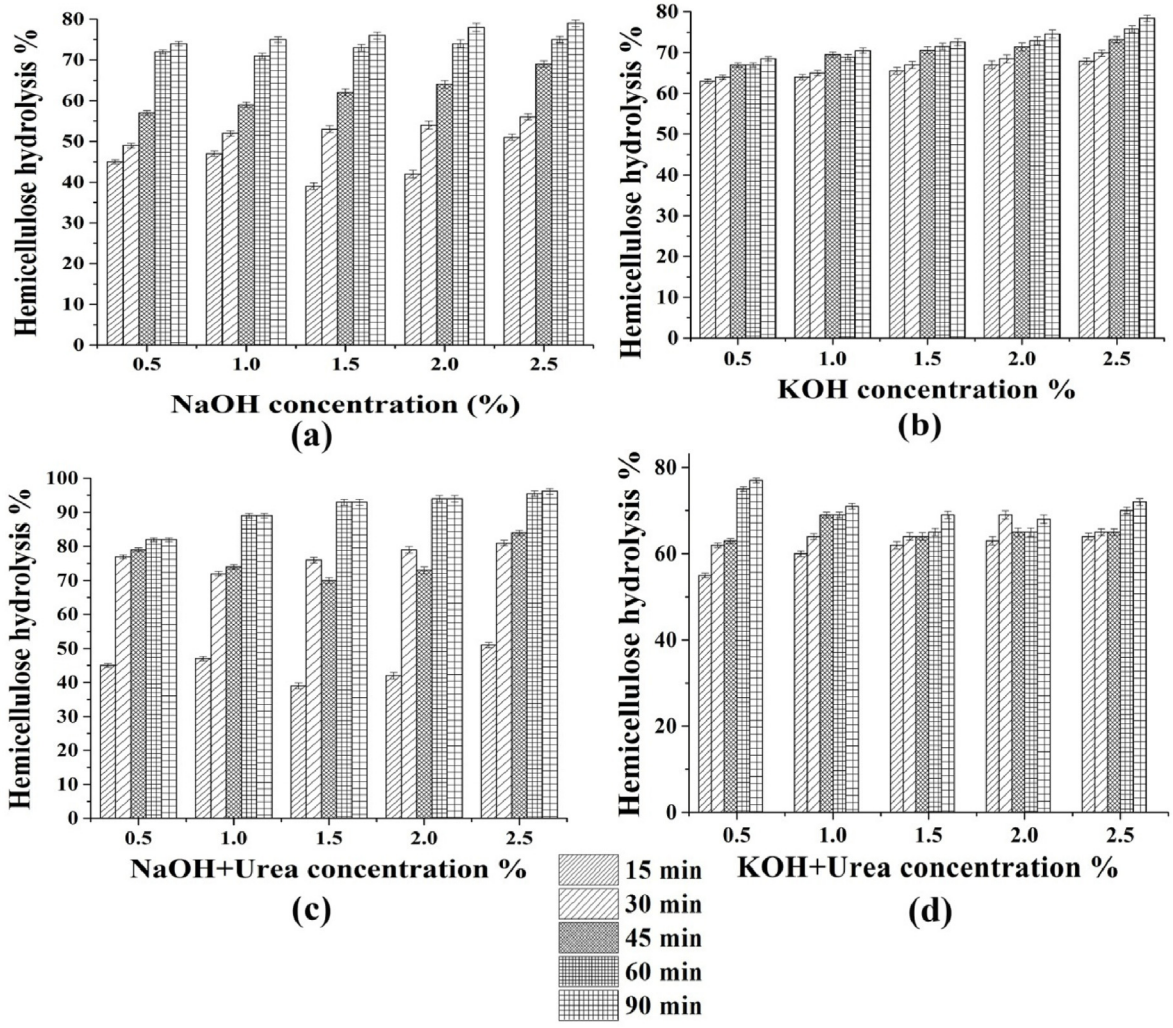


Fig. 1. Effect of thermo-chemical pre-treatment on hemicelluloses removal from *A. ascalonicum* leaf.

this period had been ignored for consequent studies. As a result, 60 min of time interval was considered to be optimum for hemicellulose hydrolysis from $NaClO_2$-delignified biomass.

$NaClO_2$-delignification of biomass followed by dilute alkaline treatment enhanced the cellulose accessibility [32]. The hemicellulose solubilization was greatly improved as increasing the alkali concentration until the treatment period was 60 min. The alkalis such as NaOH, KOH, $Ca(OH)_2$, NH_4OH and urea have been employed to treat lignocelluloseic biomass to eliminate lignin and hemicelluloses elevated temperature and pressure [33,34]. Among the various alkalis, NaOH, KOH, NaOH + urea and KOH + urea had preferred for the hydrolysis of hemicellulose from delignified biomass. Based on the triplicate investigation, maximum hemicellulose solubilization was observed in the alkali dose of 2% and thereafter remained the same in all cases (Fig. 1). It had been justified based on the calculated p-value for the dosage of 2 and 2.5%. It was calculated to be 0.8103, 0.7901, 0.9021 and 0.8601 for NaOH, KOH, NaOH + urea and KOH + urea, respectively. Hence, 2% of alkali dosage was selected for further investigations. In this present study, the maximum hemicellulose removal was observed in NaOH + urea treatment, leading to holocellulose disruption and hemicellulose solubilization. Under the optimal condition of 2% NaOH + urea, 100 °C for 60 min, the observed maximum hemicellulose removal was 85.25% (Fig. 1). This investigation had revealed that ambient temperature and time with increased alkali dosage efficiently removed hemicellulose from delignified biomass. Yao et al. 2015 observed 43.47% of hemicellulose removal with 4.42% of NaOH concentration.

3.1.2. RSM optimization of pre-treatment condition

The pre-treatment conditions such as NaOH + urea concentration (%v/v), solid loading (g/100 mL) and time (min) having an impact on hemicellulose hydrolysis results in cellulose that utilized as the substrate for bioethanol fermentation was investigated using RSM-CCRD. Table 2 summarizes the comparative result of experimental and predicted values and analysis of variance for pre-treatment conditions. The value of <0.0001 and 17.31 of p-value and F value of the model respectively were shows the significance at 95% confidence level ($p < 0.05$) [25]. Eq. (4) denotes the second-order polynomial regression equation for xylose yield. The coefficient of determination (R^2) of the model to be 0.9397 shows the model fitness. The individual effects X_1 and X_2, the interactive effects X_1 X_2 and the quadratic effects X_1^2 and X_2^2 were significant and the individual effect X_3 was insignificant that can be observed from Table 2. In this present investigation, pre-treatment time shows insignificant effect on hemicellulose solubilization because of the shorter time period. The increasing pre-treatment time would show significant effect on hemicellulose solubilization. Adeq.

