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Application of Cellulase From Mutated *Aspergillus* sp. for the Production of Sustainable 2G Ethanol From Sugarcane Bagasse

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Abstract
Sugarcane bagasse (SB) is a lucrative feedstock for sustainable fuel production, but economical conversion into fermentable sugars and ethanol presents challenges from a biorefinery perspective. The present study aimed to screen and identify a robust cellulase-producing fungal strain to improve the saccharification of SB. *Aspergillus fumigatus* exhibited enhanced cellulase activity when untreated SB served as the substrate. The EMS mediated chemical mutagenesis of *A. fumigatus* with 200 mM EMS further enhanced the cellulase production by approximately 23.47%, relative to the wild-type strain. The process optimization method demonstrated peak cellulase activity on the 6th day of incubation, at 33 °C, and with an inoculum size of 5×10^7 spores. The optimization of the filter paper assay enhanced the maximum activity to 2.5 U/mL by maintaining 6 pH and 55 °C, along with the subsequent addition of $MnCl_2$ ions to the reaction mixture. The Taguchi orthogonal array was employed to optimize the process parameters of enzymatic hydrolysis of alkali-pretreated SB, demonstrating highest efficiency when hydrolysis parameters were set to pH 4, 55 °C, 10 U enzyme, and 20 g/L substrate (SB) loading. The hydrolysate was utilized to evaluate bioethanol production employing *Saccharomyces cerevisiae* MTCC 824. The strain generated 4.2 g/L of ethanol with a total yield of 0.21 g/g. This study seeks to manage agricultural residues and wastes, generating a nutrient-rich hydrolysate that can be utilized by yeast strains for bioethanol production, thus rendering the entire process sustainable, environmentally friendly, and cost-effective.

Keywords Sugarcane bagasse · *Aspergillus fumigatus* · EMS mutagenesis · Crude Cellulase · 2G Bioethanol · Biorefinery

Introduction

Energy demand has massive surged in recent decades due to rapid industrialization and urbanization, and, associated with exponential rise in greenhouse gases (GHG) and sharp decline in crude oil reservoirs, pose as a significant threat to humanity. To alleviate this hurdle, various countries have proposed stringent policies to reduce the reliance on fossil fuels and search for a sustainable alternative.

Lignocellulosic biomass, a second-generation renewable feedstock, is a remarkable carbon source that offers benefits like carbon sequestration, bioenergy and bio-products generation, environmental impact reduction, and improvement of circular bio-economy. Currently, 1.3 billion tons/year of LCB is generated worldwide, but only 3% is utilized to produce value-added chemicals [1]. The significant gap in generation versus utilization, leads to plant matter accumulation, and, environmental pollution. In order to establish the sustainable development goals (SDGs) of clean energy and climate change, enforcement of a sustainable, bio-based economy featuring an environment friendly biorefinery process is imperative.

Ethanol (C_2H_5OH) is a resilient fossil fuel substitute for due to its higher octane number (91–96), heat of vaporisation (840 kJ/Kg), evaporation enthalpy and 15% higher combustion efficiency than gasoline due to the oxygen molecules

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in its structure. It has also emits low particulate matter and harmful gases like nitrogen oxide, sulphur oxide, and carbon monoxide when blended with gasoline making it an appropriate alternative for methyl tertiary butyl ether (MTBE), a potent carcinogen and groundwater contaminant [2].

First and second generation feedstocks (sugar, starch, and LCB) are generally used for bioethanol production. Brazil produces 79% of the bioethanol using sugarcane, while India prefers molasses. The usage of first-generation renewable feedstock however, has led to the food versus feed debate. Hence, LCB has become the favoured substrate due to its abundance, high carbon content and affordability. Bioethanol from maize and sugarcane juice costs 0.30–0.40 USD/L in the US and Brazil. The cost rises to 0.90 USD/L when softwood LCB is used as feedstock due to its inherent recalcitrance, making its use difficult, and shifting the highlight to agricultural residues and bagasse. A recent study in India reported cellulosic bioethanol from maize straw to be priced at 79–81INR/L at a 70 million-L production facility.

Sugarcane "*Saccharum officinarum*" is the most preferred LCB for biorefinery applications, comprising of abundant carbon containing polymers, including cellulose (40–50%), hemicellulose (25–30%) and lignin (15–20%) of total dry weight, which is repurposed for the production of several value-added products such as succinic acid, xylitol, lactic acid, ethanol, 2,3-butanediol, enzymes, bio-lipids etc. [3]. Brazil and India are the primary producers of sugarcane, with an annual production rate of approximately 1000 million tonnes and 25–30 wt% /kg of sugarcane processed is leftover as bagasse [4]. Currently, most industries are utilizing bagasse for electricity, paper production, animal and poultry feed, charcoal briquettes and gas production. The decimation of complex three dimensional polymers into monomeric sugars is cumbersome because of the inherent recalcitrance property. Various physical and chemical processes have been developed to destabilize the polymeric structure of SB, however, despite the attempts, efficient valorisation is still far from being achieved, and remains one of the main challenges involved in efficient biovalorization.

Cellulose is the most copious carbon source in the SB and can be hydrolysed to simple sugars and subsequent value-added product generation. Currently, acid pre-treatment is a widely employed method complex polysaccharide hydrolysis by breaking the hydrogen bonds and converting the crystalline form of LCB to an amorphous form. Nonetheless, these methods have massive drawbacks like high energy requirements, corrosive nature, loss of fermentable sugars, formation of inhibitory compounds and huge effluent generation, thus limiting their usage. In contrast, enzymatic saccharification has low energy requirement and requires milder conditions for the efficient hydrolysis of LCB, with the generation of lower volumes of less toxic byproducts [5]. The efficient enzymatic digestion of LCB requires specific hydrolytic enzymes produced by fungi, bacteria, yeast and some protozoans. Cellulase is a consortia of three enzymes that initiate cellulolysis (hydrolysis of cellulose); endoglucanase (CMCase) (EC 3.2.1.4), exo-glucanase (cellobiohydrolases) (EC 3.2.1.91), and β-glucosidase (cellobiases) (EC 3.2.1.21), which work synergistically for the conversion of the complex polymers of cellulose to glucose units. Meanwhile, the costs associated with digestion of cellulose and cellulosic bioethanol production, account for 40% of overall process expenses [6]. The cost-intensive nature of the process can be reduced significantly by changing the carbon source used for cellulase production to thoroughly available cheap substrates, which will, in turn, positively impact the overall economy. Adapting solid-state fermentation (SSF) methods using LCB provides a rich source of essential carbon as well as other nutrients for the growth of microbial cell factories, which are known producers of a plethora of robust hydrolytic enzymes [7].

In this context, the present work has been designed to screen and select a potent cellulase-producing fungal strain and evaluate its potential to secrete robust cellulase enzymes under SSF using SB as the feedstock. Pretreatment of lignocellulose has been employed to improve the accessibility and digestibility of cellulose, as well as to decrease biomass recalcitrance. The selection of a robust fungal strain entails thorough screening of strains isolated from local soil using cellulose as the sole carbon source. The cellulase production ability of the selected strains were tested on raw, acid, and alkali pre-treated SB, to understand the potential effects of any leftover pretreatment intermediates on the enzyme function, or production. The one selected strain was further enhanced via EMS mediated random mutagenesis. The cultivation parameters were also optimized for the enhancement of cellulase production. Further, the saccharification of SB was also optimized via statistical design, using the Taguchi orthogonal Array method. The hydrolysate, containing glucose, and other fermentable sugars, was then used as the substrate for bioethanol production using a well known ethanol producing yeast strain.

Materials and Methods

Microorganism

The strain *Saccharomyces cerevisiae* MTCC 824 was purchased from MTCC, Chandigarh, India for ethanol production, and maintained by culturing in Yeast Peptone Dextrose (YPD) (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Glucose) media and agar plates (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Glucose, 18 g/L Agar). The strain was subcultured on YPD agar plates at regular intervals and stored in a 4 °C refrigerator.

Materials

The analytical (AR) grade chemicals used in this study were purchased from HiMedia laboratories in Mumbai (India). Gel extraction kits were purchased from New England Biolabs (USA) for molecular analysis. The sugarcane bagasse (SB) was collected from a shop selling sugarcane juice, located outside the NIT Warangal campus.

Collection and Drying of SB

The SB (*Saccharum* spp.) was collected from a local sugarcane juice shop in a large quantity, and cut down into smaller pieces around 1–3 cm long [8] for the ease of drying. The bagasse was then kept in a hot air oven (Laboratory Drying Oven, INSIF INDIA) at 60 °C overnight, with intermittent shaking for even drying, throughout.

