Statistical Optimization of Cellulase Production from *Bacillus paramycoides* and its Role in Saccharification of Pre-treated *Brachiaria mutica* (Para grass) Biomass

Ameer Khusro,[a,*] Ellojita Rout,[b] Ramakrishnan Venkatesh Prabu,[c] Muhammad Umar Khayam Sahibzada,[d,*] Roba Alatawy,[e,f] and Saad Alghamdi [g]

The hyper-cellulase producing bacterium *Bacillus paramycoides* strain BTH was isolated and characterized by 16S rRNA sequencing. Its potential for saccharification of *Brachiaria mutica* (para grass), a lignocellulosic aquatic weed, was examined. Cellulase production from strain BTH was enhanced by optimizing various parameters in the presence of goat dung as feedstock using One factor at a time (OFAT) and Response Surface Methodology (RSM) methods. The OFAT-based non-statistical method improved cellulase activity up to 1280.32±27.3 U/g. Box-Behnken Design of RSM-based optimization exhibited 1.3-fold enhancement in cellulase activity (1725.54±32.63 U/g) as compared to OFAT technique in the presence of goat dung medium (pH 8.0), incorporated with 1.5% (w/w) CMC and incubated at 37°C. Para grass biomass was further pre-treated *via* hydrothermal, alkali, acid, hydrogen peroxide, and microwave heating methods and subjected to strain BTH-associated cellulase-based hydrolysis. The alkali pre-treated biomass exhibited maximum total reducing sugar production of 6.73±0.2, 9.25±0.16, 11.6±0.17, 14.11±0.16, and 11.54±0.16 mg/g in the presence of 4% (w/v) NaOH from 12 to 96 h. Likewise, 4% (w/v) NaOH pre-treated biomass showed maximum saccharification efficiency of 30.28±0.8, 41.62±0.6, 52.2±0.7, 63.49±0.6, and 51.93±0.8% from 12 to 96 h. The findings validated the role of *B. paramycoides*-associated cellulase in the saccharification of para grass.

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Contact information: a: Directorate of Research, Malla Reddy Vishwavidyapeeth, Suraram, Hyderabad - 500055, Telangana, India; b: Dept. of Microbiology, Malla Reddy Medical College for Women, Malla Reddy Vishwavidyapeeth, Suraram, Hyderabad - 500055, Telangana, India; c: Dept. of Microbiology, Salem Kongunaadu Arts and Science College for Women, Mamangam, Salem - 636302, Tamil Nadu, India; d: Dept. of Pharmacy, The Sahara College Narowal, Narowal, Punjab; e: Dept. of Medical Microbiology, Faculty of Medicine, University of Tabuk, Tabuk 71491, Saudi Arabia; f: Molecular Microbiology and Infectious Diseases Research Unit, University of Tabuk, Tabuk 71491, Saudi Arabia; g: Dept. of Clinical Laboratory Sciences, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah, Saudi Arabia;
** Corresponding authors: Ameer Khusro: armankhan0301@gmail.com; Muhammad Umar Khayam Sahibzada: umar.sahibzada@gmail.com*

INTRODUCTION

Lignocellulose is the most abundant renewable biomass on earth, offering a cost-effective and readily available raw material for producing various biotechnological products (Chandra and Madakka 2019; Sulis *et al*. 2025). In recent years, the growing

global energy demand has spotlighted lignocellulosic biomass for its potential as a renewable source for second-generation biofuel production (Mujtaba *et al*. 2023). Lignocellulose serves as a unique carbon source that can be transformed into biofuels through varied processes (Rastogi and Shrivastava 2017; Aarti *et al*. 2022a).

Brachiaria mutica (Family – Poaceae), commonly known as "para grass", is a lignocellulosic semi-aquatic weed found in Manipur (North-Eastern India). This semi-prostrate perennial grass, with creeping stolons, is primarily cultivated for livestock feed due to its high-quality forage for ruminants (Aarti *et al*. 2022b). Para grass contains approximately 42% cellulose and 20% hemicellulose, which, upon hydrolysis, yields fermentable sugars. This makes it an excellent feedstock for bioethanol production, contributing to sustainable biofuel initiatives (Sahoo *et al*. 2017).

The hydrolysis of cellulose into monosaccharides such as glucose is a key step in converting lignocellulosic biomass into biofuels (Higai *et al*. 2021). Enzymatic saccharification, which breaks down lignocellulose and depolymerizes biomass, is considered a crucial yet costly step in this bioconversion process (Abdulsattar *et al*. 2020). Among diversified enzymes, cellulases (EC 3.2.1.4) are a group of enzymes, comprising endo-1,4-β-D-glucanase (EC 3.2.1.4), exo-1,4-β-D-glucanase (EC 3.2.1.74), and β-glucosidase (EC 3.2.1.21) (Ejaz *et al.* 2021). Cellulase breaks β-1,4 glycosidic bonds of cellulose and produces fermentable glucose monomers (Maravi and Kumar, 2021). In fact, cellulases convert cellulose into glucose, which is then used to produce biofuels, making them essential in cellulose hydrolysis.