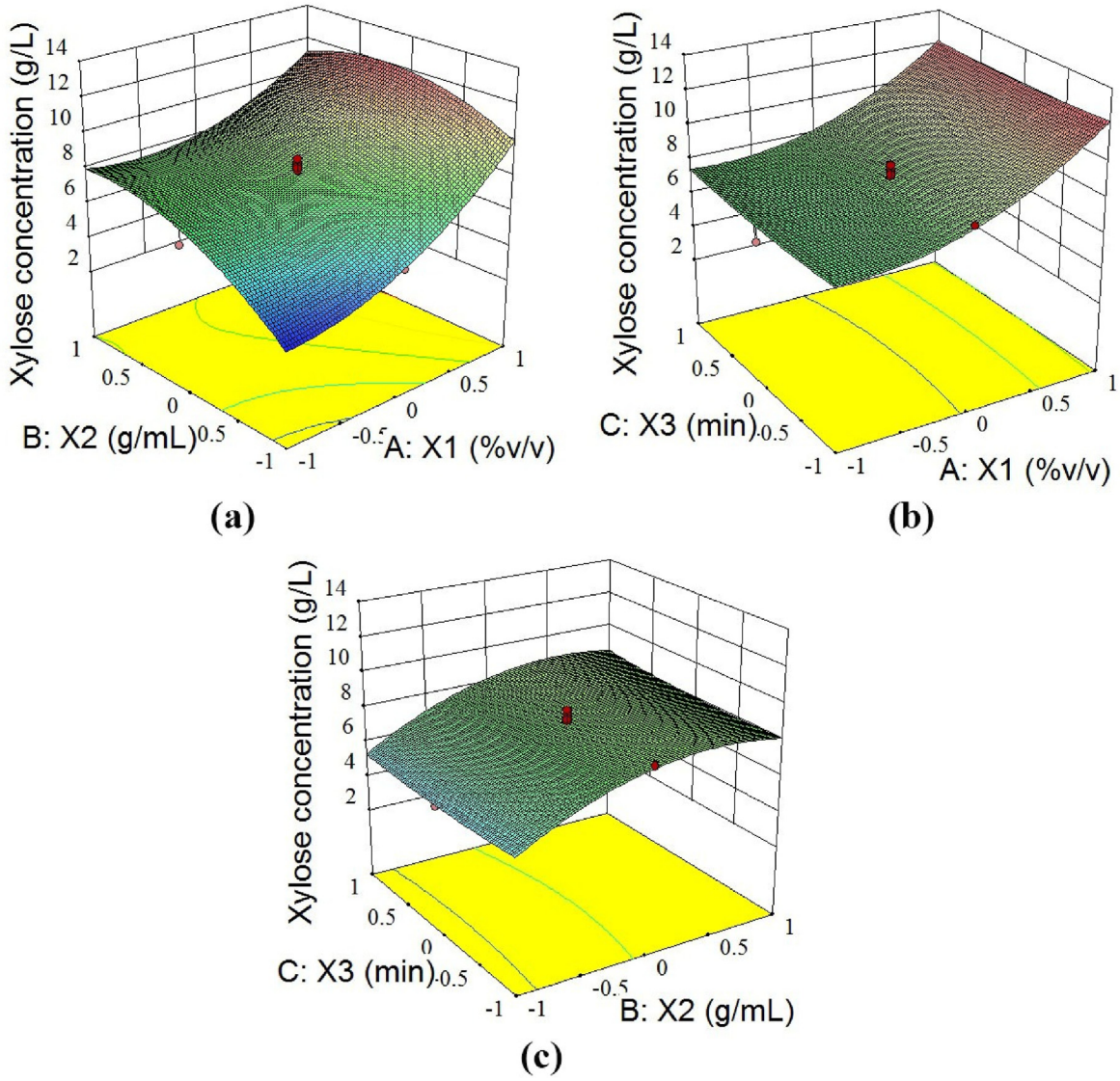


Fig. 2. Interactive response surface graphs for effects of pre-treatment on *A. ascalonicum* leaf.

Precision and standard deviation were 15.254 and 0.79, respectively, for the investigated model.

$$Y_{\text{xylose concentration}} = +8.07 + 2.46^{*} X_1 + 1.48^{*} X_2 + 0.062^{*} X_3 - 0.86^{*} X_1X_2 - 0.11^{*} X_1X_3 + 0.12^{*} X_2X_3 + 1.47^{*}X_1^2 - 1.41^{*} X_2^2 + 0.20^{*} X_3^2 \quad (4)$$

A three-dimensional response surface plot of the effect of NaOH + urea concentration (%v/v) (X_1), solid loading (g/100 mL) (X_2) and time (min) (X_3) on hemicellulose hydrolysis in terms of xylose concentration is represented in Fig. 2. It can be observed from Fig. 2, the interactive effects of parameters on hemicellulose hydrolysis that were varying as a function and other variables kept as constant. It was observed from Fig. 2, an increase in alkali concentration would result in enhanced hemicellulose solubilization and the maximum xylose concentration was observed at 3% (v/v). From the response surface plots, xylose concentration had significantly decreased when the solid loading increased above 5 g/100 mL. Figure A1 shows the experimental data plotted against RSM predicted data for pre-treatment conditions for the CCD designed experimental runs. It is apparently shown that there is a significant relationship between the experimental and predicted data. As well a similar observation had made on the time period, which was found to be 60 min and had further considered as optimum. The optimized pre-treatment conditions were found to be 2.89 of NaOH + urea concentration (%v/v), 3.75 g/100 mL of solid loading and 66.5 min of the time period with the hemicellulose hydrolysis of 86.87% from $NaClO_2$-delignified *A. ascalonicum* leaf biomass. Yao et al. [35] obtained a similar result of 43.47% hemicellulose hydrolysis with 172.12 °C, 62.27 min, NaOH dosage of 4.42%.