Pretreatment of SB

Chemical pretreatment aims to address issues such as cellulose crystallinity, the structural complexity of the cell wall, and the presence of lignin in SB to facilitate its efficient utilization in biorefineries. Based on intensive literature review, the chemical methods (acid, and alkali pretreatment) were selected for the pretreatment of bagasse in this study. The bagasse was divided into three sections: one for acidic pretreatment, one for alkaline, and another one was left untreated as the control batch.

Acid Pretreatment of SB

Acidic pretreatment of bagasse was carried out using 0.5 M sulphuric acid (H_2SO_4). The components were added at a substrate-to-liquid ratio 1:10; 1 L of 0.5 M sulphuric acid was added to 100 g of dried SB. The SB was submerged in the acid for 1 h at room temperature, and the whole mixture was autoclaved at 121 °C at 15 psi pressure for 20 min [9]. The bagasse was then allowed to cool and washed thoroughly several times with double distilled water until no traces of sulphuric acid were left behind and the pH of the discarded water turned neutral (pH 7). After cleaning, the bagasse was autoclaved, cooled, carefully sealed, and then stored in a freezer at −20 °C until it was required for further experiments.

Alkali Pretreatment of SB

For the alkaline pretreatment, 0.1 M sodium hydroxide (NaOH) was selected. The components were mixed in a substrate-to-liquid ratio 1:10; 1 L of 0.1 M sodium hydroxide was added to 100 g of dried SB. The bagasse was submerged in the alkali for 1 h and then autoclaved at 121 °C at 15 psi pressure for 20 min [10]. Upon cooling, the bagasse was washed repeatedly with double distilled water until the pH turned neutral (pH 7). The cleaned SB was subjected to autoclaving, carefully sealed, and kept in a freezer at −20 °C until needed.

Untreated SB

In order to comprehensively analyze the impact of pretreatment, a control batch consisting of 100 g of untreated SB batch was used for comparative studies. The untreated SB was also autoclaved, sealed and stored in a −20 °C freezer until required.

Isolation and Screening of Cellulolytic Fungal Strain

A soil sample was obtained from a food waste disposal site on the NIT Warangal campus and maintained under sterile conditions. The soil was serially diluted with 0.9% saline to achieve a dilution of 10^{-8}. 100 µl of the final dilution was then spread onto carboxymethyl cellulose (CMC) Agar (10 g/L CMC, 1 g/L $(NH_3)_2PO_4$, 0.2 g/L KCl, 1 g/L $MgSO_4$, 1 g/L Yeast Extract and 20 g/L Agar) plates for the identification of fungal strains with high cellulolytic potential. The plates were incubated at 30 °C in a static incubator (IGENE LABSERVE, India) for 5 days and stained with 1% Congo Red stain to identify potential cellulose hydrolyzing fungal strains [11]. Yellowish tranlucent halo-like zones were observed, and the selected fungal strains were further subcultured to obtain pure cultures. Nine different fungi (SP1-SP9) were selected based on the zones of cellulose hydrolysis, and they were subjected to submerged fermentation in CMC media for 7 days to determine their cellulolytic abilities. Samples were collected at intervals of 24 h, and the cellulase enzyme produced was tested using the Filter Paper assay [12].

Identification of the Fungal Strain

The genomic DNA from the fungal strains was isolated using a modified version of standard genomic DNA isolation procedures [13]. PCR amplification of the genomic DNA was done using primers specific to the internal transcribed spacer (ITS) conserved region in fungi (Forward ITS1- 5′ TCC GTAGGTGAACCTGCGG-3′) and (Reverse ITS4-5′-TCC TCCGCTTATTGATATGC-3′) [14] (Applied Biosystems VeritiPro DX Thermal Cycler, Thermo Fisher Scientific). Upon confirmation, the genomic DNA was subjected to gel electrophoresis on a 0.8% agarose gel and purified using the New England Biolabs (NEB) gel extraction kit. After that, the purified fungal genomic DNAs were sent for sequencing to Eurofins CCMB. The derived DNA sequences were analyzed using the NCBI Basic Local Alignment Search

Tool (BLAST) for the molecular-level identification of the strains. Mega 11 software was used to prepare a phylogenetic tree using the ITS sequences of the test strains and other cellulase-producing filamentous fungi.

Selection of Highest Cellulase Producer via Solid-state Fermentation of SB

The SB was used for solid state fermentation for the production of cellulase by the fungal strains. Fungal spores were grown and harvested, followed by inoculation of pretreated and untreated samples of SB with equivalent number of spores for estimation of the cellulase enzyme production under different conditions.

Inoculum Preparation

Two of the selected fungal strains, SP5 and SP9, with high cellulolytic activity, were cultured on Potato Dextrose Agar (PDA) (24 g/L Potato Dextrose Broth, 20 g/L Agar) plates for 5 days at 30 °C in a static incubator, and their spores were harvested by scraping the plates with a sterile inoculation loop and dissolving in 0.9% saline solution. After staining the conidiospores with Lactophenol Cotton Blue stain, spore counting was done using a hemocytometer.

Solid State Fermentation of SB

The acid-treated, alkali-treated, and untreated SB was used to test Cellulase enzyme production by both SP5 and SP9 strains. 10 g each of all three types of bagasse were kept in autoclaved glass petriplates and inoculated with 1.35×10^7 spores of both the strains along with 7 mL double distilled sterile water and 2 mL Mineral Salt Medium (MSM) (0.8 g/L KCl, 0.1 g/L $CaCl_2$, 0.8 g/L NaCl, 8.0 g/L Glucose, 0.1 g/L $FeSO_4$, 2.0 g/L NH_4Cl, 2.0 g/L Na_2HPO_4, and 0.2 g/L $MgSO_4$) [15]. These were incubated at 30 °C, and samples were collected at regular intervals to analyze the cellulase enzyme being produced using carboxymethyl cellulose assay, β-glucosidase assay and filter paper assay. 2 g of sample was collected from each set, added to 20 mL of double distilled sterile water, and mixed well by manual crushing and vortexing. After centrifugation at 11,500 rpm for 5 min, the supernatants were used for analyzing cellulase enzyme activity.

EMS Mediated Chemical Mutagenesis for Strain Improvement

Chemical Mutagenesis

Chemical mutagenesis using Ethyl methanesulphonate (EMS) [14, 16] was attempted for the higher cellulase-producing strain SP9. The wild-type strain was cultivated on PDA plates for 5 days in a 30 °C static incubator to induce sporulation. The spores were collected by dissolving in Phosphate Buffered Saline (PBS). A range of EMS concentrations (50 mM, 100 mM, 150 mM, 200 mM and 250 mM) were used for incubating 1 mL of spores for 2.5 h in a shaking incubator at 30 °C. After incubation, the spores were centrifuged and washed repeatedly with PBS. The spores subjected to EMS and a group of wild-type spores serving as a control were diluted by a factor of 100 and evenly spread on CMC Agar plates. The plates were kept at 30 °C for 5 days to observe the mutant strains.

Selection of Hyperproducing Mutant via Solid-state Fermentation

The mutants exposed to 200 mM (M2), 150 mM (M3) and 100 mM (M4) were selected and subcultured on PDA plates and incubated at 30 °C for 5 days to induce sporulation. The spores were harvested, and 1.35×10^7 spores of each mutant strain were used to inoculate SB, along with 7 mL double-distilled sterile water and 2 mL MSM. The bagasses were incubated in a 30 °C static incubator for 7–10 days, and samples were collected daily for measurement of total cellulase activity using the filter paper assay.

Effects of Cultivation Conditions On Cellulase Production

The growth parameters required for the highest cellulase-producing mutant strain M2 were subjected to optimization. The moisture content of sugarcane bagasse, the number of fungal spores used for inoculation, and the time and temperature for incubation of the strain for optimal cellulase production were chosen for further experiments [17]. Samples containing 2 g of bagasse were collected regularly and mixed with 20 mL of sterilized double distilled water. After vigorous vortexing and manual mashing, centrifugation was done at 11,500 rpm for 5 min, and the supernatants were analyzed for cellulase enzyme activity using filter paper assay.

Effect of Available Moisture

The mutant strain M2 was grown on SB containing varying moisture contents (45, 50, 55, 60, 65 and 70% w/v), i.e., variable amount of available moisture supplied as MSM, and spore solution. The strain was incubated for 7 days at 30 °C for growth on bagasse and assessed for highest cellulase enzyme activity. Analysis of samples was done using the filter paper assay.

Effect of Inoculum Size

Sugarcane bagasse was inoculated with a variable number of spores of M2 (0.5×10^7, 5×10^7 and 10×10^7 spores /mL) and incubated at 30 °C for 7 days to determine the ideal number of fungal spores needed to achieve the maximum synthesis and activity of cellulase enzymes.

