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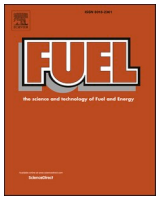

Full Length Article

Concomitant inhibitor-tolerant cellulase and xylanase production towards sustainable bioethanol production by *Zasmidiumcellare* CBS 146.36

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ABSTRACT

In the present study, a potential fungal strain (*Zasmidiumcellare* CBS 146.36) was isolated to efficiently produce a cocktail of enzymes critical for hydrolysis of lignocellulosic biomass. Various parameters were optimized for concurrent cellulase and xylanase production, which showed the highest activity for *exo*-glucanase3.48 FPU/mL, *endo*-glucanase8.69 IU/mL and xylanase12.41 IU/mL. For cellulase production a Mamdani-based fuzzy model was developed with a prediction accuracy of 0.033 mean squared error (MSE) for test data sets. Enzymatic hydrolysis of pre-treated mustard biomass generated maximum fermentable sugar (78.19 mg/mL total sugar consisting of xylose 17.28 mg/mL, and glucose 40.91 mg/mL) at 18 % substrate loading. Enzymatic hydrolysis in the presence of various pre-treatment inhibitors revealed the tolerance and catalytic efficiency of the enzyme. The saccharification efficiency increased from 75.91 % to 83.5 % in presence of non-ionic surfactant (triton x-100). Fermentation of non-detoxified sugar hydrolysate produced 9.98 g/L of ethanol (0.38 g/g ethanol yield) using co-culture of *Saccharomyces cerevisiae* MTCC 170 and *Candida vishwanathii* JCM 9567.

1. Introduction

Lignocellulosic biomass (LCB) is a plentiful energy source that significantly addresses the world's present energy dilemma and has been realized as a sustainable alternative for second-generation biofuel production. However, the heterogeneous nature of LCB is one of the major caveats in its complete degradation, thus affecting its application in biorefinery-based approaches [1]. The hydrolysis products of LCB have essential applications in textile, food, pharmaceutical, biofuel, and other fields. Enzymatic treatment is more acceptable and environmentally friendly as it offers cost-effective hydrolysis under mild conditions without toxic chemical generation [2]. The high cost of enzyme production at a large scale led to a search for alternative low-cost substrates for enzyme production using high-yielding microbes [3]. LCB mainly contains the polysaccharides cellulose and hemicellulose, along with a substantial amount of lignin, forming the matrix around the sugar polymer; however, the compositions of the three major constituents vary among various lignocellulosic biomasses. The choice of biomass is a critical factor affecting the techno-economics of a bioprocess. In the present study, Mustard stalk and straw were utilized as the source of sugars for bioethanol production. Mustard stalk and straw (MSS) in India account for approximately 70 % of the total mustard plant, excluding the seed. These plant remnants are considered agricultural waste and are neither utilized as livestock feed nor appropriately managed; they are either left in the field to decompose naturally or disposed of through burning, contributing to environmental pollution. In comparison to other conventionally used agro-wastes like rice straw (cellulose 28.5 %, hemicellulose 24.7 %) and wheat bran (cellulose 30 %, hemicellulose 27.2 %), MSS exhibits higher cellulose (48.5 %) and hemicellulose content (29.6 %) while being economically cheaper and thus can serve

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as a better choice as an economically feasible agro-residue for 2G bio-ethanol production [4]. Bio-conversion of LCB to biofuels involves three main steps: pre-treatment, saccharification and fermentation [5]. A cocktail of hydrolytic enzymes is required for improved saccharification of major complex polysaccharides, glucan and xylan, to monomeric sugar glucose and xylose, respectively [6]. Among different microbes for hydrolytic enzyme production, fungi are prominent due to the concomitant multiple extracellular enzyme production [7]. Cellulase is composed of *endo*-glucanase, *exo*-glucanase and β-glucosidase), which act synergistically for complete cellulose hydrolysis. In contrast, xylanases are specific for the hydrolysis of xylan (hemicellulose) to xylose. Therefore, combined actions of cellulase and xylanase can improve the hydrolysis of LCB to glucose and xylose, which can be further used as a carbon source by microbes for other bioproducts formation [8]. The enzyme cocktail production can be a viable option for the cost-effective production of biofuels and biochemicals in sugar-based biorefineries [9,10].Since cellulases and xylanases are high-value-added microbial products for biofuel production, this study aimed to isolate a fungal strain for the concurrent production of cellulase and xylanase using LCB as a carbon source. The inability to accurately handling of non-linear and imprecise data by statistical modelling, several researchers used soft computing-based approaches for process modelling [11,12]. Fuzzy logic is one of the artificial intelligence-based process modelling approaches that turn imprecise (fuzzy) data in to a decision-making tool by stimulating the brain's cognitive process [13]. The successful implementation of fuzzy logic modelling for biological approaches was greatly recognized [14]. In the present study, a Mamdani-based fuzzy model was developed for cellulase production.

Different physiological parameters necessary for enzyme production were optimized and the physicochemical properties of crude enzyme were studied. Further, the effect of various inhibitors and non-ionic surfactants on the enzymatic hydrolysis of biomass was studied. The non-detoxified sugar hydrolysate obtained after saccharification was analysed for ethanol production using the co-culture of *Saccharomyces cerevisiae* MTCC 170 and *Candida vishwanathii* JCM 9567.Since the present work is also the first report evaluating the potential of *Zasmidiumcellare* CBS 146.36 for producing hydrolytic enzymes, the authors hope to contribute to the existing database of hydrolytic strains.

2. Materials and methods

2.1. Isolation and screening of cellulase and xylanase-producing fungal strain

The fungus was isolated from termite mound soil collected from the local area of Banasthali Vidyapith. The sample was serially diluted and cultured on potato dextrose agar (PDA) plates, incubated at 30°Cfor 10 days. The isolated fungal strains were screened for cellulase and xylanase production by agar plate assay on mineral salt medium (MSM) (2.5 g/L $NaNO_3$, 1 g/L KH_2PO_4,0.5 g/L $MgSO_4$, 0.5 g/L KCl, 4 % agar) supplemented with 0.5 % CMC (Carboxy methyl cellulose) and beechwood xylan, respectively. After 72 h, the CMC and beechwood xylan agar plates were stained with Congo red (0.1 %) for 15 min, then de-staining with 1 M NaCl for 10 mins [15]. The CMC and beechwood xylan plates were observed for the hydrolysis zone and the positive isolates were further evaluated for enzyme production under submerged conditions using wheat straw as substrate. The inoculum was prepared by inoculating a loop full of 5-day-old culture in autoclaved potato dextrose broth (50 mL) in an Erlenmeyer flask (250 mL) and incubated for 5 days at 30 °C.The isolate was sub-cultured weekly on PDA slants and stored at 4 °C [16].

2.2. Molecular characterization

Molecular identification of isolated fungal strains was carried out based on the 18S ribosomal sequencing at NCCS (National Centre for Cell Science), Pune, facilities complying with the standard protocol [17].

2.3. Enzyme production

Cellulase and xylanase enzyme production was carried out under the same culture conditions in 500 mL Erlenmeyer flasks containing 100 mL of MSM supplemented with 10 % wheat straw as the sole carbon source. The 5-day-old fungal culture was inoculated (2 % inoculum volume) in the production medium and kept under static conditions for 5 days at 30 °C. After 5 days, the enzyme was extracted from the fermented broth with muslin cloth and consequently, the filtrate was further centrifuged (10,000 rpm, 15 mins, 4 °C). After centrifugation, clear supernatant was used as a crude enzyme [18]. The crude enzyme was examined for exoglucanase (FPase), endoglucanase (CMCase), β-glucosidase and xylanase activities using standard methods [19,20].

2.4. Optimization of enzyme production

Enzyme production was optimized to increase its activities by varying different parameters. The effects of incubation time (1–7 days), pH (3–9), and temperature (25–45 °C) werestudied on the simultaneous production of cellulase and xylanase in an unoptimized production medium containing MSM and 10 % wheat straw [21].Further, the production medium was optimized by varying substrate concentration (2–20 % wheat straw), 0.2 % inducers (glucose, xylose, lactose, cellulose, cellobiose) and 0.2 % nitrogen source (Yeast extract, peptone, urea, malt extract, soybean meal, ammonium nitrate, NH_4Cl, $(NH_4)_2SO_4$). All the experiments were conducted in an Erlenmeyer flask (500 mL) containing production medium (100 mL), following a strategy of one factor at a time approach.