3.1.3. Analysis of efficiency of A. ascalonicum leaf pre-treatment

The efficiency of $NaClO_2$-delignified *A. ascalonicum* leaf biomass pre-treatment for enhanced hemicellulose hydrolysis was carried at optimal conditions such as 2.89 of NaOH + urea concentration (% v/v), 3.75 g/100 mL of solid loading and 66.5 min of time interval. The maximum hemicellulose removal of 87.17 ± 1.12% from $NaClO_2$-delignified *A. ascalonicum* leaf biomass had attained. Fig. 3 shows the result of FTIR analysis of pre-treated biomass in compared with untreated biomass.

From Fig. 3, the photon energy with the wavenumbers 1461.2 and 1419.6 cm^{-1} was assigned to C—H deformation in pre-treated

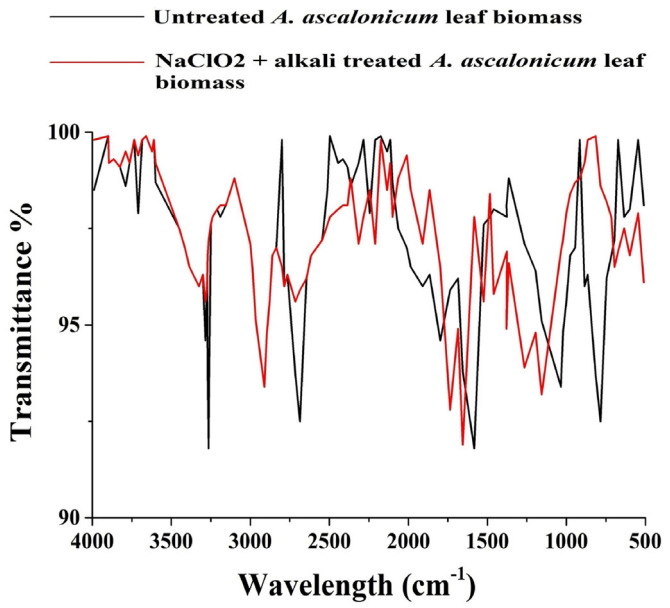


Fig. 3. FTIR analysis of pre-treated *A. ascalonicum* leaf biomass.

and untreated *A. ascalonicum* leaf biomass. The peak presented at 1734.1/1636.2 cm^{-1} in the spectrum for untreated biomass corresponding to the C═O stretching of carbonyl group represented the presence of hemicelluloses. As well as the peak at 1654.3 cm^{-1} for treated biomass revealed the existence of a lesser amount of hemicellulose. The peaks 2912.5/2859.5 and 2878.1 cm^{-1} respectively for treated and untreated *A. ascalonicum* leaf biomass corresponded to the stretching vibration of (-CH) groups in cellulose and hemicellulose [36]. The peaks that occurred in the range between 3600 and 3100 cm^{-1} indicated the presence of the hydroxyl group. The wavenumbers 3321.1 and 3310.2 respectively for treated and untreated biomass were attributed to the OH stretching on the cellulose surface. It had revealed that the $NaClO_2$ treatment removed the extractives (protein, lipid, aromatic components, etc.) from *A. ascalonicum* leaf biomass and followed by mild alkaline treatment hydrolysis hemicellulose fractions. According to the results, under the optimal conditions (2.89 of NaOH + urea concentration (%v/v), 3.75 g/100 mL of solid loading and 66.5 min), 87.17 ± 1.12% of the hemicellulose was hydrolyzed and resulted in semi-crystalline cellulosic fractions.

3.2. Optimization of medium components for H. thermocellum KSMK1203 and consortium of C. fimi MTCC 24 and Z. mobilis MTCC 92

The essential medium components for bioethanol production from pre-treated *A. ascalonicum* leaf biomass were optimized using PBD followed by CCRD-RSM. The PBD was employed to select the essential medium components and their effect on microbial growth with respect to the experimental study based on Table 1. It is a time-saving screening method that significantly minimizes the number of experimental trials and explores the most efficient and influential factors on bioethanol productivity using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92. RSM, followed by RSM, was the most widely used statistical method of independent variables optimization for bioethanol production from pre-treated *A. ascalonicum* leaf biomass using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 a comparative analysis. It could efficiently be used to construct the cost-effective fermentation of pre-treated biomass into bioethanol through consolidated bioprocessing.

Table 4
RSM-CCRD comparative result of experimental and predicted values for medium components.

Variables				Code	Levels		
					−1	0	+1
Pre-treated biomass (g/L)				X_1	3	4	5
Malt extract (g/L)				X_2	3	4	5
KH_2PO_4 (g/L)				X_3	1	2	3
NaCl (g/L)				X_4	1	2	3

Run	X_1	X_2	X_3	X_4	Ethanol concentration (g/L)			
					EC		ECc	
					Exp.	RSM	Exp.	RSM
1	1	−1	−1	−1	4.924	5.584	1.284	2.274
2	−1	1	−1	−1	3.819	4.184	1.764	2.754
3	1	1	1	1	4.934	5.924	4.114	4.204
4	0	0	0	1	8.654	9.144	7.184	7.674
5	−1	−1	1	−1	3.724	3.714	1.744	1.734
6	0	0	−1	0	6.224	6.214	6.464	7.054
7	−1	−1	−1	1	1.414	2.404	1.434	2.024
8	0	0	0	−1	6.394	7.384	5.324	5.514
9	0	0	0	0	5.484	6.474	6.844	7.934
10	1	−1	−1	1	3.964	4.354	2.454	2.444
11	1	0	0	0	9.064	8.954	4.694	4.684
12	1	−1	1	1	5.234	6.224	3.304	4.294
13	0	0	0	0	9.184	9.174	6.964	6.954
14	0	0	0	0	9.974	9.164	5.954	5.944
15	1	−1	1	−1	3.124	3.514	2.244	2.234
16	0	1	0	0	8.424	8.614	4.064	4.054
17	−1	1	1	−1	3.814	4.104	2.604	2.594
18	0	−1	0	0	6.844	7.234	3.664	4.654
19	−1	1	1	1	2.794	3.184	2.104	3.094
20	−1	−1	1	1	3.984	4.974	3.104	3.094
21	−1	0	0	0	6.094	6.184	6.574	6.864
22	−1	−1	−1	−1	2.014	3.004	1.584	1.574
23	1	1	−1	−1	4.854	4.844	5.714	6.164
24	0	0	0	0	8.704	8.694	6.124	6.114
25	0	0	0	0	7.704	7.694	6.114	6.104
26	0	0	1	0	6.034	6.024	6.804	6.794
27	−1	1	−1	1	2.684	2.974	1.234	2.224
28	1	1	−1	−1	4.624	3.614	4.174	5.164
29	0	0	0	0	9.654	9.644	7.054	7.144
30	1	1	1	−1	1.523	2.514	1.234	1.624

The values presented in the table are average values of three repetitions, Exp.: Experimental values, RSM: RSM predicted values; EC_C: Bioethanol concentration for the consortium (*C. fimi* MTCC24 + *Z. mobilis* MTCC92) (g/L); EC: Bioethanol concentration for *H. thermocellum* KSMK 1203 (g/L).