Effect of Incubation Temperature

The selected mutant strain M2 was cultivated on SB and incubated at varying temperatures (25 °C, 30 °C, 33 °C and 37 °C) for 7 days in a static incubator (IGENE LABSERVE, India) for the identification of the temperature required by the strain for highest cellulase enzyme production by the strain.

Effect of Incubation Time

The mutant strain M2 was grown on SB for 7 days to identify the optimum time for obtaining high cellulase enzyme titer. The samples were collected on a daily basis to ascertain the day with the highest enzyme production.

Characterization of Crude Cellulase

Based on an intensive literature survey, the parameters temperature, pH, and effect of metal ions were chosen to characterize crude cellulase enzyme produced by mutant M2.

Effect of pH

To optimise the pH required for the highest activity of cellulase enzyme, 0.05 M Sodium Acetate buffer in the following pH range of 3, 4, 5, 6 and 7 was prepared. 20 mg of Whatman no. 1 filter paper was added to 0.5 mL of the sodium acetate buffer of varying pH, and 0.5 mL of crude cellulase was added. The filter paper assay was then used to measure total cellulase enzyme activity.

Effect of Temperature

The crude cellulase enzyme was added to 20 mg of Whatman no. 1 filter paper and incubated at a wide range of temperatures: 40 °C, 45 °C, 50 °C, 55 °C and 60 °C for 60 min. The optimum activity of the cellulase enzyme was then measured using a filter paper assay.

Effect of Metal ions

Different metal ions were tested for their effects on the activity of the crude cellulase enzyme produced by the mutant strain M2. 0.05 M Sodium Acetate buffer (pH 6) was used to prepare 0.2 M stocks of NaCl, KCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $FeCl_3$, $CoCl_2$ and $CuCl_2$. The metal ions were added to the filter paper-crude cellulase mixture to achieve a final 2 mM metal ion concentration. The effects of the metal ions were observed on the activity of the cellulase enzyme.

Optimization of Saccharification using Taguchi Orthogonal Array

Taguchi orthogonal array $L_9(3^4)$ was used for the optimization of saccharification of sugarcane bagasse using crude cellulase enzyme produced by mutant strain M2 [18]. pH, Temperature (^{0}C), Substrate loading (g), and Enzyme loading (U) were chosen as the parameters for optimization based on literature survey and previous experiments. The levels are shown in Table S1. 250 mL Erlenmeyer flasks were used to carry out the experiments and samples were collected every 8 h for analysis. Mean values from triplicate data were used for analysis. The acquired data was arranged in the MINITAB statistical software (version 16, PA, USA). Table 1 contains the experimental design used in this study. The Signal to Noise Ratio, or the S/N Ratio, refers to the relationship between the control factors and the noise. The S/N Ratio was measured using the quality characteristic "Larger is better". The S/N Ratio is represented by ***Eq.*** 1.

Table 1 L9 (3^4) Orthogonal array of Taguchi experimental design for optimization of Glucose released by saccharification of sugarcane bagasse

Experiment Number	pH	Temperature (°C)	Biomass loading (g)	Enzyme loading (g)	Glucose (g/g)	S/N Ratio
1	4	40	10	1	0.15 ± 0.007	−16.47
2	4	50	15	5	0.19 ± 0.009	−14.42
3	4	55	20	10	0.24 ± 0.012	−12.39
4	5	40	15	10	0.13 ± 0.006	−17.72
5	5	50	20	1	0.19 ± 0.012	−14.42
6	5	55	10	5	0.2 ± 0.01	−13.97
7	6	40	20	5	0.15 ± 0.007	−18.41
8	6	50	10	10	0.19 ± 0.01	−16.47
9	6	55	15	1	0.19 ± 0.012	−14.42

$$S/N = 10log_{10}(\beta^2/\sigma^2) \quad (1)$$

The mean value (β) is the signal, and the variance (σ) is the noise. Analysis of variance (ANOVA) is used to determine and elucidate the statistical significance of all the parameters individually or in combination. Mean square deviation (MSD) measures the deviation of the values from the target values. MSD was calculated using-

$$\eta = -10\log\left[\frac{1}{n}\sum_{i=1}^{n}\frac{1}{Y_i^2}\right] \quad (2)$$

where n represents the number of replications, and Y_i represents the response (objective function). Combining the parameter levels led to the maximum output (glucose yield in g/g).

Bioethanol Production Using Saccharomyces Cerevisiae MTCC 824

Saccharomyces cerevisiae MTCC 824 was cultured on Yeast Peptone Dextrose (YPD) Agar plates (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Glucose, 18 g/L Agar) and grown for 3–5 days at 30 °C. Bioethanol production by the strain was tested via submerged fermentation using Yeast Peptone Dextrose (YPD) as well as Yeast Peptone Hydrolysate (YPH) media containing 20 g/L of commercially purchased Dextrose and 20 g/L of Glucose obtained after saccharification of SB using the mutant fungal strain M2 post-Taguchi optimization respectively. Seed cultures were prepared by inoculating one single colony in 10 mL of YPD and YPH media in 100 mL conical flasks and incubating at 30 °C at 100 rpm for 24 h. 2% (v/v) of the seed cultures (preinoculums) were used to inoculate 100 mL of YPD and YPH media, respectively, in 250 mL conical flasks. The flasks were incubated in a 30 °C shaking incubator at 100 rpm, and samples were collected periodically over 60 h. The cell pellets' absorbance were measured to analyze yeast cell growth. The supernatants were filtered using PVDF 0.22 mm membrane filters and used for HPLC for ethanol estimation (Shimadzu, Kyoto, Japan) using a Rezex ROA Organic Acid H + column (Phenomenex, USA) attached to a refractive index detector (RID 20A) at 60 ℃ [19]. The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.4 mL/min. The glucose levels in the fermentation samples were measured using the GOD POD assay in triplicates, and the mean values were used for analysis.

Analytical Methods

The crude cellulase enzyme activity was measured using the Filter paper assay and the CMC Assay. All the samples were centrifuged using Eppendorf. The Filter paper assay [11] was used to measure the hydrolytic potential of all components of the cellulase enzyme in the unit FPU (filter paper unit). 20 mg of dry Whatman No. 1 filter paper was used as a substrate, and 0.5 mL of the crude cellulase enzyme was added. The mixtures were incubated at 50 ℃ for 60 min. 0.5 mL 3,5-dinitrosalicylic acid (DNS) reagent [20] was added to the mixtures immediately after incubation. The mixtures were then incubated in a boiling water bath for 5 min and cooled to room temperature. The samples were analyzed by measuring absorbance at 540 nm in a double-beam UV–visible spectrophotometer (Shimadzu) [21]. The activity of the endoglucanase enzyme was assessed using carboxymethyl cellulose (CMC) as the substrate. 0.05 M Sodium Acetate buffer was used to prepare a 1% CMC solution, and 0.45 mL of the solution was added to 0.05 mL aliquot of the crude cellulase enzyme [22]. The mixture was incubated at a temperature of 50 ℃ for 10 min. Subsequently, 0.5 mL of DNS reagent was promptly added to the reaction mixture, which was then heated in a boiling water bath for 5 min. Afterwards, the mixture was allowed to cool to room temperature, and the absorbance was measured at 540 nm using a double-beam UV–visible spectrophotometer. The enzyme activity was calculated using a suitable formula based on the obtained absorbance values. The enzyme activity in this study is defined as the amount of cellulase enzyme required to create 1 µmol of glucose per minute under standard experimental conditions, expressed as U / mL. The cellulase enzyme activity throughout this study was calculated using the following formula (Eq. 3).

$$\frac{Units}{mL}of\ Cellulase\ Enzyme = \frac{Dilution\ factor \times \mu moles\ of\ product \times Volume\ of\ Assay}{(Time \times Volume\ of\ Enzyme)} \quad (3)$$

The aim of this study was to saccharify SB efficiently, hence the total cellulase activity has been considered throughout, using the filter paper assay.

A quick and rapid Glucooxidase Peroxidase (GOD POD) assay was used to calculate Glucose units released after saccharification. 1 mL of GOD POD reagent [Excel Diagnostics (India)] was taken, and 10 µl of the sample was added to it and incubated at 37 °C for 10 min. The analysis was done by measurement of absorbance at 505 nm in a double-beam UV spectrophotometer. The Glucose units were expressed in g/g and g/L throughout the study.

The cell growth and biomass accumulation of *Saccharomyces cerevisiae* MTCC 824 was measured by absorbance analysis. The samples collected during ethanol fermentation were centrifuged at 11,000 rpm for 5 min, and separate Eppendorf tubes were used to collect the supernatants. The cell pellets were

dissolved in 1 mL of double distilled sterile water each and used to analyze biomass accumulation and yeast cell growth by measuring absorbance at 600 nm. The experiments were conducted in triplicate, and the mean values were used for analysis. The standard deviation was computed and presented as the standard error in the graphical depictions of the experimental findings.