2.5. Fuzzy logic modelling for cellulase production

In the present work, a Mamdani-type fuzzy inference system was utilized to implement IF-THEN cellulase production (IU/mL) rules. The various modules, namely fuzzification, fuzzy inference engine, and defuzzification of the fuzzy system (Fig. 1a). In the fuzzification step, the variables (input and output) of cellulase production were assigned as low (L), medium (M), higher (H) and determined the corresponding membership functions. The chosen triangular membership functions for cellulase production variables (input and output) were shown in Fig. 1b. The temperature, pH, substrate concentration and incubation time ranged from 25 °C-35 °C, 4.5—6.5, 6 % w/v – 10 % w/v and3-5 days, respectively. The main output of the process is the cellulose activity.

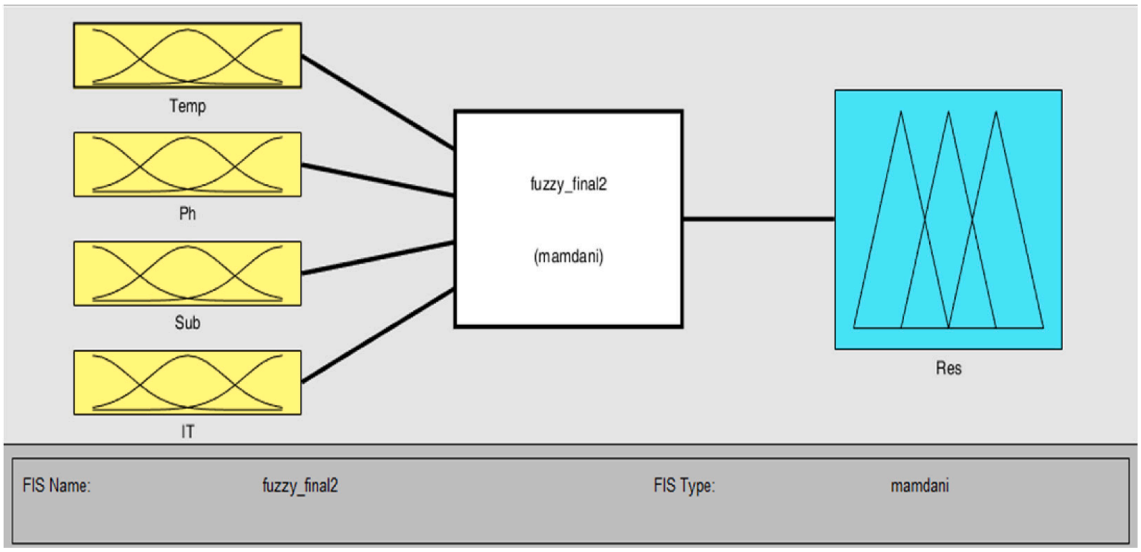
In the present work, 17 fuzzy rules were generated, using cellulase production variables (input and output). Finally, the defuzzification of the output variable was done, depicted in Fig. 1c.

2.6. Preliminary characterization of crude enzyme

The crude enzyme was used to determine the optimum temperature, pH, thermal and pH stability of the exoglucanase, endoglucanase and xylanase. The optimum temperature for enzyme activities was assessed in the range of 35–60 °C.The thermal stability was evaluated by incubating the enzyme at different temperatures (35–60 °C) for 12 h in 0.05 M acetate buffer at pH 7.0. To determine the optimum pH of cellulase and xylanase activity, enzymatic assays were conducted at 50°Cusing substrate dissolved in 0.05 M acetate buffer of varied pH of 3.0–9.0. The enzyme was dissolved in 0.05 M acetate buffer with varying pH (3–9) and incubated for 24hat room temperature to determine the pH stability. Samples were withdrawn to examine residual enzyme activity [22].

2.7. Saccharification of alkali pre-treated mustard biomass

Mustard biomass was thermo-chemically treated with NaOH (0.2 M)



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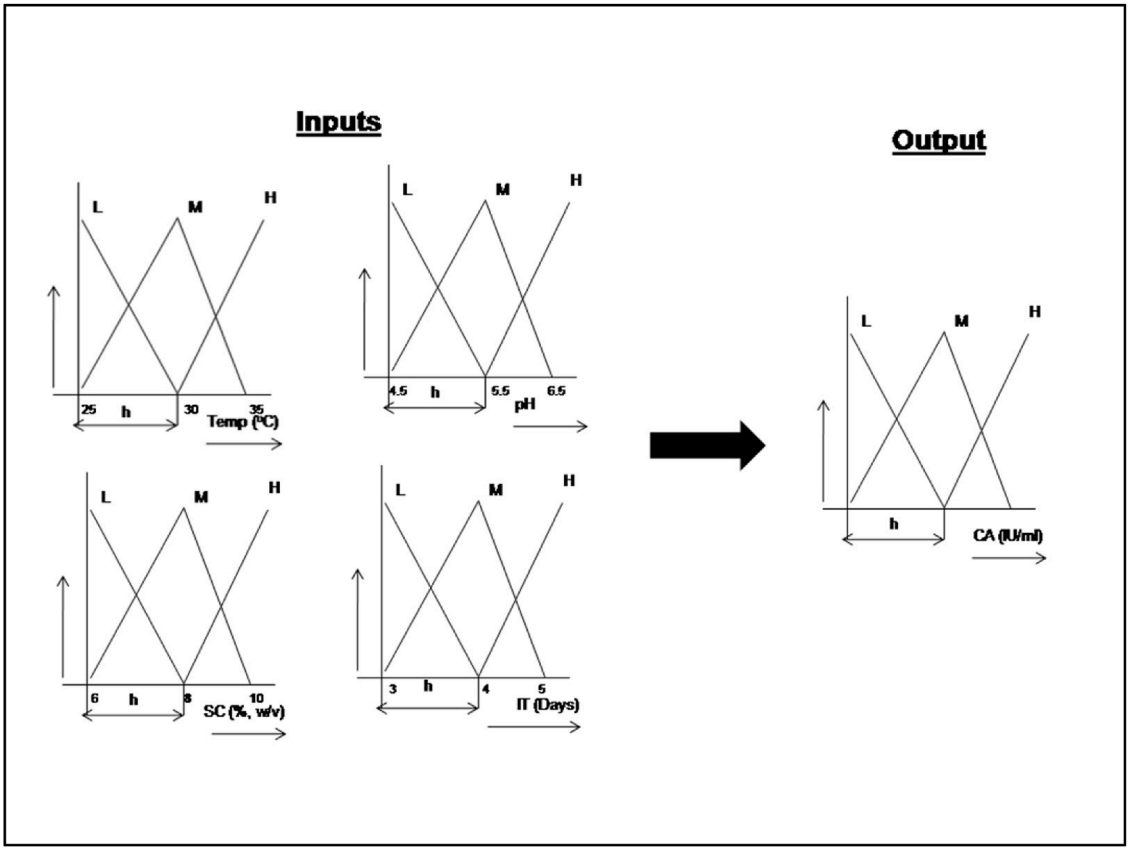
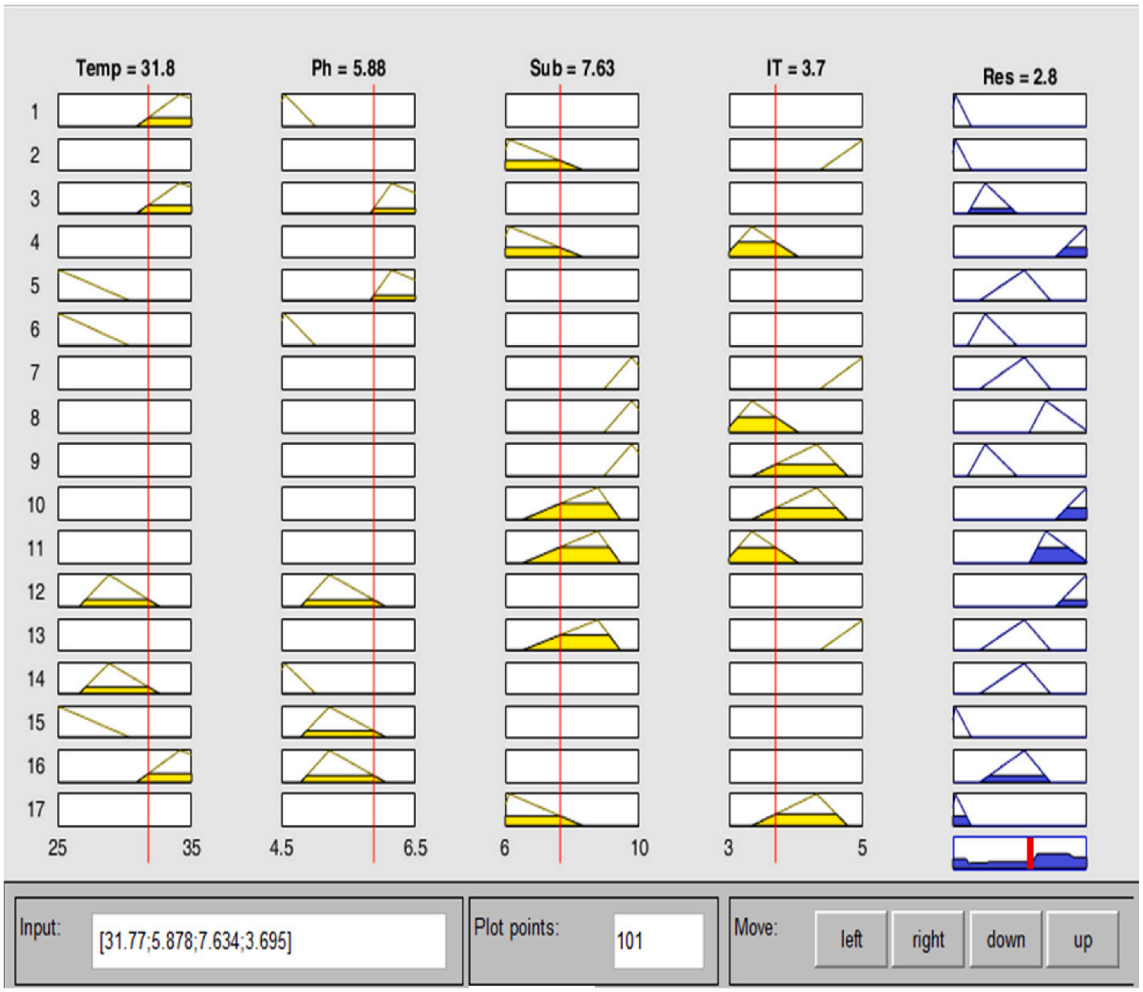