3.2.1. Plackett-Burman design

The Plackett-Burman design is a statistical tool for screening the effective variables based on the effects on yield and reduces the number of trials in a further optimization study using RSM [6,37,38]. The variable's effect on bioethanol productivity was determined by conducting twelve experimental trials as given by the model mentioned in Table 3. The coefficient R^2 value 0.985 and 0.971 respectively for *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 were confirmed that the design was significant in predicting the effects of the variables on bioethanol productivity. The p-value of $p \geq 0.05$ indicated that the models for bioethanol production were significant and A (pre-treated biomass), C (malt extract), F (sodium chloride) and G (KH_2PO_4) were identified as significant variables (Table 3). The regression equations for *H. thermocellum* KSMK1203 (Y_{EC}) and consortium (Y_{ECC}) were obtained using the design that had predicted based on the effects of variables on bioethanol production. It has given as Eqns. (5) and (6).

$$Y_{EC} = 6.10-1.71\ A - 0.29\ B - 0.037\ C - 0.89\ D - 0.29E + 0.67\ F + 0.44\ G - 0.72\ H - 0.47\ I + 0.76\ J + 0.32\ K \quad (5)$$

$$Y_{ECC} = 5.23-1.41\ A + - 0.14\ B + - 0.32\ C - 0.84\ D - 0.46E + 0.33\ F - 0.045\ G - 1.03\ H - 0.90\ I + 1.08\ J + 0.33\ K \quad (6)$$

The PBD was used to select the effective medium components for fermentation with their main effects. Among the chosen eleven variables, the four variables mentioned earlier were found to be significant. Analysis of the different components showed that pre-treated *A. ascalonicum* leaf biomass and sodium chloride were showed a high percentage contribution with 93 and 80% respectively on the bioethanol production from both the process using *H. thermocellum* KSMK1203 and consortium. The carbon source in the production medium plays a major role in the biosynthesis regulation [39] and bioethanol productivity from different microorganisms [9].

Figure A2 and Figure A3 show normal plot of standardized effect for ethanol concentration for *H. thermocellum* KSMK 1203 and consortium, respectively. In this investigation, the carbon source pre-treated biomass constitutes cellulose showed a highly significant effect on bioethanol production, and it possibly up-regulated the yield of cellulases. The previous investigations reported that cellulose was the excellent source for consolidated bioprocessing of bioethanol bioconversion using *H. thermocellum* KSMK1203 [9] and a consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 [6].

3.2.2. RSM-CCRD optimization

The essential medium components such as pre-treated biomass, malt extract, potassium dihydrogen phosphate and sodium chloride were screened from PBD having the capability to secrete cellulase that bioethanol yield through CBP using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92. It was further examined through RSM-CCRD to determine the optimal fermentation conditions among the consecutive interactions of independent variables in three levels with six centre points. The quadratic polynomial regression model was evaluated by ANOVA, in order to find the statistical significance of the model [9]. The p-value of the model was found <0.0001 for the model performed using *H. thermocellum* KSMK1203 and consortium, which confirmed the significance of the model at 95% confidence [4]. Also, Fisher's test of the model was 17.27 and 31.48, respectively, for *H. thermocellum* KSMK1203 and consortium, indicating that the resultant coefficients were significant [6]. The correlated empirical relationship for both the type and second-order polynomial regression equations with coded variables in terms of *H. thermocellum* KSMK1203 and consortium, respectively, were given by the following Eqs. (7) and (8).

$$Y_{EC} = +12.14 + 0.62{*}\ X_1 + 0.61{*}\ X_2 + 0.024{*}\ X_3 + 0.71{*}\ X_4 - 0.19{*}\ X_1X_2 - 0.67{*}\ X_1X_3 + 0.096{*}\ X_1X_4 - 0.56{*}\ X_2X_3 + 0.40{*}\ X_2X_4 + 0.44{*}\ X_3X_4 - 1.59{*}\ X_1^2 - 3.04{*}\ X_2^2 - 1.54{*}\ X_3^2 - 1.90{*}\ X_4^2 \quad (7)$$

$$Y_{ECC} = +11.31 + 0.74{*}\ X_1 + 0.81{*}\ X_2 + 0.27{*}\ X_3 + 0.99{*}\ X_4 + 0.46{*}\ X_1X_2 - 0.60{*}\ X_1X_3 + 0.098{*}\ X_1X_4 - 0.74{*}\ X_2X_3 + 0.066{*}\ X_2X_4 + 0.73{*}\ X_3X_4 - 2.81{*}\ X_1^2 - 2.58{*}\ X_2^2 - 1.81{*}\ X_3^2 - 0.69{*}\ X_4^2 \quad (8)$$

Where, X_1-pre-treated biomass, X_2-malt extract, X_3-NaCl and X_4 -KH_2PO_4. The R^2 value was found to be 0.9516 and 0.971, respectively, for *H. thermocellum* KSMK1203 and consortium, indicating 95.16 and 97.1% confidence of the model response. The experimental and predicted values for bioethanol production were comparably similar to each other for both the strain types that promise the accuracy of the design shown in Table 4. As well as, the statistical analysis of variance (ANOVA) showed in Table A1. It was observed that the model was significant for bioethanol production using two types with regards to individual variables effect. The individual effects X_1 and X_4, and the quadratic effects X_1^2, X_2^2 and X_4^2 were significant for the growth of *H. thermocellum* KSMK1203. Likewise, for the consortium, the individual effects X_1 X_2 and X_4, the interactive effects X_1X_3, X_2X_3 and X_3X_4, and the quadratic effects X_1^2, X_2^2 and X_3^2 were having significant effects on co-production of cellulase and bioethanol. For the investigated models, Adeq. Precision and standard deviation were 11.795 and 1.31 for *H. thermocellum* KSMK1203 and 0.8060 and 0.96 for consortium.