Results and Discussion

Evaluating the Potent Cellulase-producing Fungi

The soil samples from nearby food waste dump sites were collected, and the soil was subjected to serial dilution and plated on the agar plate with CMC as a sole carbon source for evaluating its cellulase activity (Fig. 1A). 1% Congo Red stain was used to stain the CMC agar plates for observation of the zones of hydrolysis formed around the fungal colonies due to hydrolysis of cellulose in the agar medium by the hydrolytic enzymes secreted by the fungi. The enzymatic index (EI) was calculated based on the diameters of the halo like clear zones formed around the colonies. EI is a semi-quantitative parameter used to measure the extracellular enzyme production by microbial species on a solid medium, calculated using the Eq. 4 mentioned hereafter.

$$Enzymatic\ Index\ (EI) = \frac{Diameter\ of\ the\ zone\ of\ hydrolysis}{Diameter\ of\ microbial\ colony} \quad (4)$$

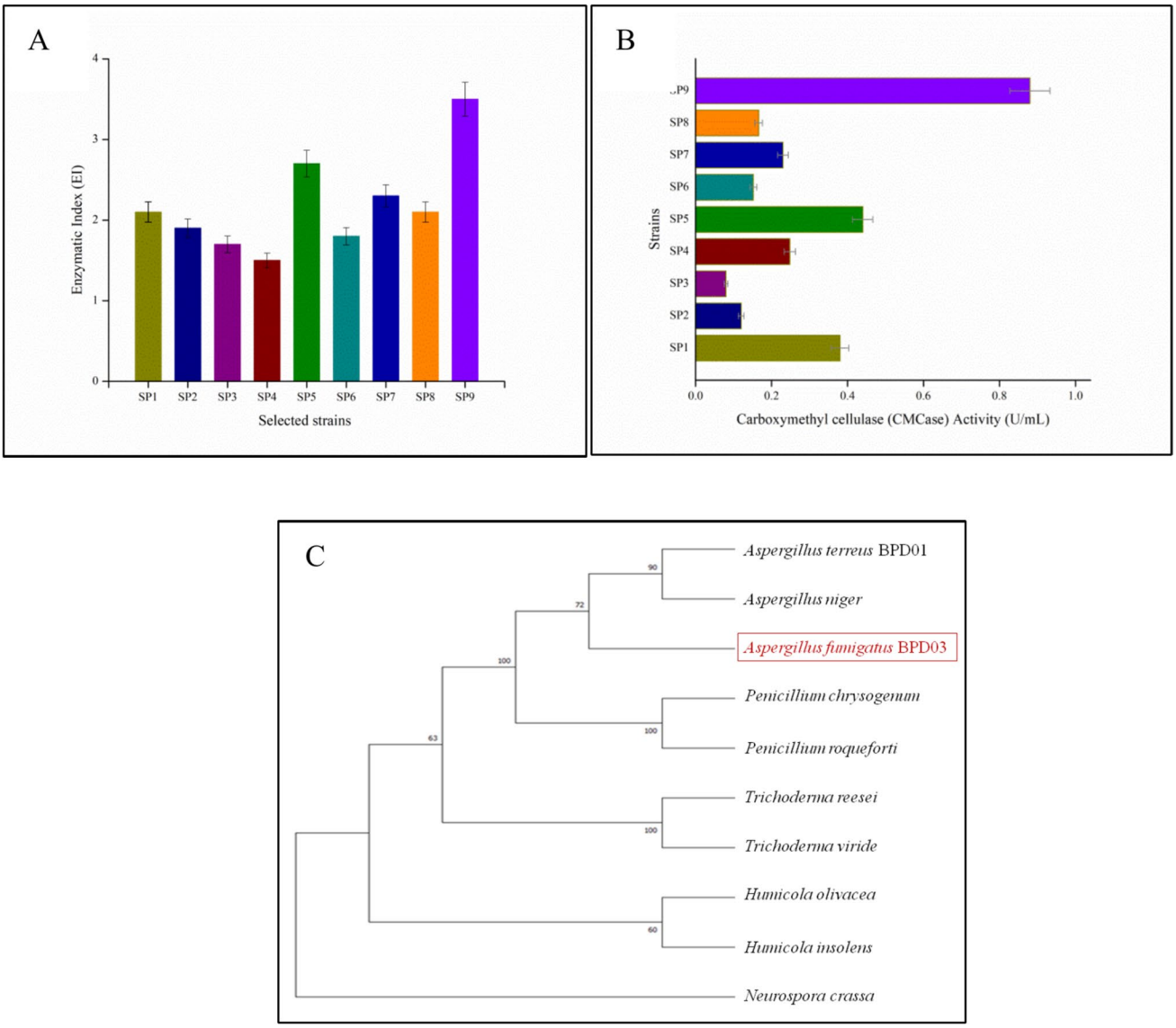

Fig. 1 Screening and selection of cellulolytic strain; Enzymatic index of selected strains (A), quantitative screening of cellulase-producing organisms using CMC media (B), phylogenetic tree prepared using ITS region sequence of Aspergillus fumigatus BPD03 and other Cellulase enzyme-producing filamentous fungi

Nine colonies were selected based on EI (> 1.4) and further evaluated for their cellulase activity (Fig. 1A).

The selected strains were further estimated for qualitative production in liquid culture using CMC as a carbon source. The strains SP9 and SP5 showed rationally good carboxymethyl cellulase (CMCase) activity of 0.9 U/mL and 0.5 U/mL, respectively (Fig. 1B). Further, the SP9 and SP5 strain genomes were sequenced using ITS primers. The SP9 strain was confirmed as *Aspergillus fumigatus* (NCBI Accession and No- OQ618999.1), while the SP5 strain was identified as *Aspergillus terreus* with 99% similarity, after performing the sequence analysis using NCBI BLAST. The same strains also displayed glucoamylase activity reported in previous studies [14]. A phylogenetic tree was constructed by adapting the Neighbour-joining method for the ITS sequence for cellulase-producing fungi and the isolated species to understand the evolutionary distance between them (Fig. 1C).

The *A. fumigatus* and *A. terreus* strains were tested further to identify the better cellulase producer. The strains were inoculated on untreated, alkali-treated, and acid-treated SB, and their proficiency in cellulase secretion through SSF was estimated. SSF draws special attention, especially for enzyme production, due to its superior qualities, like, low cost, high enzyme yield, low water usage, and efficient use of a range of agricultural wastes, residues, and byproducts [23]. The *A. fumigatus* outperformed *A. terreus* showing a noteworthy production of 1.08 U/mL of Endoglucanase (carboxymethyl cellulase or CMCase) activity (Fig. 2A), 0.245 U/mL of total cellulase (Filter Paper Cellulase or FPase) activity (Fig. 2B) and 0.87 U/mL of β-glucosidase activity (Fig. 2C) using untreated SB. Both alkali (0.24 U/mL of CMCase activity, 0.12 U/mL of FPase activity and 0.43 U/mL of β-glucosidase) and acid-treated (1.08 U/mL of CMCase activity, 0.245 U/mL of FPase activity and 0.87 U/mL of β-glucosidase) SB did not contribute for the improvement in cellulolytic activity. Despite the presence of crystalline cellulose and lignin in untreated SB, fungal growth was not impeded. Filamentous soil-dwelling fungi are recognized as exceptional reservoirs of numerous hydrolytic enzymes, including cellulases and hemicellulases, as well as lignin-degrading enzymes [24], which likely contributed to the optimal growth of the fungal strain on untreated SB. A further explanation for the reduced enzyme titers in pretreated SB may be the presence of toxic intermediates such as furfurals, vanillin and hydroxymethylfurfural (HMF), which are known for inhibiting microbial growth [25]. These results hold good for the *A. terreus* strain where the CMCase, FPase and β-glucosidase were high in untreated SB compared to alkali and acid-treated SB. To date, limited literature is available on cellulase production from *A. fumigatus* using untreated SB. In another study, the production of 95.2 U/mL of CMCase and 0.17 U/mL of β-glucosidase by *Aspergillus fumigatus* JCM 10253 was reported using ragi husk as substrate [26].