(b)

Fig. 1. Mamdani-based Fuzzy logic model for cellulase production: (a) Fuzzy System (b) Membership function plot of input and output variable (c) Rule set (Where "Res" means "Cellulase activity").

for 30 min at 160 °C following the previously optimized process [23]. Alkali treated biomass was washed with water till a neutral pH was obtained. The saccharification experiment was carried out in 250 mL Erlenmeyer flask of 50 mL working volume containing thermochemically pretreated mustard biomass and the crude enzyme at a loading of 20 FPU/gds. To prevent any microbial contamination, a minute



(c)

Fig. 1. (*continued*).

concentration (0.015 %) of sodium azide was added to each reaction flask. Saccharification was optimized concerning various parameters such as (2–30 % biomass concentration, pH 3–9 and 12–120 h incubation period) at 50 °C.After specific time intervals, the biomass hydrolysate was centrifuged (10,000 rpm, 10 min) and the supernatant was used for sugars estimation through the HPLC method [23].

2.8. Effect of inhibitors and non-ionic surfactants on enzymatic saccharification

The tolerance of enzymes against various inhibitors (generated during pretreatment) was also evaluated. The hydrolysis reaction mixture containing 18 % biomass and 50 mL crude enzyme at aloading of (20FPU/gds) was supplemented with inhibitors at different concentrations, i.e., furfural (0.01—0.1 % v/v); acetic acid (0.1–0.5 % v/v); HMF (0.05——0.2 % v/v); ethanol (1–5 % v/v). The effect of various non-ionic surfactants (0.1 % v/v) (Tween 20, Tween 40, Tween 60, triton X-100, PEG-4000) on saccharification was also tested. The HPLC method was used to estimate the amount of glucose and xylose generated after enzymatic hydrolysis [24].

2.9. Fermentation of non-detoxified sugar hydrolysate for ethanol production

The non-detoxified biomass hydrolysate obtained after enzymatic hydrolysis was evaluated for its fermentation to ethanol. For the fermentation experiments, mono and co-culture of Saccharomyces cerevisiae MTCC 170 and Candida viswanathii JCM 9567 were examined. The sugar hydrolysate containing 4 % of total sugar (40 g/L) supplemented with 0.5 % yeast extract, 1 % peptone (pH-5.5) was inoculated with 2 % (v/v) inoculum volume of 18 h old culture of *S. cerevisiae*, *C. viswanathii* and co-culture of both in 1:1 ratio [23]. Sugar utilization and ethanol production was evaluated at 35 °C for 120 h under partial anaerobic conditions. The ethanol and residual sugar were measured every 24 h interval by the HPLC method [24].

3. Results and discussion

3.1. Screening and identification of cellulase and xylanase producing fungal strain

In this study, 13 fungal isolates were obtained from termite mound soil. Nine isolates with marked distinct colony characteristics were further screened for hydrolysis zone on CMC and xylanagar plates. Four of these isolates were found positive for only CMC hydrolysis, whereas

sixisolates were able to degrade both CMC and beechwood xylan as the sole carbon source. Based on the hydrolysis zone, these six positive isolates were further tested for cellulase and xylanase production under submerged fermentation. All positive isolates were capable of producing xylanase, *exo*-and *endo*-glucanases (Fig. 2).

Among six isolates, only two were producing β-glucosidase (0.042 IU/mL and 0.034 IU/mL, respectively), but with lower xylanase (3.21 IU/mL), exo- and *endo*-glucanase activities (0.42 FPU/mL and 1.24 IU/mL, respectively). However, out of six positive isolates, one strain(TS2) was chosen for further studies based on its highest cumulative activities for xylanase (7.12 IU/mL), exo- and *endo*-glucanases (2.16 FPU/mL and4.62 IU/mL, respectively).Basic Local Alignment Search Tool (BLAST) results of the 18S rRNA gene nucleotide sequence of isolated strain TS2 revealed it to have 100 % similarity with *Zasmidiumcellare* CBS 146.36 with accession number EF137362.1.*Zasmidiumcellare* CBS 146.36 is a slow-growing fungus capable of metabolizing volatile organic compounds, also known as wine cellar mould. It was first isolated from the wall of the wine cellar by H. Schander in 1936. It belongs to the dothideomycetes class of Ascomycota [25]. Though the strain has long been identified, it was not evaluated for cellulolytic enzyme production for the degradation of LCB. For efficient biomass saccharification, the synergistic actions of cellulase, xylanase and auxiliary enzymes are required. Therefore, multifunctional enzymes owingto cellulase and xylanase activities could be more effective. In the same context, the present study is the first report on the concomitant production of cellulase and xylanase enzyme from *Zasmidium cellare* CBS 146.36 using wheat straw as a substrate.

3.2. Optimization of enzyme production

3.2.1. Effect of fermentation time, temperature, and pH

Optimization studies were conducted using wheat straw as the sole carbon source to achieve concomitant cellulase and xylanase production. Fermentation time plays a vital role in extracellular enzyme production. Fig. 3a represented the highest production of cellulase (2.09 FPU/mL exoglucanase, 5.34 IU/mL endoglucanase) and xylanase (8.34 IU/mL) was achieved after 120 h of incubation time. There was a steady increase in fungal growth and enzyme production with time. After 120 h, there was a slight decrease in enzyme production due to the depletion of essential nutrients in the fermentation medium resulting in the inactivation of enzyme secretion systems under stress conditions. Similarly, Rana et al. [26] studied cellulase and xylanase production from *Fusarium oxysporum* MTCC 7229 using wheat bran and rice bran, at 28 °C. After 5 days of incubation, they reported the highest CMCase 4.10 U/g and xylanase activities (13.77 U/g). The physiological factors pH and temperature play a crucial role in enzyme production. Fungi are generally mesophiles with optimum temperature between 25–35 °C for maximal growth and enzyme production. *Zasmidium cellare* CBS 146.36 was also found to be a mesophile showing maximum growth (1.74 g/L DCW) and enzyme production at 30 °C depicted in Fig. 3b,which agrees with the existing literature [27,28].The enzyme production and fungal growth decreased with further increase in temperature showing minimum enzyme activities and growth (1.01 g/L DCW) at 45 °C. Ezeilo et al. [29] reported 30 °C as optimum temperature for *Trichoderma asperellum* UC1 with maximum CMCase, FPase, β-glucosidase and xylanase activities 136.16 IU/g, 26.03 U/g, 130.09 IU/g and 255.01 U/g, respectively. Optimum pH can be an important determinant of gene expression responsible for substrate utilization and enzyme production. The maximum growth was at pH 5.0 (1.81 g/L DCW) but the fungal strain maintained a steady growth rate in a broad range of pH 3.0–9.0 presented in Fig. 3c. Changes in the pH had no significant effect on fungal growth, but enzyme production was greatly affected. Endoglucanase and xylanase production was significantly affected by pH changes in the production medium, exhibiting the lowest activities at acidic pH 3.0–4.0 and the highest endoglucanase (6.01 IU/mL) and xylanase activity (10.84 IU/mL) was at pH 5.0. Although, the highest *exo*-glucanase activity (3.09 FPU/mL) was found at pH 8.0, similar activity was observed at pH 5.0 (2.64 FPU/mL). Therefore, no significant effect of pH on exoglucanase production was observed and pH 5.0 was chosen as optimum for enzyme production which is in accordance with previous literature [30,31]. The fungal isolate was tolerant toward a broad pH range but sensitive to high temperatures.