A three-dimensional response surface plots of the effect of pre-treated biomass (X_1), malt extract (X_2), NaCl (X_3) and KH_2PO_4 (X_4) on the growth of *H. thermocellum* KSMK1203 and consortium were predicted using RSM-CCRD modeling and presented in Figs. 4 and 5. From Figs. 4 and 5, it was observed that how the growth of individual strain and consortium were affected been represented with the bioethanol yield as a function of two independent variables with all other variables set as constant. The growth of *H. thermocellum* KSMK1203 was significantly affected by pre-treated biomass, malt extract and NaCl and consortium growth affected by pre-treated biomass, malt extract, and KH_2PO_4 concentration.

Figure A4 and Figure A5 show experimental values plotted against RSM model predicted data for ethanol concentration for *H. thermocellum* KSMK 1203 and consortium, respectively. Hence, the growth of *H. thermocellum* KSMK1203 and consortium were significantly affected by pre-treated biomass that constitutes cellulose (a carbon source) and malt extract (nitrogen source). As well as NaCl and KH_2PO_4 were affected by the growth of *H. thermocellum* KSMK1203 and the consortium, respectively, which served minerals. Kavitha et al. [9] reported that 7 g/L of pre-treated biomass, 4 g/L of malt extract, 3 g/L of ammonium chloride and 2 g/L of sodium chloride was the predicted optimal condition with the yield of 12.90 ± 0.987 g/L in pre-treated *Nannochloropsis gaditana* biomass using *H. thermocellum* KSMK1203.

Likewise, Selvakumar et al. [6] stated that under the optimal conditions, bioethanol could be commercially produced using *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 through CBP from lignocellulosic biomass. The optimized medium components for *H. thermocellum* KSMK1203 was found to be pre-treated biomass 4.59 g/L, malt extract 2.61 g/L, sodium chloride 1.34 g/L and potassium dihydrogen phosphate 2.43 g/L and for the consortium, pre-treated biomass 4.85 g/L, malt extract 2.36 g/L, sodium chloride 2.96 g/L and potassium dihydrogen phosphate 1.85 g/L. The maximum bioethanol yield was obtained under the optimal conditions to be 8.91 g/L and 7.09 g/L, respectively, for *H. thermocellum* KSMK1203 and consortium from pre-treated *A. ascalonicum* leaf biomass through CBP with the desirability of 1.0.

3.3. Efficiency of cellulose (pre-treated biomass) hydrolysis

The efficiency of $NaClO_2$+ alkali-treated and $NaClO_2$ treated *A. ascalonicum* leaf biomass hydrolysis was evaluated using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 for 36 h. *H. thermocellum* KSMK1203 strain produced cellulases to hydrolyze the $NaClO_2$+ alkali-treated and $NaClO_2$ treated biomass with the pre-treated biomass 4.59 g/L, malt extract 2.61 g/L, NaCl 1.34 g/L and KH_2PO_4 2.43 g/L. As well as during the hydrolysis of biomass using consortium *C. fimi* MTCC 24 was produced cellulases with pre-treated biomass 4.85 g/L, malt extract 2.36 g/L, NaCl 2.96 g/L and KH_2PO_4 1.85 g/L to mediate hydrolysis of $NaClO_2$+ alkali-treated and $NaClO_2$ treated biomass. The rate of hydrolysis was analyzed every hour. The results of this study revealed that biomass hydrolysis was begun to stabilize after 24 h of fermentation process. The maximum hydrolytic yields for

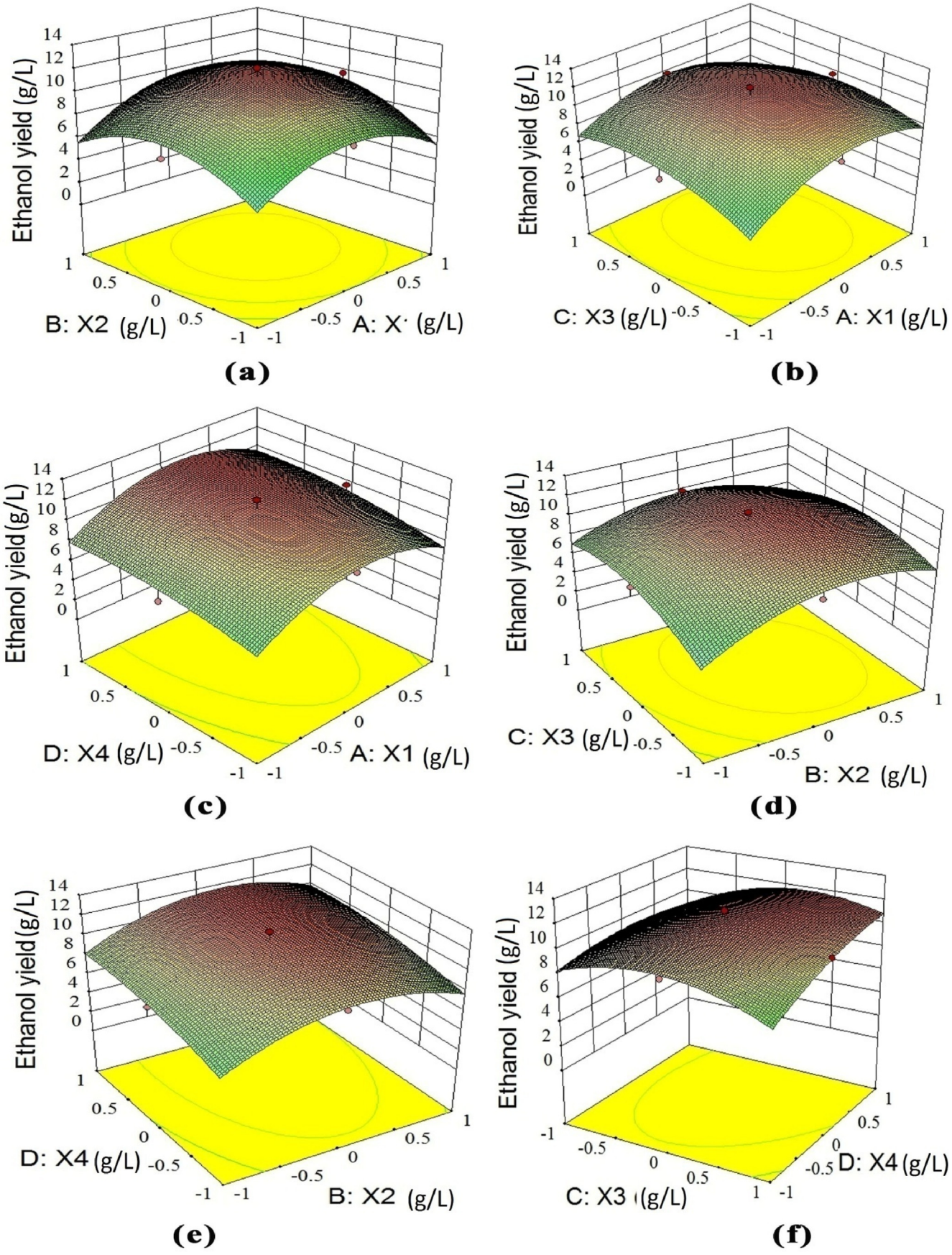


Fig. 4. Interactive effects of medium components on bioethanol yield for *H. thermocellum* KSMK 1203.