Strain Improvement Using EMS- Mediated Chemical Mutagenesis

Genetic manipulation in fungal systems is an intricate and laborious procedure, owing to multiple factors like the eukaryotic cellular machinery, thicker cell walls, genomic complexity, etc. Random mutagenesis introduces modifications across the entire genome, potentially resulting in the development of mutant organisms with enhanced functions. The mutations generated due to random mutagenesis are non-specific and do not use foreign genetic material, thereby making it suitable for industrial usage. Methods such as UV irradiation, microwave, X-ray, or Gamma irradiation, as well as potent chemical mutagens like Ethyl methanesulphonate (EMS), Ethylene bromide, Nitrous Acid, and Benzene, can be used to deliberately cause random mutations. It is, however, essential to acknowledge that although random mutagenesis is effective, it can also generate unwanted mutations, necessitating meticulous screening to select the most advantageous variations. Increased enzyme production by fungi has been reported by several literatures post-random mutagenesis. Ethyl methanesulfonate (EMS), a potent alkylating mutagen, reacts with nitrogen in the guanine bases of DNA, explicitly targeting the N7 position of guanine, leading to the formation of the modified base O6-ethylamine. DNA polymerase fails to recognize O6-ethylguanine during DNA replication due to its altered structure and consequently, mispairs O6-ethylguanine with thymine instead of the complementary cytosine. This mispairing event ultimately results in a G: C to A: T transition mutation at the affected site in the newly synthesized DNA strand [27].

To identify mutants with enhanced glucoamylase production, fungal spores were treated with EMS at 50 to 250 mM concentrations for 2.5 h. Following mutagenesis, qualitative screening was performed using a CMC plate hydrolysis assay with Congo Red dye. Among the generated mutants (M1-M5) and the wild-type strain (SP9), mutant M2 (derived from 200 mM EMS treatment) exhibited the largest halo, or zone of CMC hydrolysis diameter (data not shown). Subsequently, all mutants were evaluated for quantitative cellulase activity using solid-state fermentation on SB. Mutant M2 displayed the highest cellulase enzyme activity (1.145 ± 0.045 U/mL) (Fig. 3). Due to its superior performance, M2 was chosen for further investigation. To verify the stability of the randomly induced mutation, M2 was repeatedly sub-cultured. Finally, glycerol stocks of M2 were prepared and stored at −80 °C for future use. Extensive literature has demonstrated the effectiveness of EMS mutagenesis in producing cellulase hyperproducing mutants in filamentous fungi. Random mutagenesis generating enzyme hyperproducing strains are estimated to work in either of the two ways. The process either induces genetic mutations that promote increased growth of the strain, resulting in heightened enzyme production and activity, or

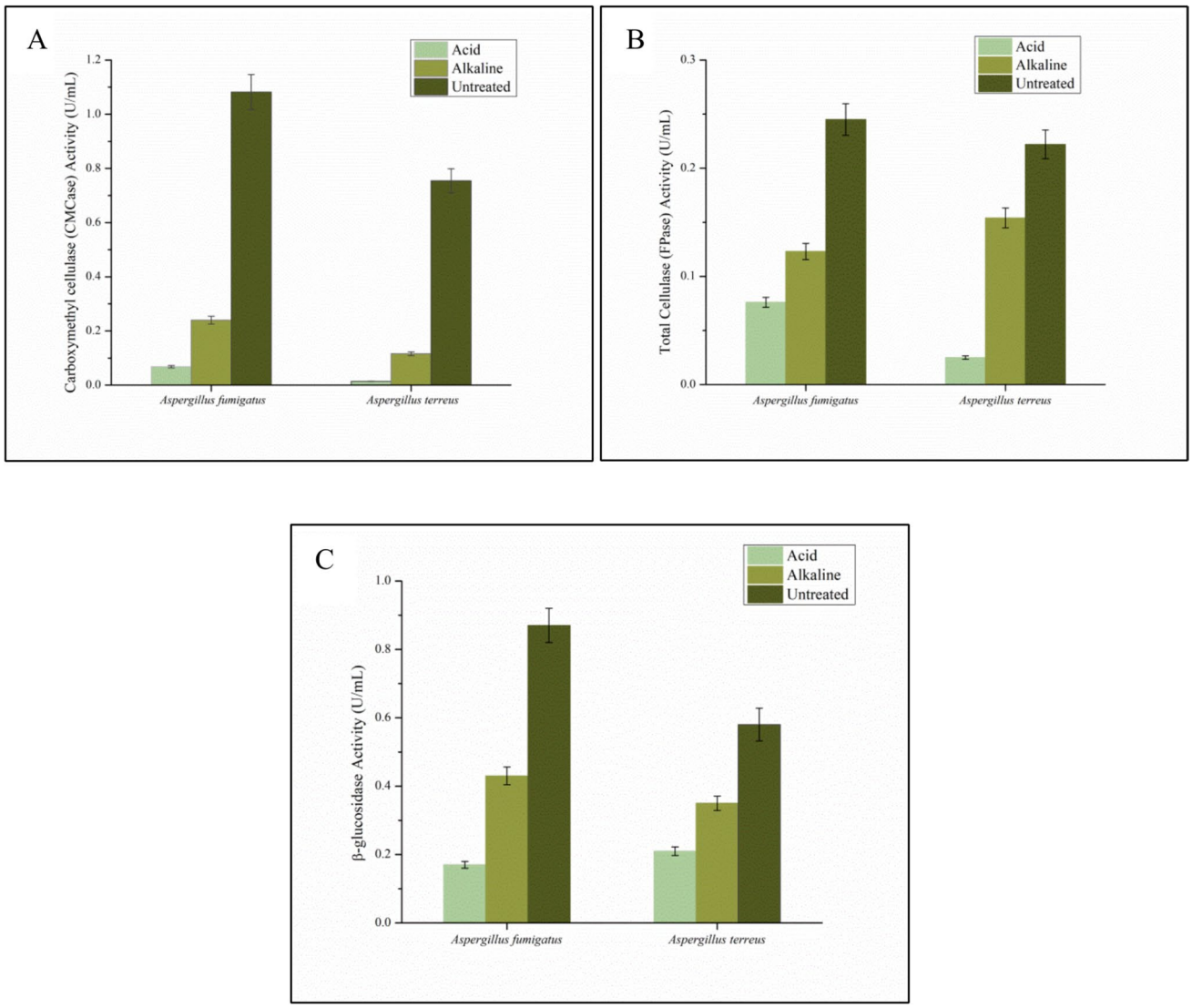

Fig. 2 Comparison of *Aspergillus fumigatus* and *Aspergillus terreus* for CMCase activity (**A**), FPase activity (**B**), and β-glucosidase activity (**C**) using pre-treated and untreated SB

it causes mutations that enhance the secretion of extracellular enzymes and modify the secretory pathways, producing supersecretor mutants [28]. A 4.9-fold rise in the activity of β-glucosidase in a mutant strain of *Aspergillus terreus* D34 was observed after EMS treatment [29] and the same in an *Aspergillus niger* FCBP-02 strain reported a substantial enhancement in total cellulase activity [16].

Effect of Cultivation Parameters On the Production of Cellulase Production From Mutant A. Fumigatus

Environmental factors such as pH, temperature, moisture content and metal ion concentration play a decisive role in the growth and product formation in SSF. Hence, it is vital to optimize these parameters to get the maximum yield of enzymes.

Effect of Moisture Content On Cellulase Production

The moisture content plays a significant role in the enzyme production using SSF. The moisture content affects the water tension and swelling of the substrate, thereby altering the rate of gas exchange and solubility in the fermentation process. Further, it also impacts the uptake of nutrients by imposing the mass transfer of the solid phase, thereby impeding microbial growth and bioproduction [17]. Usually, at higher moisture levels, the contact between the particle surface and microbes is reduced, escalating the viscosity and curtailing the substrate's air supply, thereby hindering product formation and growth [7]. In most of the literature, the moisture content varies from 30–80% under SSF, and disparities in the cellulase activity have been reported. In the present study, the moisture content

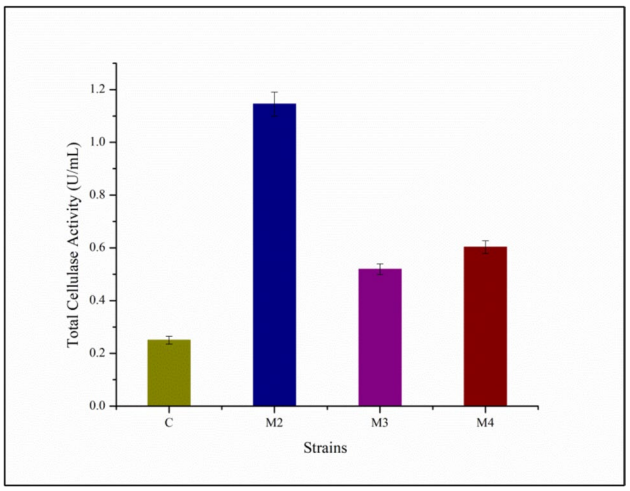

Fig. 3 Effect of EMS treatment on the cellulase production of the wild type and mutant *Aspergillus fumigatus*

varied from 45–70% (w/v); at lower moisture content, the cellulase production drastically affected the substrate solubilization, water absorption, swelling and, in turn, impacting the microbial growth. Maximum cellulase activity (1.95 ± 0.133 U/mL) was witnessed by maintaining 65% moisture content and reduced enzyme productivity (Fig. 4A). A maximum cellulase production of 18.74 U/g from *A. fumigatus* was reported by maintaining 60% moisture content with oil palm leaves as substrate [30]. This study reports similar information showing that 65% moisture is requisite for optimal cellulase production.