3.2.2. Effect of substrate concentration, inducer, and nitrogen source

After optimization of physiological parameters, the effect of substrate concentration (wheat straw) on extracellular enzyme production was studied. Fig. 4a represented that cellulase and xylanase production gradually increased as the substrate concentration increased from 2 % to 14 %, where the minimal and maximum enzyme production was at 2 % and 14 % substrate concentrations, respectively. The highest enzyme activities, 2.83 FPU/mL, 6.34 IU/mL, and 11.47 IU/mL, were achieved for exo-, *endo*-glucanases and xylanase, respectively. Further, increase in substrate concentration resulted in a decline in the growth and accumulation of enzymes in the production medium. That could be due to a decrease in moisture content which results in a higher viscosity of the medium, subsequently leading to decreased mass exchange and poor

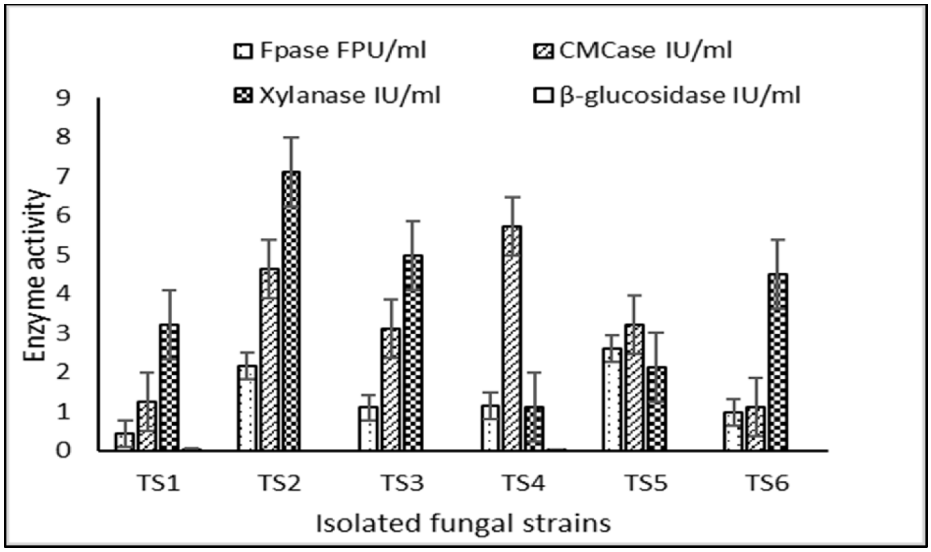
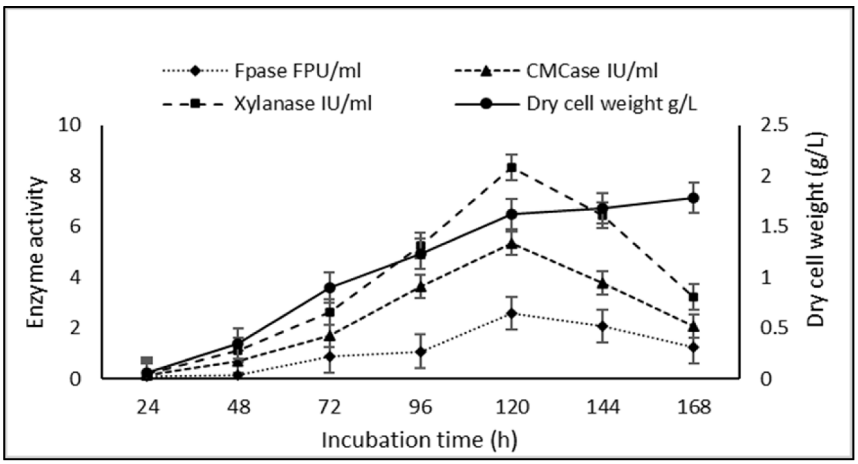


Fig. 2. Cellulase and xylanase activities of screened fungal strains in MSM containing wheat straw as substrate.



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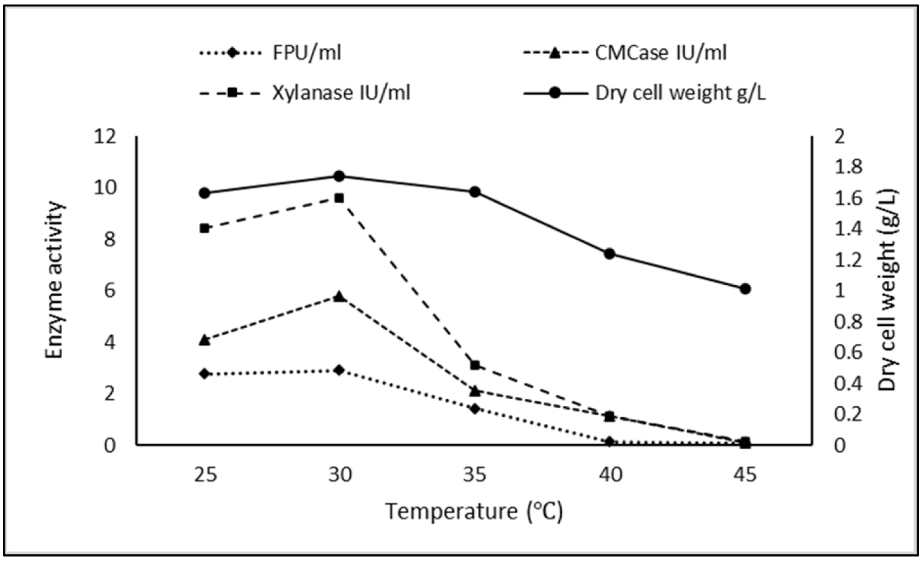

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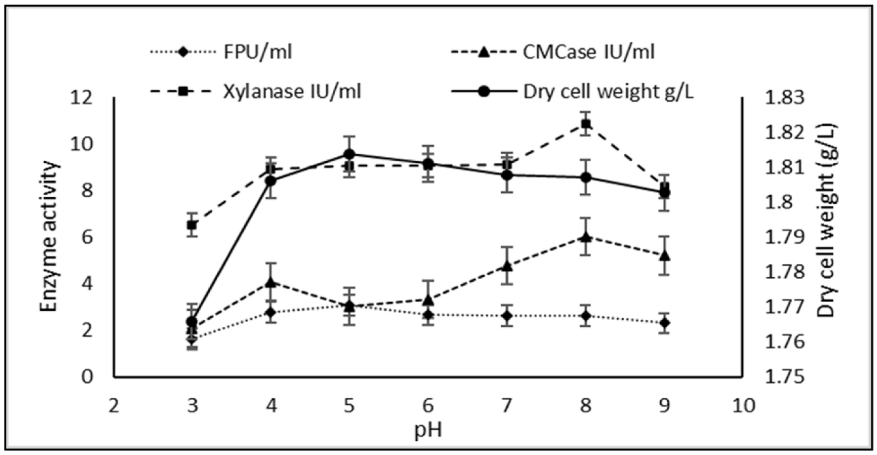

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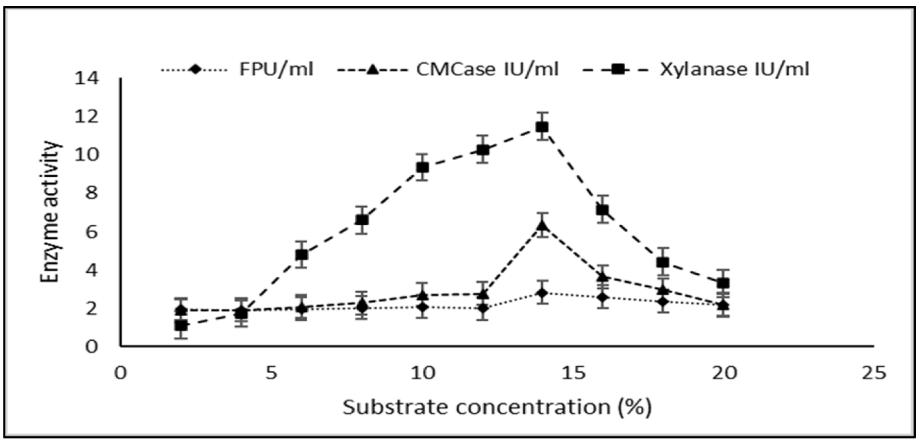
Fig. 3. Effect of different physiological parameters on enzyme production and fungal growth (a) Incubation time (b) Temperature (c) pH.