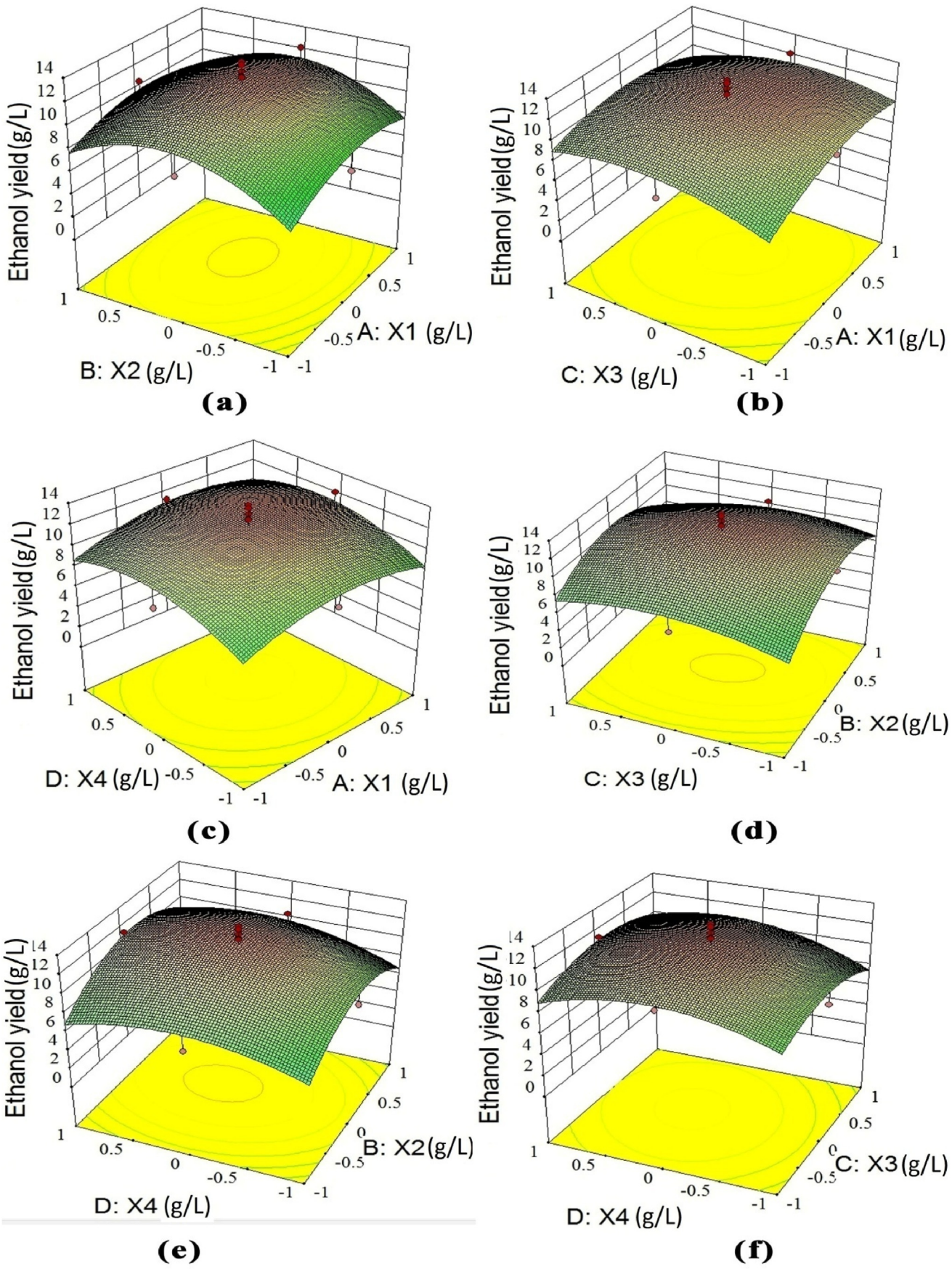


Fig. 5. Interactive effects of medium components on bioethanol yield for consortium (*C. fimi* MTCC24 and *Z. mobilis* MTCC92).

Table 5
Fermentation kinetics of consortium (*C. fimi* MTCC 24 + *Z. mobilis* MTCC 92) and *H. thermocellum* KSMK1203 in $NaClO_2$ + alkali-treated and $NaClO_2$ treated *A. ascalonicum* medium for 24 h.

Parameters investigated	*H. thermocellum* KSMK 1203		Consortium	
	$NaClO_2$ + alkali treated medium	$NaClO_2$ treated medium	$NaClO_2$ + alkali treated medium	$NaClO_2$ treated medium
Y_1(%)	22.81 ± 1.51	10.55 ± 1.69	25.33 ± 2.01	9.18 ± 1.35
Y_2(%)	62.71 ± 2.31	45.82 ± 2.56	64.39 ± 1.12	38.78 ± 2.14
$Y_{p/s}$(%)	52.54 ± 2.47	35.13 ± 2.12	40.93 ± 1.9	37.91 ± 1.01
rs($gL^{-1}h^{-1}$)	0.454 ± 0.09	0.246 ± 0.08	0.492 ± 0.06	0.296 ± 0.05
rp($gL^{-1}h^{-1}$)	0.559 ± 0.02	0.258 ± 0.09	0.621 ± 0.08	0.225 ± 0.08
rx(log cell $mL^{-1}h^{-1}$)	0.635 ± 0.12	0.197 ± 0.02	0.603 ± 0.09	0.219 ± 0.06
Cellulase activity (IU/mL)	11.54 ± 1.89	3.26 ± 0.98	12.65 ± 1.29	4.62 ± 1.36

Y_1-bioethanol yields over cellulose; Y_2-total cellulose consumption; $Y_{p/s}$ -total cellulose in bioethanol conversion; rs-total cellulose consumption rate; rp-bioethanol production rate; rx-cell generation rate.