Effect of Inoculum Size On Cellulase Production

Several studies suggest that inoculum size is decisive for maximum fungal growth and boosting enzyme production during SSF [31]. The inoculum size was varied from 0.5×10^7—10^8 spores/mL, keeping other parameters constant. It was evident that the highest cellulase production was 2.15 ± 0.86 U/mL on the 5th day of the fermentation while maintaining 5×10^7 spores/mL (Fig. 4B). Another study also reported maximum cellulase production by *Aspergillus fumigatus* NITDGPKA3 grown on alkali hydrolyzed bagasse at 7% v/v inoculum size with 6.53 U/mL on the 5th day [32]. With a higher inoculum of 10^8 spores/mL, the cellulase production reached around 1.39 ± 0.05 U/mL. The higher dose of spores may stimulate a higher fungal growth rate. However, it could induce the production of other undesirable metabolites, and increase the competition for available surface area, nutrients, and moisture for optimal fungal growth and enzyme production [14].

Effect of Temperature On Cellulase Production

Incubation temperature has a profound effect on cellulase production. It was observed that lower temperatures around 25 ℃, yielded minimum cellulase activity (0.89 ± 0.02 U/mL). This might be a result of lower substrate transportation across the cells at lower temperatures, ultimately resulting in minimal product yield [33]. As the temperature was increased to 33 ℃, the cellulase activity was drastically elevated to 2.5 ± 0.1 U/mL, displaying the optimum temperature for the *A. fumigatus* (Fig. 4C). Further, with an increase in the temperature to 37 ℃, the cellulase production was briskly reduced to 1.11 ± 0.44 U/mL. At higher temperatures, enzyme denaturation takes place, consequently reducing the energy required for cell maintenance. The results of this work are in correlation with a report of 33 ℃ to be the temperature most suitable for cellulase production using *Trichoderma reesei* RUT C30, with sugarcane bagasse as substrate [34].

Effect of Incubation Time On Cellulase Production

It was observed that a maximum cellulase production of 2.5 U/mL was obtained on the 5th day of the fermentation (Fig. 4D). Further extending the incubation period resulted in a steep depletion in cellulase production, with 1.63 and 1.12 U/mL on the 6th and 7th days, respectively. The reason for the depletion of cellulase with the increase in incubation time could be severe depletion in nutrients and accumulation of toxic by-products, which impact fungal growth and enzyme production [35]. Most of the studies indicated that fungi usually exhibit elevated cellulase production during the 7th—8th day of incubation with different substrates under SSF [26, 36]. 6.53 U/mL of cellulase activity was reported using *A. fumigatus* NITDGPKA3, similar to the findings of this research. However, in the current study, the strain produced better cellulase productivity within 5 days, which is a crucial attribute for industrial production and bio-economy.

Characterization of Crude Cellulase Enzyme

Parameters such as pH, temperature, and metal ions exhibit a dominant role in maintaining the 3D structural integrity of the enzyme and enhancing the chance of binding the substrate to the active site. The pH was varied from 3 – 7, and the effect was observed on the cellulase activity. At pH 3, the cellulase activity was observed to be 0.98 ± 0.03 U/mL, because at lower pH values, the tertiary structure of the protein may be disrupted and lead to low substrate binding to the active site [37]. The optimum cellulase activity was found at pH 6 with 2.5 ± 0.1 U/mL (Fig. 5A). The pI of the cellulase for different species was reported between 4–7 [38]. The *Aspergillus* sp displays the optimal Filter Paper cellulase (FPase) activity at around pH 5. Another study also reported the highest FPase activity at 34 U/gds by maintaining pH 6 [39]. When the pH was increased to 7, there was a slight decline in the FPase activity.

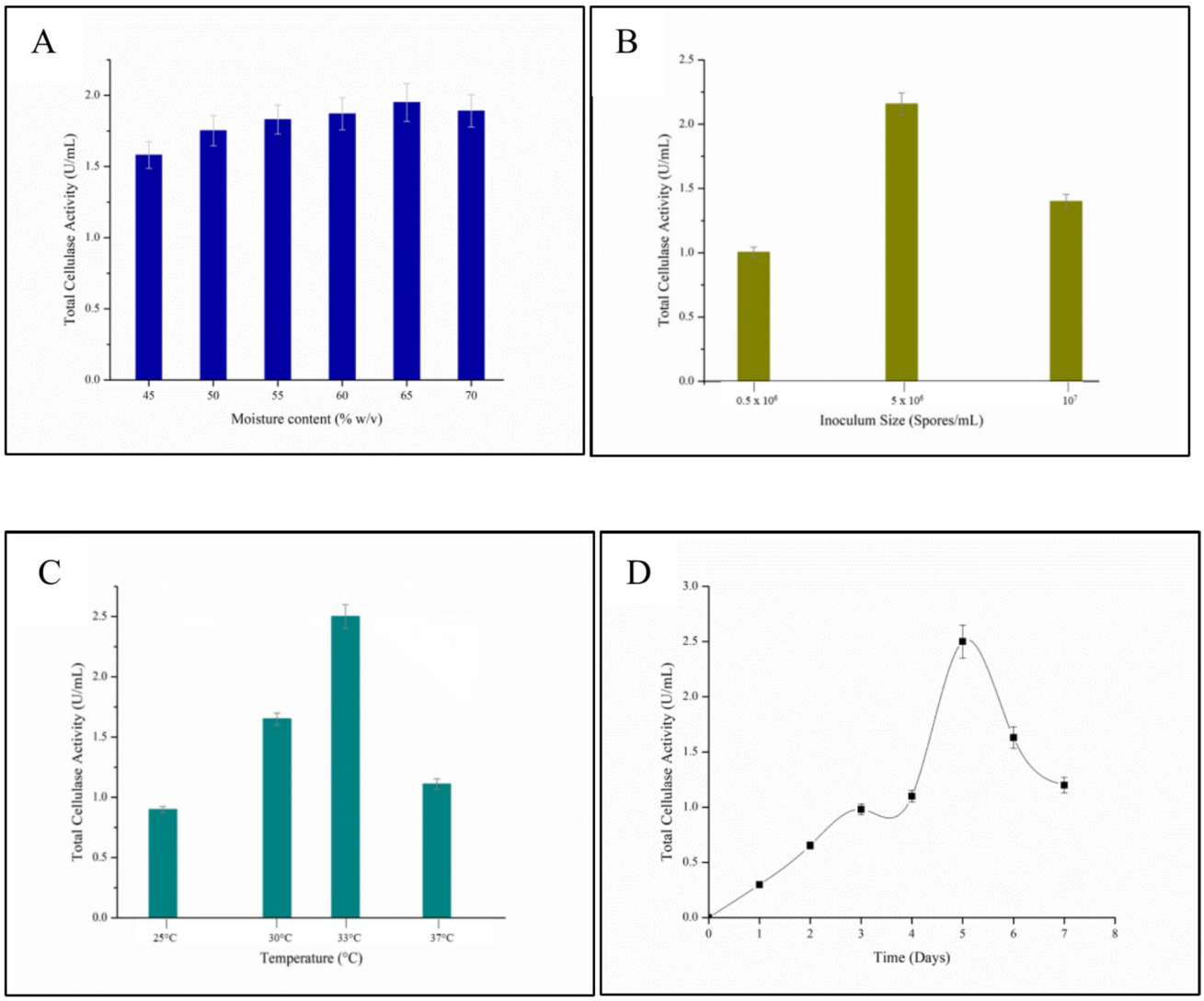

Fig. 4 Optimization of cultivation conditions for cellulase production from *A. fumigatus* using sugarcane bagasse under SSF conditions; Optimization of available moisture content (**A**), inoculum size (**B**), incubation temperature (**C**), and incubation time (**D**)

The hydrolytic activity of cellulase using filter paper was assessed under varied temperatures of 40- 60 ℃. As the temperature was increased to 55 ℃, the cellulase activity reached the peak of 2.529 U/mL (Fig. 5B). Increase in temperature increases the kinetic activity and improves the collision between substrate and enzymes, which leads to enhanced activity [14]. In a study as well, maximum exocellulase activity was observed at 55 ℃ for *Aspergillus niger* using *Arachis hypogaea* shells as substrate [40]. At 60 ℃, the activity was reduced to 1.95 U/mL; assuming that the enzyme underwent partial denaturation, the active sites were unable to bind the substrate at its proximity. The thermal deactivation kinetics displayed that even at 80 ℃, 70% activity was retained (Data not shown).