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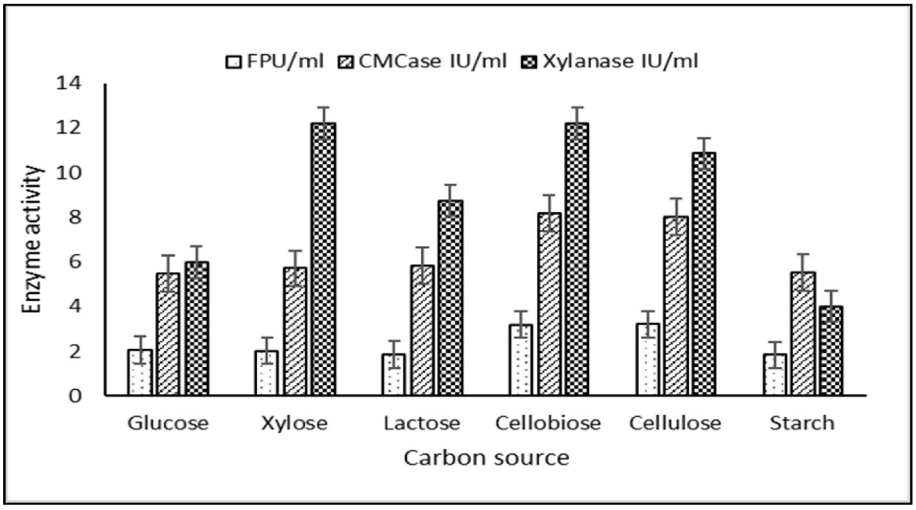

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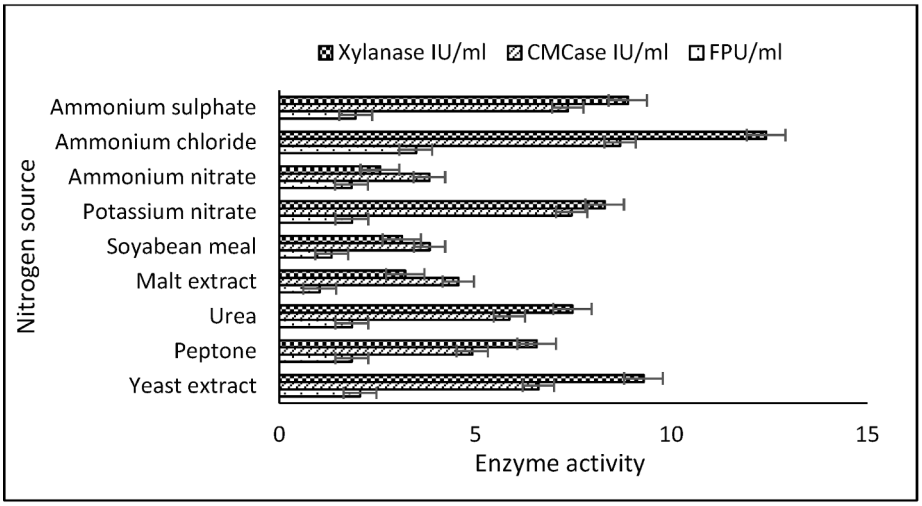


(c)

Fig. 4. Optimization of enzyme production with respect to (a) Substrate concentration (b) Carbon source (c) Nitrogen source.

aeration. Production of hydrolytic enzymes is activated only when an inducer or easily metabolizable carbon sources are present along with lignocellulosic biomass. Because complex and insoluble lignocellulosic polymers cannot be readily utilized by fungal cells, the small amount of simple or complex saccharides like glucose, xylose, lactose, cellobiose and cellulose in the production medium can act as inducers to initiate the transcription of cellulase and xylanase genes [32]. The present study obtained the highest exoglucanase of 3.20 FPU/mL and endoglucanase activity of 8.17 IU/mL with 0.2 % cellobioseas inducer. Xylanase production was observed to be induced by both xylose and cellobiose with the highest activity for both xylose (12.20 IU/mL) and cellobiose (12.19 IU/mL) represented in Fig. 4b.In the absence of inducers, the highest exoglucanse activity of 2.05 FPU/mL, endoglucanase activity of 5.38 IU/mL and xylanase activity of 9.35 IU/mLwere obtained, which suggested that the cellulase and xylanase production is inducer-dependent. All the inducers promoted fungal growth and efficiently induced the production of enzymes. Among the tested carbon sources, 0.2 % w/v cellobiose was used as inducer for further studies because it efficiently increased the activities of exoglucanase, endoglucanase, and xylanase. Kunitake et al [33] studied a signalling pathway in *Aspergillus aculeatus.* They reported the prominent role of cellobiose insignalling pathway activation of cellulase and hemicellulase gene expression under the control of the XlnR-independent regulation through CeRE.Schuerg et al[34] reported a 6.2-fold increase in CMCase and an 11-fold increase in xylanase activities using *Thermoascus aurantiacus* cultivated on xylose-enriched hydrolysate. When the effect of nitrogen source was studied, 0.2 % ammonium chloride was found best among all the tested nitrogen sources depicting the highest activities for exo-, *endo*-glucanases and xylanases were 3.48 FPU/mL, 8.69 IU/mL, and 12.41 IU/mL, respectively (Fig. 4c). Das and Ray [35] reported maximum xylanase production ((253.98 IU/gds) from *Aspergillus aculeatus* on wheat bran supplemented with 1 % ammonium chloride. The secretion of extracellular enzymes is greatly affected by nitrogen sources in the production medium, as it is the ultimate precursor for enzymeproduction.

3.3. Fuzzy logic modelling for cellulase production

As fuzzy logic is a valuable tool for dealing the inaccurate, incomplete, and uncertain data, in the present work, 17 rules have been generated in the IF-Then format of the Fuzzy logic model. The model was evaluated with the test runs (Table 1). The generated 17 rules and the corresponding input and output variable surface view were shown in Fig. 3b. For the FL operation, AND, and OR, the min and max criterion was used. Other fuzzy operation activities, i.e., Implication and aggregation, were performed with min and max functions. The final step of defuzzification was done with the centroid function method. The results of test runs have been summarized in Table 1 along with the FL model predicted values and % deviation of fuzzy value with the exp. values have been depicted in Fig. 5.

From the tested data analysis through FL modelling, it was found that

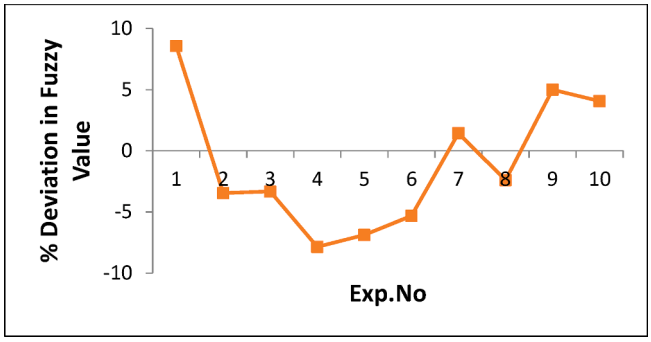


Fig. 5. Percentage deviation of tested data with FL model for cellulase production.

the derived fuzzy values are in close agreement with the exp. values, and the MSE (Mean Squared Error) of the Fuzzy model was found to be low, i.e., 0.033. By observing the testing results, it is concluded that the developed fuzzy model fitted well for all other experimental training datasets.