Table 6
Observation of fermentation end products with exogenous addition of bioethanol and sodium acetate for 36 h of fermentation.

		H. thermocellum KSMK1203				Consortium			
		Bioethanol	Acetate	Formate	Lactate	Bioethanol	Acetate	Formate	Lactate
Bioethanol addition (%v/v)	0	8.01 ± 0.23	2.26 ± 0.56	0.17 ± 0.03	0.38 ± 0.09	6.81 ± 0.36	1.29 ± 0.16	0.09 ± 0.02	0.38 ± 0.06
	2	5.26 ± 0.56	1.12 ± 0.35	0.20 ± 0.04	0.13 ± 0.06	4.21 ± 0.41	0.45 ± 0.11	0.02 ± 0.01	0.13 ± 0.03
	4	2.62 ± 0.23	0.78 ± 0.56	0.16 ± 0.02	0.03 ± 0.01	1.91 ± 0.35	0.21 ± 0.08	0.00 ± 0.00	0.03 ± 0.01
	6	1.01 ± 0.45	0.21 ± 0.61	0.02 ± 0.01	0.00 ± 0.00	0.12 ± 0.10	0.17 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
	8	0.23 ± 0.25	0.03 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.07	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	10	0.11 ± 0.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetate addition (mM/L)	0	7.62 ± 1.02	0.81 ± 0.13	0.12 ± 0.02	0.28 ± 0.09	6.91 ± 0.87	0.68 ± 0.13	0.11 ± 0.03	0.12 ± 0.05
	15	8.21 ± 0.98	0.89 ± 0.09	0.05 ± 0.01	0.11 ± 0.02	7.01 ± 0.89	0.72 ± 0.11	0.02 ± 0.01	0.06 ± 0.02
	30	6.21 ± 0.89	0.31 ± 0.11	0.00 ± 0.00	0.01 ± 0.01	7.21 ± 0.65	0.78 ± 0.12	0.00 ± 0.00	0.01 ± 0.01
	60	2.14 ± 0.68	0.61 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	3.31 ± 0.25	0.31 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
	120	0.98 ± 0.23	0.21 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	1.01 ± 0.06	0.16 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
	180	0.31 ± 0.03	0.09 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

H. thermocellum KSMK1203 strain were 61.25 ± 2.1% and 33.45 ± 3.2% respectively for $NaClO_2$+ alkali-treated and $NaClO_2$ treated *A. ascalonicum* leaf biomass. Also, the hydrolytic yields for the consortium were obtained to be 39.14 ± 2.6% and 19.89 ± 3.1% respectively for $NaClO_2$+ alkali-treated and $NaClO_2$ treated *A. ascalonicum* leaf biomass. In this present study, it has been observed from the results that pre-treatment of the biomass increases the rate of hydrolysis. Kavitha et al. [6] were obtained the hydrolytic yield of 57.84 ± 1.9% using *H. thermocellum* KSMK1203 strain from acid-treated *N. gaditana* biomass. Similarly, 41 ± 0.16% of hydrolysis yield was attained from *M. esculenta* Crantz YTP1 stem biomass using co-culture of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 [6]. Rettenmaier et al. [40] investigated the characterization of *H. thermocellum* 253c-K6 isolate and reported that the cellulose hydrolysis with 84% during the production of metabolites.

3.4. Kinetic parameters and fermentation yield of $NaClO_2$ + alkali treated and $NaClO_2$ treated biomass medium

In order to find kinetic parameters and fermentation yield, 50 g/L of $NaClO_2$+ alkali-treated and $NaClO_2$ treated *A. ascalonicum* leaf biomass medium with single *H. thermocellum* KSMK1203 strain and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 separately. Table 6 demonstrates the fermentation kinetics of and *H. thermocellum* KSMK1203 in $NaClO_2$+alkali treated and $NaClO_2$ treated *A. ascalonicum* leaf medium for 24 h. The maximum cellulose consumption was 62.71 and 45.82%, respectively, from $NaClO_2$ + alkali-treated and $NaClO_2$ treated biomass for *H. thermocellum* KSMK1203 strain and 64.39 and 38.78% from treated and untreated biomass for the consortium. The yield over cellulose was observed to be 22.814 and 10.55% for *H. thermocellum* KSMK1203 and 25.33 and 9.18% for consortium from $NaClO_2$+alkali treated and $NaClO_2$ treated biomass, respectively. These results showed that the rate of the microbial population was increased with the decreasing the cellulose in the medium [9,26]. For *H. thermocellum* KSMK1203 strain, the bioethanol yield ($Y_{p/s}$) from $NaClO_2$+ alkali-treated and $NaClO_2$ treated *A. ascalonicum* leaf biomass was 52.54 and 35.13%, respectively. Similarly, for consortium, $Y_{p/s}$ was obtained 40.93 and 37.91 from $NaClO_2$+alkali treated and $NaClO_2$ treated biomass that showed the amount of bioethanol produced from initial cellulose (biomass). From Table 5 it was observed that from $NaClO_2$+alkali treated biomass medium, the rate of cellulose consumption (rs) and yield formation rate (rp) were 0.454 and 0.559 g/L.h, and 0.492 and 0.621 g/L.h, respectively for *H. thermocellum* KSMK1203 and consortium. Similarly, for $NaClO_2$ treated biomass medium rs and rp were 0.246 and 0.258 g/L.h, and 0.296 and 0.225 g/L.h for *H. thermocellum* KSMK1203 and consortium. The cellulase activity for *H. thermocellum* KSMK1203 was observed to be 11.54 ± 1.89 and 3.26 ± 0.98 IU/mL, respectively, for treated and untreated biomass. Similarly, for the consortium, cellulase activity was observed as 12.65 ± 1.29 and 4.62 ± 1.36 IU/mL for treated and untreated medium, respectively.