The effect of various metal ions on the cellulase activity was examined. It was found that Mg^{2+}, Fe^{2+}, and Mn^{2+} showed a prominent impact on enzyme activity, whereas Cu^{2+}, Zn^{2+}, and Ca^{2+} negatively affected enzyme activity. Among the metals, Mn^{2+} has a notable effect on enzyme activity (2.73 ± 0.1 U/mL) (Fig. 5C). Mn^{2+} profoundly increases the cellulase production in *Aspergillus* species. Increase in Mn^{2+} increases the cytosolic Ca^{2+} concentrations, which plays a crucial role cellulase regulation [41].

Optimization of Saccharification using Taguchi Orthogonal Array

Sugarcane bagasse has been shown to contain approximately 30–42% cellulose, 20–32% hemicellulose, 15–30% lignin along with some extractives and ashes [3]. The high quantity of cellulose, cost efficiency, and abundant availability make the bagasse an ideal substrate for exploiting

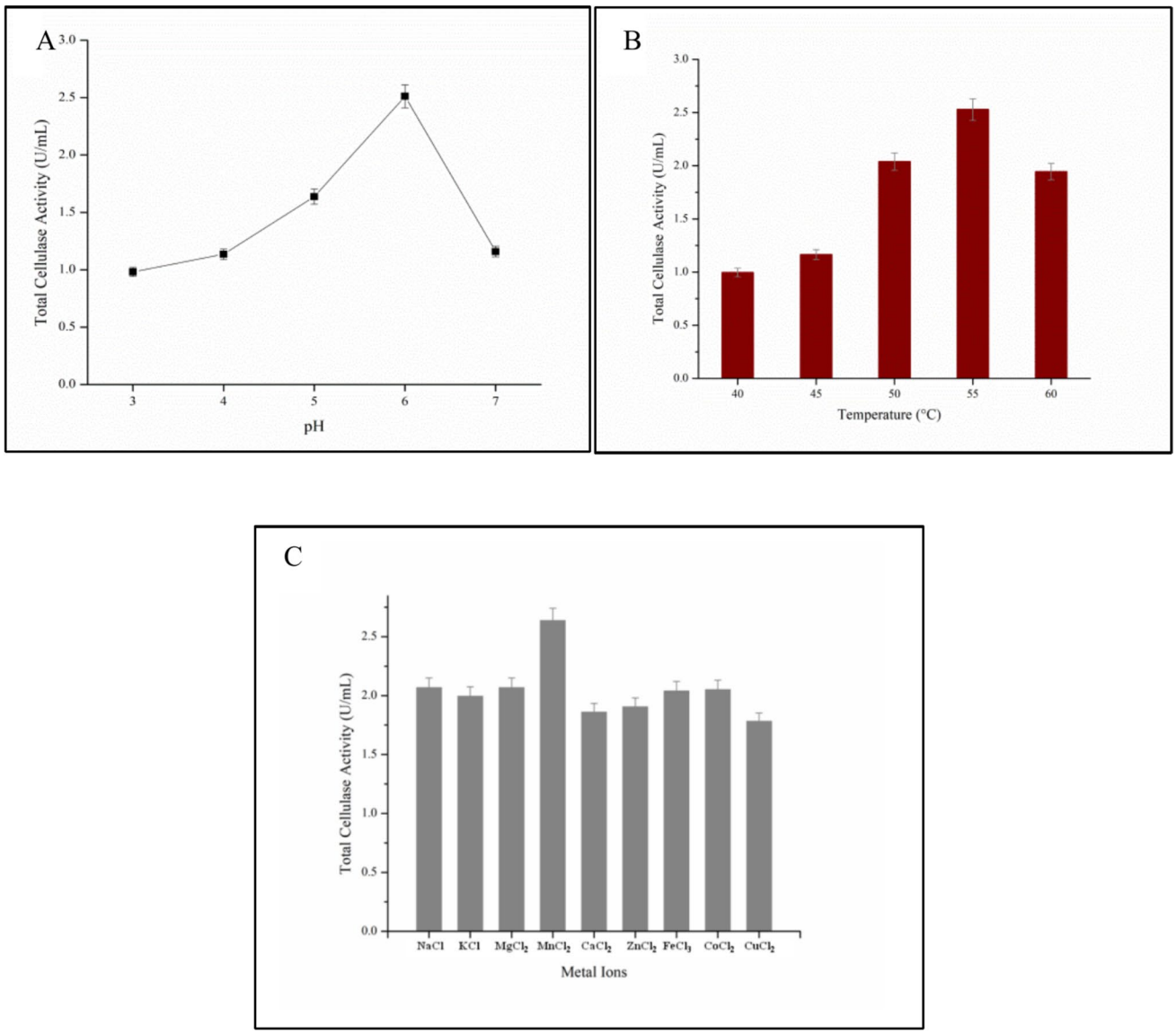

Fig. 5 Characterization of the crude cellulase enzyme; optimization of pH (**A**), Temperature (**B**), and the effect of metal ions (**C**)

glucose production by utilizing fungal cellulase enzymes. The Taguchi orthogonal array for statistical design was thus chosen for optimization of the enzymatic saccharification of SB. The selected design comprised four factors, three levels each, and nine experiments. The saccharification of SB is critically influenced by the parameters chosen for optimization, such as pH, temperature, substrate, and enzyme loading. The response table of SB saccharification under different process parameters is shown in Table 1. The pH of the medium can alter the conformation of an enzyme and modify the charge characteristics of the substrate, resulting in differences in the interactions. At a specific pH value, these enzyme substrate complexes attain the appropriate qualities necessary for maximum enzyme activity. Nevertheless, extreme pH levels might cause the enzyme to undergo denaturation, resulting in a loss of its functional properties. Literature suggests that fungal cellulases are active within a pH range from 3.5 to 6.5, with very few exceptions. In a study, the effect of pH on the ionization of the active centre of the enzyme–substrate complex was studied in the range of 3–8, and minimal variation was observed, which indicated that a broad pH range could be used without significant loss of cellulase activity [42].

According to Arrhenius's theory, an increase in temperature raises the level of kinetic energy in the system, enhancing the chemical potential of molecules, and thereby increasing the rate of the enzyme-catalyzed reaction up to a specific point. Therefore, higher kinetic energy is associated greater chances of collisions and increased substrate to product conversion [14]. However, higher saccharification temperatures might lead

to partial or absolute denaturation of the sensitive structures of the enzyme, thereby leading to loss of enzyme activity [14]. Higher temperatures might also break the hydrogen bonds critical in maintaining the water network around the intricately folded enzyme structure. Based on the prior characterization of the fungal cellulase, a range from 40 to 55 ℃ was chosen for optimization via statistical design. Owing to ample evidence from the literature, the other two factors, substrate and enzyme loading, were also chosen for Taguchi optimization. The range was decided by relevant scientific literature based on the characterization and hydrolysis parameter optimization.

Generally, greater enzyme concentrations enhance glucose production from lignocellulosic biomass, in this case, SB, due to increased contact and interaction between the enzyme and substrate molecules.

The interaction between the factors and the variance caused by each factor was calculated using ANOVA (Table 2). The model showed significance with F value of 52.58 and $p < 0.01$. The study investigated the influence of the parameters on glucose yield by determining the disparity between the maximum and lowest S/N ratios for each factor. The parameter with the most significant delta S/N Ratio was designated as Rank 1, signifying its predominant impact on the Glucose yield from SB. In the present study, Temperature was observed to have the highest delta S/N Ratio (3.94), followed by pH (2.01), Substrate loading (0.57), and Enzyme loading (0.50), indicating that the temperature had the maximum effect on saccharification of SB using cellulase enzyme. The parameters and their levels are tabulated and shown in supplementary materials (Table S2). The factors showed high precision, having $p < 0.05$, thus indicating the statistical significance of the model. The coefficient of determination (R^2) was 98.1%, indicating that the model fits the experimental data well.