3.4. Thermotolerance and pH tolerance of crude enzyme

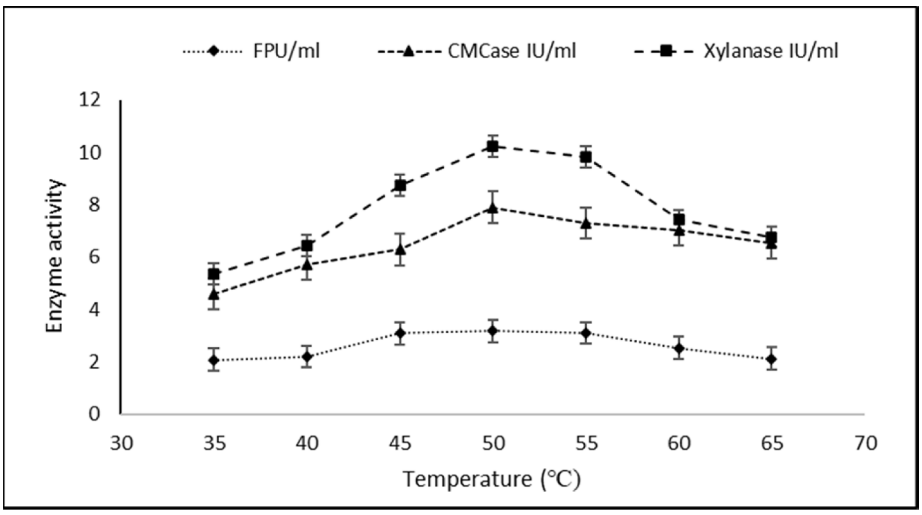
The crude enzyme was assessed for temperature, pH optima and thermal and pH stability. The optimum temperature for the enzymes was 50 °C (Fig. 6a), but the optimum pH was different (Fig. 6b). For exoglucanase and endoglucanase, pH 5.0 was optimum (3.67 FPU/mL and 8.90 IU/mL), but for xylanase, comparable activities were observed at pH 5.0 and 8.0(12.46 and 12.74 IU/mL), respectively (Fig. 6b). Exo- and *endo*-glucanaseswere stable in a broad pH range from 4.0 to 9.0, but xylanase was stable under pH 6.0 to 9.0 which make it suitable for industrial applications (Fig. 6c). The lowest residual exoglucanase and endoglucanase activity was 65.72 % and 66.58.19 % at pH 9.0. In contrast, xylanase was sensitive to acidic conditions at pH 3.0–4.0, exhibiting the lowest residual activity (21.84 %). In the case of temperature stability (Fig. 6d), the enzyme was stable up to 55 °C (approx. 82 % total residual activity), making it suitable for enzymatic saccharification at 50 °C.

3.5. Saccharification of alkali pre-treated mustard biomass

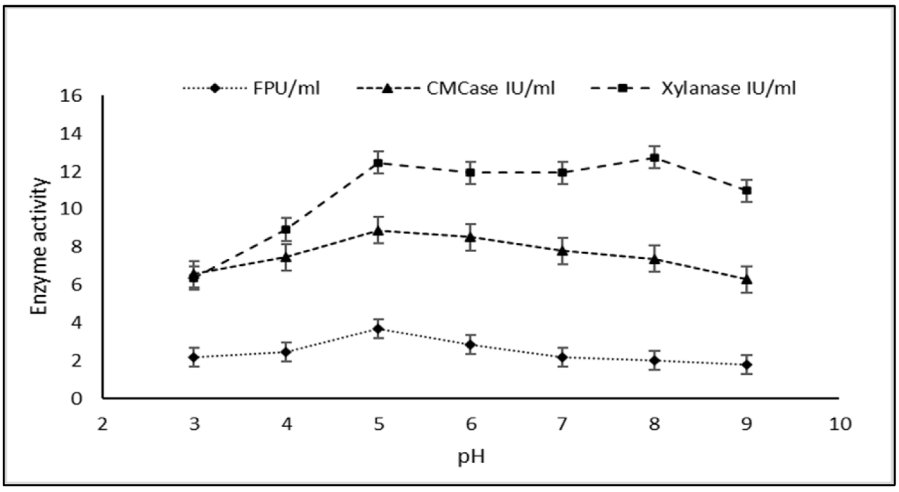
The recalcitrant nature of lignin makes cellulose and hemicellulose inaccessible for enzymatic degradation. The delignified mustard biomass with 44.9 % cellulose and 12.46 % hemicellulose was enzymatically hydrolysed by crude cellulase produced from *Zasmidium cellare* CBS 146.36. In the present study, enzymatic hydrolysis was optimized to investigate the role of pH, biomass concentration and incubation time on biomass valorisation to reducing sugars. The total reducing sugars obtained at each hydrolysis step were analysed through

Table 1
Test sets with parameters for FL model and test data analysis.

Expt. No.	Temp	pH	SC (%W/V)	IT (days)	Exp.value (Cellulase activity)	Fuzzy Value
1	27	5	9	3.5	2.1	44.851
2	25	4.5	8	3	2.6	5.656923077
3	30	4.5	6	5	2.4	−47.798125
4	28	4.8	7	4.5	2.8	−16.6225
5	32	6	7	3.5	3.2	−9.1404375
6	28	5	9	4.5	3	−14.8511333
7	30	5.5	6	5	2.1	22.21642857
8	32	6.2	7	4.5	2.5	28.4324
9	28	6	7	3.5	1.9	80.45505263
10	35	5.5	10	5	2.7	1.374074074



(a)



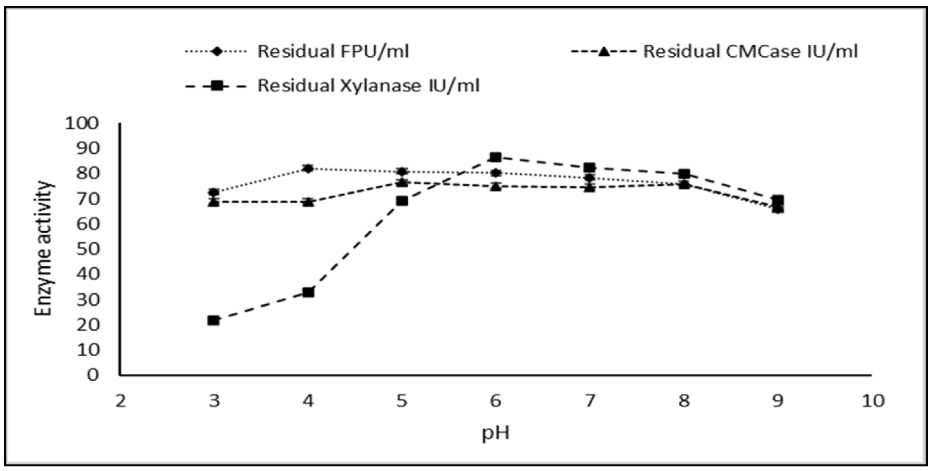
(b)

Fig. 6. Determination of (a) pH optima (b) Temperature optima (c) pH stability (d) thermostability of crude enzyme.

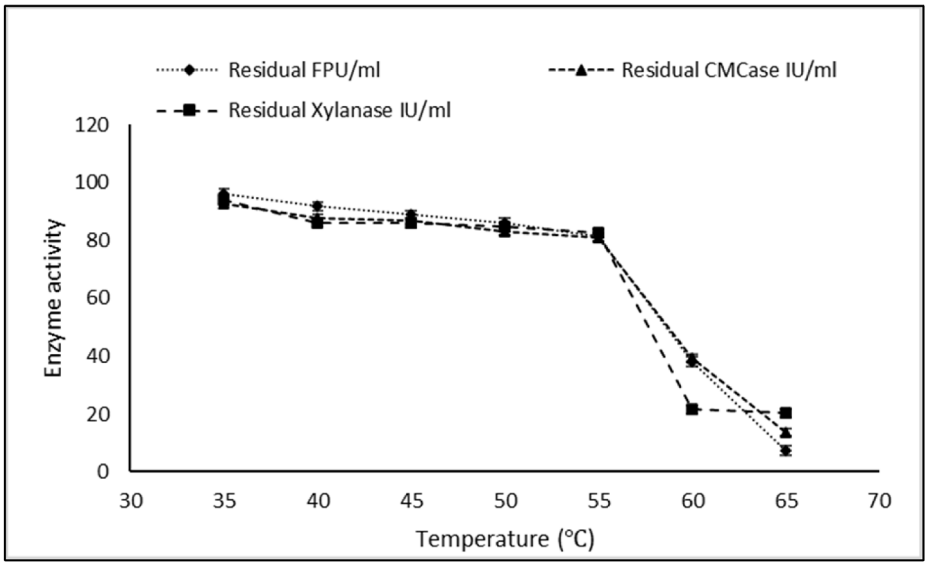
analysis of variance (ANOVA) presented in Table 2. A maximum fermentable sugar of 78.19 mg/mL (40.91 mg/mL glucose and 17.28 mg/mL xylose) was obtained with 18 %biomass loading at pH5.0 within 48 h (Table 2). Biomass concentration higher than 18 % resulted in decreased sugar yield, possibly due to a decrease in moisture content. The sugars obtained after hydrolysis were in proximity to the enzyme resulting in feedback inhibition at higher solid loading. The decline in sugar production after 48 h could be because of enzyme denaturation at 50 °C for longer period of time. There was no significant effect of pH on hydrolysis because the enzyme was stable in a broad pH range of 4.0–9.0 (Table 2). Maximum saccharification efficiency 75.91 % was observed at the above optimized conditions. Singh et al. [36] studied enzymatic saccharification of the mustard stalk and straw (MSS) using a crude enzyme (20FPU/g) produced by *T. reesei* NCIM 992 and *T. reesei* NCIM 1052. Their studies reported an increase in glucose yield from 9.69 to 10.19 g/100 g of treated MSS after hydrolysis. Several authors reported use of mustard sugar hydrolysates for of biofuel production [36–38].