Ha-Tran et al. [41] investigated *H. thermocellum* growth on various carbon sources such as cellobiose, glucose and fructose containing medium and observed significant bioethanol yield of on cellobiose adapted medium. They obtained bioethanol yield of 42.61 ± 1.74 mmol L^{-1}, cellobiose consumption of 25.36 ± 0.79 mmol L^{-1}, and Volumetric bioethanol productivity of 0.710 ± 0.003 mmol $L^{-1}h^{-1}$ on cellobiose adapted medium. Kavitha et al. [9] reported the fermentation kinetic parameters such as sugar consumption rate of 0.5175 ± 0.12 and 0.3708 ± 0.61 g/L.h, the bioethanol production rate of

0.8065 ± 0.32 and 0.4333 ± 0.67 g/L.h, were observed for treated and untreated *N. gaditana* biomass. Selvakumar et al. [6] investigated co-culture of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 for consolidated bioprocessing of pre-treated *M. esculenta* Crantz YTP1 stem biomass into bioethanol and reported the maximum cellulose consumption and bioethanol yield were found to be 20.7 ± 1.4 and 9.39 ± 0.33 g/L respectively under optimal conditions. They stated that co-cultures of C. fimi MTCC 24 and Z. mobilis MTCC 92 effectively consume cellulose from pre-treated biomass to produce cellulase and bioethanol with the maximum cellulase and bioethanol yield 11.63 ± 1.23 IU/mL and 9.39 ± 0.33 g/L, respectively.

3.5. Effect of exogenous bioethanol addition on fermentation

To determine the level of bioethanol tolerance of *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92, exogenously bioethanol was added to the production medium ranges from 0 to 12% (v/v) on pre-treated biomass fermentation for 36 h. It was observed that metabolic pathway was altered with the external addition of bioethanol for both the cases and variation in metabolite profile shown in Table 6. About 41% and 36% decrease in bioethanol productivity had been observed while the addition of 2% (v/v) bioethanol exogenously to the production medium for *H. thermocellum* KSMK1203 and consortium, respectively. However, cell growth and bioethanol accumulation had approximately reduced to 90% at the addition of above 6% (v/v) of externally added bioethanol for both cases. The further addition of bioethanol concentration beyond 6%, bioethanol yield decreased to zero for *H. thermocellum* KSMK1203 and consortium was observed at 12% that connected with decreased cell growth. Singh et al. [42] investigated bioethanol tolerance of *Clostridium thermocellum* ATCC 31924 strain and observed that beyond 4.5% of external bioethanol concentration, bioethanol yield and microbial growth were ceased. The bacterium results in high bioethanol productivity and high bioethanol tolerance [43]. In the synthetic consortium, the growth of *C. fimi* MTCC 24 was restricted with the exogenous addition of bioethanol to the medium that mitigated cellulose hydrolysis and minimized bioethanol yield.

3.6. Effect of exogenous acetate addition on fermentation

During the cellulosic bioconversion, acetate has been produced as the major metabolic end product. An elevated acetate concentration has a subtle effect on CBP candidates in cellulosic bioethanol fermentation [44]. To determine effect of acetate accumulation on metabolite profile of *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92, varying concentration (0–180 mM) of sodium acetate was added to the optimized medium. In this present study, *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 were performed under stress conditions to determine the ability of an acetate-tolerance of these strains resistant to exogenously added sodium acetate. These experiments were carried out in 50 g/L of pre-treated *A. ascalonicum* leaf biomass (cellulose) concentrations with supplementation of sodium acetate (NaAc) ranging from 0, 15, 30, 60, 120 and 180 mM. For *H. thermocellum* KSMK1203, an initial addition of NaAc up to 15 mM resulted 9% increased bioethanol yield of 8.21 g/L was obtained compared to medium without exogenous addition of acetate conversely, formate and lactate production were completely inhibited beyond 30 mM NaAc addition (Table 6). Also, for consortium, bioethanol yield was increased about 6% till 30 mM addition of NaAc to the medium and after 15 mM addition formate and lactate production had been ceased (Table 6).

In both cases, lower concentration of NaAc addition resulted slight increase in bioethanol yield. The less amount of NaAc addition redirects the metabolic flux towards more ATP generation for cell growth resulted increase in bioethanol yield [45]. The further addition of NaAc concentration beyond 30 mM, bioethanol yield decreased from 6.21 to 0.31 g/L for *H. thermocellum* KSMK1203 and 7.21 to 0.29 g/L for consortium was observed at the elevated NaAc addition that accompanied by reduced cell growth. Singh et al. [42] obtained 20% increases in bioethanol production with 30 mM NaAc addition for *Clostridium thermocellum* ATCC 31924 strain. Puspita et al. [47] stated *Z. Mobilis* AcR/2–12 (mutant) had the ability to withstand and grow up to 250 mM of NaAc and in contrast the growth of *Z. mobilis* ZM481(wild-type) was inhibited above 150 mM NaAc addition. The increasing level of NaAc addition to the production medium inhibits sugar consumption and reduced bioethanol production.

4. Conclusion

This present study led to producing bioethanol from pre-treated *A. ascalonicum* leaf biomass through consolidated bioprocessing that was achieved using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92. Pre-treatment of biomass was performed for screening of efficient alkali and their combinations with low dosage for better hemicellulose solubilization and enhanced cellulose recovery and optimization vis RSM. Among different alkalis and their combinations, NaOH + urea showed better results and under the optimal condition of 2% NaOH + urea, 100 °C for 60 min, the observed maximum hemicellulose removal was 85.25% and produced semi-crystalline cellulosic material. The essential medium components (pre-treated biomass, malt extract, KH_2PO_4 and NaCl) and were screened from PBD and further optimized with RSM to secrete cellulase that produces bioethanol through CBP using *H. thermocellum* KSMK1203 and consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92. The CBP was achieved through a single bacterial isolate and synthetic consortium with an excellent growing capability in the optimized medium, which produced bioethanol efficiently from pre-treated *A. ascalonicum* leaf biomass. Hence, this investigation results suggest that wild-type *H. thermocellum* KSMK1203 strain and a synthetic consortium of *C. fimi* MTCC 24 and *Z. mobilis* MTCC 92 could be exploited for the cellulose secretion and simultaneous bioethanol bioconversion using pre-treated *A. ascalonicum* leaf biomass in a single reactor.

CRediT authorship contribution statement

S. Kavitha: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **T. Gajendran:** Conceptualization, Supervision, Data curation, Formal analysis, Methodology. **K. Saranya:** Supervision, Writing – review & editing. **P. Selvakumar:** Conceptualization, Data curation, Formal analysis, Methodology. **V. Manivasagan:** Validation, Writing – review & editing. **S. Jeevitha:** Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2022.01.047.

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