The regression equation for glucose yield was denoted as Eq. 5.

$$\text{Glucose}\left(\frac{g}{g}\right) = 0.0084 - 0.000328\ \text{Enzyme loading (U)} + 0.00167\ \text{Substrate loading}\ (g) + 0.00500\ \text{Temperature}\ (^{\circ}\text{C}) - 0.0200\ \text{pH} \quad (5)$$

The main effects plot (Fig. S2) illustrates the patterns in the impact of individual parameters on the saccharification process. The plot demonstrates that the glucose yield decreased with an increase in pH value, and the most acidic pH of 4 had the maximum impact on the glucose release. The glucose yield also increased with the rise in saccharification temperature, and 55 ℃ was found to have the most effect on the hydrolysis. The current study obtained the highest yield of 0.24 g/g glucose at 55 ℃, with pH 4, 10 U enzyme, and 20 g substrate loading (Table 1). Extensive literature review revealed that sugarcane bagasse generally consists of about 30–42% cellulose, 20–32% hemicellulose and 15–30% lignin [3]. In this study, around 0.24 g/g glucose was obtained after hydrolysis of SB, suggesting around 60–80% conversion of cellulosic material into glucose monomers by the robust fungal cellulase enzyme. pH 4 has also been reported to be the optimum for the activity of a cellulase produced by *Aspergillus niger* on corncobs [43]. Another study reported the optimum activity of an *Aspergillus japonicus* endocellulase at pH 4 in a range of 50–55 ℃ [44]. The percentage of saccharification of cellulose-rich bagasse ultimately depends on the interaction between the factors and their levels chosen for the design and the type of bagasse and cellulase enzyme used [45]. Another study reported using a blend of *Penicillium funiculosum* and *Trichoderma harzianum* cellulases, which led to the release of 15.21 g/L sugar from SB [46]. Conversion of LCBs into smaller sugars has been reported to be enhanced by optimization using statistical design.

Table 2 Analysis of variance for the enzymatic saccharification of sugarcane bagasse

Source	DF	SS	MS	F	P
Regression	4	0.01158	0.00289	52.58	0.001
Residual Error	4	0.00022	5.5E-05		
Total	8	0.0118			

Bioethanol Production Using Saccharomyces Cerevisiae MTCC 824

Saccharomyces cerevisiae is a well-known strain for ethanol production because of its strong alcohol dehydrogenase gene and can uptake wide spectra of sugars. The metabolic pathway for ethanol production from glucose is depicted in Fig. S1. In this study, *S. cerevisiae* MTCC 824 strain was cultured on YPD plates from glycerol stock and incubated at 30 ℃ for 3–5 days for single colonies. Two media types were considered to test the ethanol production ability via submerged fermentation. A modified YPD medium containing untreated sugarcane bagasse hydrolysate (YPH media) instead of commercially available glucose was considered. Both media contained glucose at a concentration of 20 g/L. Apart from the glucose, the hydrolysate also comprised 0.12 g/g of xylose and 0.01 g/L of furfural. Saccharification of sugarcane bagasse using fungal enzymes has been documented to contain considerably high amounts of pentose and hexose sugars and some other disaccharides. Along with saccharides, the hydrolysate was also reported to contain other byproducts like free amino nitrogen (FAN), phosphate, and other compounds associated with enhancing the growth of other microorganisms. The FAN content refers collectively to the amino acids, short peptides, and ammonium ions generated during the pre-treatment and hydrolysis of SB. FAN

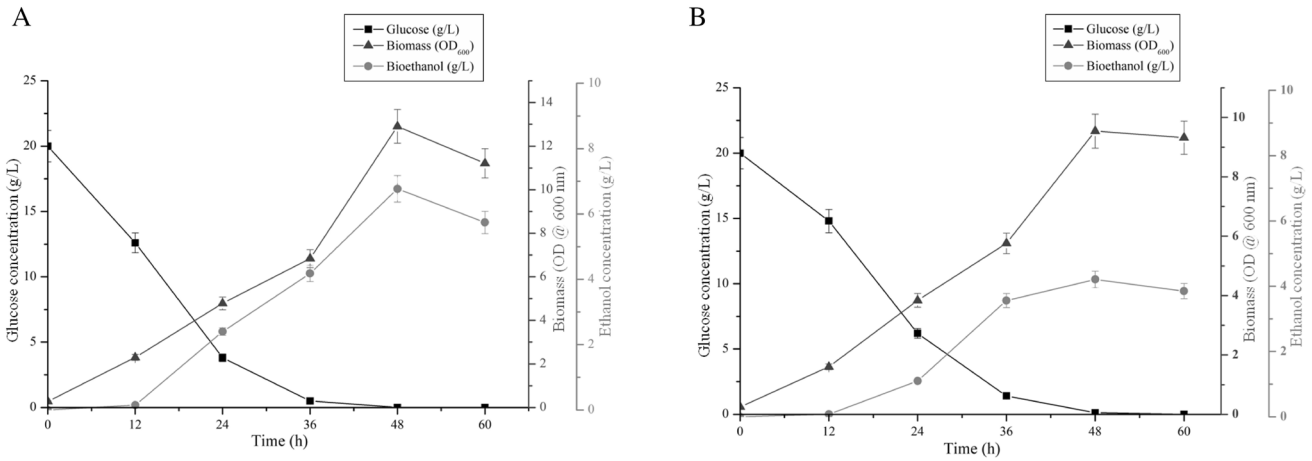

Fig. 6 Fermentation time profile of *S. cerevisiae* MTCC 824 for the production of biomass formation, glucose consumption and ethanol formation using glucose as substrate (**A**) and using sugarcane bagasse hydrolysate as substrate (**B**)

released in SB hydrolysate after protein degradation plays a crucial role in enhancing the metabolic activities of yeast cells during fermentation.

The YPD and the YPH media were used to prepare preinoculum, and then the main cultures were inoculated with 2% preinoculum for ethanol production by *S. cerevisiae* MTCC 824. The flasks were incubated at 30 °C with 100 rpm shaking to initiate an anaerobic environment. The organism exhibited a rise in biomass concentration until the 48th h, after which it decreased at the 60th h in both YPD and YPH media. The highest absorbances recorded were 12.8 and 9.23 (OD_{600}) during the 48th h. The lower cell density in the YPH media containing the SB hydrolysate can be explained by growth inhibitors produced during hydrolysis (0.01 g/L furfural). A range of inhibitory chemicals can be generated during hydrolysis of SB or other LCB, like products from degradation of sugars (furfural from pentose and hydroxymethylfurfural from hexoses), degradation of lignocellulose (acetic acid, formic acid), or degradation of lignin (aromatic, phenolic and aldehydic compounds) [47]. The presence of carbohydrates, FAN, and other compounds act as a reservoir for available nutrients that further enhance the yield of bioproduct generation by microorganisms. The *S. cerevisiae* strain enhanced ethanol production with decreasing media glucose concentration. The highest titer of 6.77 g/L and 4.21 g/L ethanol was obtained in the YPD and YPH media, respectively. The low ethanol titer is most likely attributable to the presence of inhibitors, as well as other metabolites in the hydrolysate. The highest ethanol titers were obtained at the 48th h of the fermentation (Fig. 6). The organism consumed the glucose completely within 36 h. 9.09 mg/mL ethanol production by *Saccharomyces cerevisiae* NCIM 3521 on SB hydrolysate containing 50 g/L reducing sugars was reported [48]. Another study reported maximum bioethanol concentration of 4.88 g/L from SB in a simultaneous saccharification and fermentation process by *S. cerevisiae* [49]. The lower ethanol yield in this process could be attributed to the lower glucose concentration in the fermentation media and the presence of inhibitors. However, this strain can adapt new-age techniques and strategies to scale up ethanol production. A comparative study of ethanol-producing yeast strains growing on agricultural residue and bagasse hydrolysates has been tabulated in Table S3. *Saccharomyces cerevisiae* has been identified through generations of research as one of the most optimal strains for ethanol generation. Emerging second-generation technologies for converting lignocellulosic materials like SB into ethanol and biofuels have attracted significant interest and have the potential to become commercially viable soon.

Conclusion

2G biofuel utilizing agro-industrial residues is a possible solution to the fuel crisis and environmental issues, but LCB pretreatment and high costs of commercial hydrolytic enzymes and robust strains persist as obstacles. Hence, it is imperative to address these issues to build a sustainable bio-economy. This research identified and isolated cellulolytic fungi and employed chemical mutagenesis to enhance cellulase production. Through process and parameter optimizations, a highly efficient SB saccharification process was achieved. The hydrolysate was used for bioethanol production by *S. cerevisiae*. Additional scale-up studies and economic cost analyses are required to assess process feasibility.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12155-025-10825-z.

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Authors' Contributions Satwika Das, Dharmendra Shakya, Ramadeep Kaur, and Naveen Kumar B designed and experimented with the idea for the current study. Ashish A Prabhu and Rangabhashiyam Selvasembian supervised the findings of this work. Satwika Das and Ashish A Prabhu wrote the manuscript and interpreted the results. Surajbhan Sevda and Chandukishore T assisted in proofreading the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Data Availability All data generated or analyzed during this study are included in the manuscript.

Declarations

Ethical Approval Not applicable.

Competing Interests The authors declare that they have no competing interests.

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