3.6. Effect of inhibitors and non-ionic surfactants (NIS) on enzymatic saccharification

Acetate, 5-HMF and furfural are major inhibitors generated after pretreatment of LCB that can significantly affect the catalytic efficiency of the hydrolytic enzyme resulting in poor saccharification yield. In the current study, the presence of inhibitors did not significantly impact the enzymatic hydrolysis process at low concentrations (Table 2). Acetic acid at a higher concentration (0.5 % v/v) acted as a mild inhibitor because of the extremely low pH, reducing total sugar production to 38.15 mg/mL. A higher ethanol concentration also decreased the enzyme's hydrolysis efficiency with 45.98 mg/mL of total sugar production. Alves et al. [39] reported a 1.7-fold increase in β-glucosidase activity in the presence of 10 % ethanol and 1 % HMF. All the tested NIS improved the biomass saccharification, but maximum sugar production (86.27 mg/mL total sugar consisting of xylose 20.28 mg/mL, and glucose 59.91 mg/mL) was achieved with Triton X-100 (Table 2). The saccharification efficiency was increased from 75.91 % to 83.5 % when triton x-100 was used. NIS acts as an enzyme stabilizer and effector by blocking the unproductive binding of enzymes to lignin. It also



(c)



(d)

Fig. 6. (*continued*).

facilitates the desorption of enzymes from the substrate and subsequently blocks enzyme deactivation by shear forces [40]. Therefore, triton X-100 (0.1 % v/v) was used for further studies in order to achieve maximum reducing sugar. The pre-treated biomass does not need to be detoxified before saccharification due to the crude enzyme's resistance to a variety of inhibitors and ethanol making it suitable for industrial ethanol production. Developing low-cost multifunctional enzymes with greater temperature, pH stability and inhibitor tolerance is essential for improved biomass saccharification in bioethanol production.

3.7. Fermentation of non-detoxified sugar hydrolysate for ethanol production

Dilute alkali treated mustard sugar hydrolysate was used for ethanol production in present study. The concentrated biomass hydrolysate containing 39.14 g/L of total reducing sugar encompassed 23.08 g/L glucose and 14.82 g/L xylose. *Saccharomyces cerevisiae* is well-documented for its ability to efficiently ferment hexose sugars, such as glucose and fructose, into ethanol. At the same time, *Candia viswanathii* has shown promise in fermenting pentose sugars, such as xylose, which are abundant in lignocellulosic hydrolysates. Moreover, the study published by Cao et al. [41] showed the positive effect of furfural, one of the major inhibitors generated during pretreatment of LCB, on the growth of *C. viswanathii*, thus making the strain a potential alternative for LCB-based fermentation approaches. Since high ethanol production requires fermentation of both hexose and pentose sugars, for the present study, the ethanol production from monocultures of *S. cerevisiae* and *C. viswanathii* was compared with the co-culture to explore the potential of the two strains to act synergistically for complete fermentation of biomass sugar hydrolysate subsequently leading to higher ethanol yield. Table 3 represented that the monoculture of *S. cerevisiae* utilized 22.02 g/L of reducing sugars to produce 6.12 g/L of ethanol at 72 h of fermentation time thus providing the maximum ethanol yield of 0.27 g/g. Since *S. cerevisiae* is a hexose fermenting yeast, the xylose present in hydrolysate was left unutilized after fermentation. Similarly, fermentation utilizing the monoculture of *C. viswanathii* resulted in 3.89 g/L of ethanol with 0.23 g/g ethanol yield. Ethanol production by *S. cerevisiae* was reported to be higher as compared to *C. viswanathii*; this might be due to hydrolysate composition and *S. cerevisiae* convert glucose to ethanol at faster rate compared to *C. viswanathii* converts xylose to

Table 2
Enzymatic saccharification of treated mustard biomass and the effect of inhibitors and non-ionic surfactants.

Substrate concentration (%)	Total glucose (mg/ml)	Total xylose (mg/ml)	Incubation time (h)	Total glucose (mg/ml)	Total xylose (mg/ml)	pH	Total glucose (mg/ml)	Total xylose (mg/ml)	Inhibitors (concentration)	Total glucose (mg/ml)	Total xylose (mg/ml)	Non-ionic Surfactants	Total glucose (mg/ml)	Total xylose (mg/ml)
2	3.06 ± 0.12^{a}	2.04 ± 0.67^{c}	12	15.27 ± 0.18^{a}	05.18 ± 0.25^{y}	3	25.05 ± 0.12^{k}	08.03 ± 0.31^{q}	Control	40.91 ± 0.18^{j}	17.28 ± 0.97^{h}	Control	40.91 ± 0.21^{z}	17.28 ± 0.12^{y}
4	5.83 ± 0.98^{d}	2.49 ± 0.39^{f}	24	25.69 ± 0.36^{p}	09.01 ± 0.17^{s}	4	28.30 ± 0.51^{ns}	10.56 ± 0.22^{p}	HMF (0.05 %)	31.06 ± 0.74^{k}	13.70 ± 0.11^{y}	Tween 20	49.67 ± 0.17^{p}	17.45 ± 0.25^{ns}
6	6.88 ± 0.32^{k}	4.58 ± 0.42^{ns}	48	32.22 ± 0.41^{ns}	13.80 ± 0.11^{d}	5	39.79 ± 0.20^{j}	16.34 ± 0.21^{ns}	HMF (0.1 %)	28.59 ± 0.84^{o}	11.97 ± 0.95^{k}	Tween 40	44.22 ± 0.58^{ns}	18.67 ± 0.42^{ns}
8	9.17 ± 0.21^{ns}	6.11 ± 0.11 k	72	30.52 ± 0.802^{ns}	13.48 ± 0.18^{ns}	6	39.75 ± 0.33^{ns}	16.23 ± 0.18^{ns}	HMF (0.2 %)	25.65 ± 0.42^{p}	08.46 ± 0.82^{ns}	Tween 60	45.71 ± 0.39^{ns}	18.34 ± 0.18^{ns}
10	9.61 ± 0.62^{e}	8.65 ± 0.19^{ns}	96	30.32 ± 0.98^{b}	13.71 ± 0.24^{p}	7	38.37 ± 0.11^{ns}	15.30 ± 0.57^{ns}	Furfural (0.01 %)	30.23 ± 0.88^{ns}	13.16 ± 0.12^{ns}	Triton X-100	59.91 ± 0.45^{o}	20.28 ± 0.20^{p}
12	16.09 ± 0.64^{f}	10.72 ± 0.28^{r}	120	30.18 ± 0.21^{c}	12.36 ± 0.10^{ns}	8	30.95 ± 0.88^{ns}	10.84 ± 0.19^{ns}	Furfural (0.05 %)	26.81 ± 0.21^{u}	10.35 ± 0.36^{ns}	PEG 4000	45.45 ± 0.65^{n}	18.34 ± 0.45^{w}
14	20.16 ± 0.79^{ns}	10.85 ± 0.66^{q}				9	29.16 ± 0.17^{d}	08.49 ± 0.41^{i}	Furfural (0.1 %)	24.04 ± 0.23^{ns}	08.44 ± 0.29^{ns}			
16	20.53 ± 0.84^{ns}	13.68 ± 0.12^{p}							Acetic acid (0.1 %)	31.46 ± 0.51^{ns}	12.62 ± 0.18^{q}			
18	26.81 ± 0.86^{p}	11.48 ± 0.80^{r}							Acetic acid (0.3 %)	27.58 ± 0.67^{ns}	08.39 ± 0.31^{t}			
20	16.29 ± 0.79^{r}	10.86 ± 0.2^{u}							Acetic acid (0.5 %)	22.71 ± 0.88^{ns}	05.44 ± 0.23^{g}			
25	14.16 ± 0.34^{ns}	6.07 ± 0.65^{ns}							Ethanol (1 %)	31.94 ± 0.17^{e}	12.40 ± 0.15^{ns}			
30	10.80 ± 0.47^{ns}	7.21 ± 0.87^{ns}							Ethanol (5 %)	30.49 ± 0.88^{w}	10.64 ± 0.18^{o}			
									Ethanol (10 %)	24.28 ± 0.91^{ns}	09.97 ± 0.97^{ns}			

Mean ± S.D. shown in the table. Same rows showing different values with the same letters are significantly different at $P<0.05$. ns: non-significant values, manifested as average with error bars (±) showing standard deviation of the samples prepared in triplicate.

Table 3
Comparative study of ethanol production using mono-culture and co-culture of *S. cerevisiae* and *Candida vishwanathii.*

Yeast	Initial sugar (g/L)	Sugar consumption (g/L)	Ethanol (g/L)	Total sugar conversion efficiency (%)
S.C	39.14 ± 1.15^{ns}	56.27 ± 1.47^{m}	6.12 ± 0.18^{p}	52.94 ± 2.41^{a}
C.V	39.14 ± 1.32^{t}	43.12 ± 1.39^{ns}	3.89 ± 0.37^{q}	45.18 ± 3.94^{s}
S.C+C.V	39.14 ± 1.26^{q}	65.66 ± 1.55^{ns}	9.97 ± 0.97^{r}	74.93 ± 5.16^{ns}

Mean ± S.D. shown in the table. Same rows showing different values with the same letters are significantly different at $P<0.05$. ns: non-significant values, manifested as average with error bars (±) showing standard deviation of the samples prepared in triplicate.

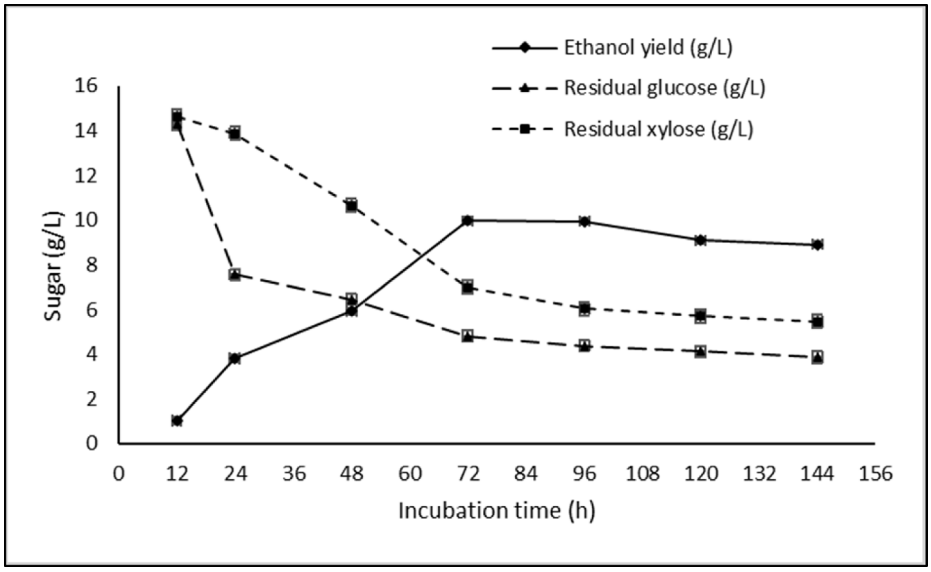


Fig. 7. Ethanol production and residual sugar after fermentation using co-culture of *S. cerevisiae* and *C. vishwanathii* with respect to fermentation time.

ethanol. Since single microbial strains often cannot efficiently ferment both types of sugars, in the next set of experiments, co-cultures of *Saccharomyces cerevisiae* (which ferments glucose) and *Candida vishwanathii* (*which ferments xylose*) was explored to ensure the complete utilization of all available sugars During co-culture process, maximum ethanol production was observed to be 9.97 g/L resulting in an ethanol yield of 0.38 g/g (Table 3). High sugar consumption and ethanol concentration during co-culture was due to conversion of both hexose and pentose sugars to ethanol. Production of ethanol from glucose is rapid in the co-culture system which might also raise the possibility of inhibition of xylose fermentation by ethanol. Fig. 7 represents the residual sugars (glucose and xylose) and ethanol produced in the fermentation medium. In the present co-culture study, 82.17 % of glucose and 61.4 % of xylose was used within 120 h. There was a linear increase in the ethanol production along with fermentation time. During the first 24 h, ethanol was exclusively produced by glucose assimilation due to carbon catabolite repression of xylose fermentation until a critical level of glucose is depleted in the medium. Initially the sugar consumption was not directed to the ethanol production but rather for the survival and growth of the microorganism. After 48 h xylose assimilation was initiated and 9.98 g/L (0.38 g/g yield) of ethanol was produced by consuming approx. 79 % (18.26 g/L) of glucose and 52.8 % (7.83 g/L) of xylose. The conversion efficiency for total sugars present in hydrolysate reached up to 74.97 % at 72 h of fermentation time. When compared to other reported studies, this yield was reported to be competitive and, in some cases, superior. While numerous research studies have already explored the use of mustard oil as a feedstock for second-generation (2G) biodiesel production, to the best of our knowledge, no research study other than our previously published article reports the production of bioethanol from the mustard stalk and straw (MSS). Currently, MSS biomass is being examined as a potential energy feedstock. However, the results obtained in the present study are similar to those of previous studies involving bioethanol production from other lignocellulosic substrates. For instance, a study conducted by Kumar and Prakash [42] optimized the environmental conditions for maximal production of bioethanol from alkali-treated wheat stalk under varying conditions and observed a maximum ethanol yield of 0.21 ml/g for a fermentation period of 11 days. In another study by Santosh et al. [43], ethanol production from sugarcane bagasse using a co-culture of *S. cerevisiae* and *Scheffersomyces stipitis* achieved a maximum fermentation efficiency of 77.92 %. Previous studies have also explored the utilization of more than two strains developing a synergistic consortium in order to enhance the ethanol yield. For instance, a study by Hashem et al. [44] applied a consortia of three yeast strains, *Saccharomyces cerevisiae*, *Pichia barkeri,* and *Candida intermedia* for production of bioethanol from starchy biowastes and obtained the final yield of 167.80 ± 0.49 g/kg. The present study not only achieved a higher ethanol yield but also utilized non-detoxified hydrolysate, which simplifies the process and reduces costs associated with detoxification. The synergistic effect of the co-culture allowed efficient utilization of both hexose and pentose sugars present in the hydrolysate, contributing to the higher yield and making it a promising approach for lignocellulosic ethanol production. Although the fermentation results revealed that both yeast strains were capable of fermenting biomass hydrolysate to ethanol it is essential to acknowledge that the residual sugars in the hydrolysate left unutilized after fermentation suggests further improvement in bioethanol production process. Ghiorghita et al. [45] reported a novel integrated process for ethanol production by co-culture of *Pecoramyces* sp. F1 and *Zymomonas mobilis* ATCC 31821 from lignocellulosic biomass using simultaneous pretreatment, saccharification, and fermentation. Their process achieved an ethanol yield of 0.32 g/g glucose, demonstrating the potential of integrated processes in optimizing ethanol production from lignocellulosic feedstocks. The similar approach could be followed in the future studies to target higher ethanol yield. Several factors might have contributed to

the prolonged fermentation time and the unutilized sugars in the biomass hydrolysate. The co-culture of yeast strains used in the study might exhibit competitive interactions that can decelerate the fermentation process. Additionally, the specific strains utilized may not have the most synergistic relationship for efficient ethanol production, leading to suboptimal fermentation performance. Therefore, further optimization of the fermentation process by either refining the co-culture conditions or exploring other fermentation strains should be explored to enhance the ethanol yield. Strains isolated in the present study hold significant promise in the quest for robust and inhibitor-tolerant microbial platforms for 2G bioethanol production. The results of the study are vital for future researchers and the bioethanol industry, as they provide valuable insights into the potential utilization of MSS as a feedstock for bioethanol production. Future research will be directed towards unlocking the full potential of mustard stalk and straw as a valuable feedstock for bioethanol production, contributing to developing a sustainable and renewable energy sector.

4. Conclusion

In the present work, Mamdani-based FL model was successfully developed for cellulase production with a 0.033 MSE. Enzymes exhibited tolerance to organic acids, solvents (ethanol), furan and phenol derivatives are desirable for simultaneous saccharification and fermentation, where inhibitor accumulation affects both saccharification and microbial fermentation processes. Furthermore, biomass hydrolysate fermentation revealed a significant increase in ethanol production using the co-culture of S. cerevisiae and C. vishwanathii compared to their monoculture.

CRediT authorship contribution statement

Ritika: Writing – original draft, Methodology. **Shailja Pant:** Writing – original draft, Methodology. **Anand Prakash:** Writing – review & editing, Formal analysis. **Pandu Ranga Vundavilli:** Writing – review & editing, Software, Methodology. **Kanhu Charan Khadanga:** Writing – review & editing, Software, Methodology. **Arindam Kuila:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Tejraj M. Aminabhavi:** Writing – review & editing, Methodology, Conceptualization. **Vijay Kumar Garlapati:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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.

Data availability

No data was used for the research described in the article.

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Declaration of generative AI in scientific writing

During the preparation of this work the author(s) not used any AI tool/service.

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