Cellulases can be produced from diverse sources; however, microorganisms remain the essential factories for cellulase production (Bhardwaj *et al*. 2021). Among microbes, fungi are commonly utilized at industrial scale for large production of cellulase due to their capability to release ample amounts of active free lignocellulose-hydrolyzing enzymes extracellularly and the ease of purification of these enzymes. Therefore, in industries, various fungal strains have been harnessed for the vast production of cellulases (Zhang *et al*. 2024). Interestingly, in recent years, bacterial cellulase has gained paramount significance because bacteria show high growth rate, high thermal stability of enzymes, better expression systems, resistivity to adverse conditions, and genetic diversity (Shyaula *et al*. 2023). Bacteria produce cellulase either by submerged fermentation (SmF) or solid state fermentation (SSF) processes. The SmF method is extensively used for cellulase production. However, this method has several drawbacks, such as it is non-economical, requires high energy input, shows susceptibility to varied factors, and is prone to being adversely affected by contaminants (Mattedi *et al*. 2023). In contrast, the SSF method is considered as an ideal alternative to the SmF process and has gained global attention over the past few decades. In SSF, bacteria are generally grown on non-soluble organic substrates in the absence or in the presence of minimal water (Soccol *et al*. 2017). Solid state fermentation method shows several advantages over SmF, such as low energy requirement, greater tolerance of contamination, high enzyme productivity, less sensitivity to substrate inhibition, inexpensive technique, and an ability to facilitate the managements of solid wastes (Yafetto 2022). Prior studies have revealed the uses of numerous substrates for the production of cellulases under SSF condition, including wheat bran, corn stover, apple pomace, rice husk, *Jatropha curcas* seed cake, and cow dung (Soccol *et al.* 2017). A recent report has shown the prominent role of goat dung as a cost-effective feedstock in order to produce cellulase from bacteria (Aarti *et al*. 2018), but it has not been extensively exploited further. It should be noted that goat dung contains high nutrients (Mnkeni and Austin 2009), which certainly makes it a promising feedstock for the production of

cellulase from bacterial sources under SSF.

Cellulase yield from bacteria depends on a variety of abiotic and nutritional factors. Therefore, it is imperative to scale up the production of cellulase by optimizing various nutritional and non-nutritional parameters. One factor at a time (OFAT) technique is often used as non-statistical technique for the optimization of enzyme production. This method of optimization is not only time-consuming, but it often shows low enhancement in enzyme activity and fails to establish the connection between two parameters simultaneously in terms of enzyme production (Aarti *et al.* 2017). In order to overcome such problems, statistical methods-based optimization, particularly Response Surface Methodology (RSM) has gained immense interest among researchers. RSM delivers fast and reliable output with comparatively higher enzyme production than the OFAT method. Most importantly, RSM-based optimization depicts inter-dependent interactions between two factors, which is crucial to understand the role of specific variable towards enhancement of enzyme activity (Khusro *et al.* 2017; Khusro *et al*. 2024).

In view of the potency of cellulase in the saccharification of lignocellulosic biomasses, the present study was aimed not only to isolate hyper-cellulase producing bacterium from soil sample and optimize its production statistically under SSF condition using goat dung as ideal feedstock but also to decipher its prominent role in the saccharification of para grass.

EXPERIMENTAL

Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade with the highest purity, and were obtained from HiMedia, India. These chemicals and reagents were stored at specific temperatures, as specified for further experimental purposes.

Soil Sample Collection

Sub-soil samples (soil beneath 2 cm from the top-soil) were collected from Neyveli Lignite Corporation (NLC; Neyveli, Tamil Nadu, India) and placed in sterile polythene bags.

Isolation of Cellulolytic Bacteria

Cellulolytic bacteria were isolated by serially diluting 1 g of collected soil sample up to 10^{-5} dilution. One millilitre from the 10^{-5} dilution was spread onto sterilized Nutrient Agar plates supplemented with 0.5% (w/v) carboxymethyl cellulose (CMC). The suspension was evenly distributed using an L-rod, and the plates were incubated aerobically at 37 °C for 24 h to promote the growth of bacterial colonies. Colonies showing distinct morphological characteristics were selected and subsequently purified using the quadrant streaking method on freshly prepared sterile Nutrient Agar plates for further analysis.

Cellulase Production

Qualitative assay

Purified bacterial cultures were inoculated into sterile Nutrient broth supplemented with 0.5% (w/v) CMC and incubated at 37 °C for 24 h. Following incubation, the cultures were centrifuged at 6000 *g* for 15 min, and the resulting supernatant was collected.

Concurrently, CMC agar medium (CMC – 5.0 g/L, agar – 20.0 g/L, pH – 7.0) was prepared, and wells were punched into the solidified medium using a cork borer. A volume of 100 µL of the supernatant was added to each well, and the plates were incubated at 37 °C for 24 h. After incubation, the plates were flooded with Lugol's iodine solution for 5 min to visualize the clear zones indicating CMC degradation. Bacterial isolates forming prominent hydrolysis zones were selected for further quantification.

Quantitative assay

Cellulase activity of each isolate was quantified following a modified protocol of Ghosh (1987). Isolates were cultured in nutrient broth supplemented with 0.5% (w/v) CMC and incubated at 37 °C for 24 h. Post-incubation, cultures were centrifuged, and 1 mL of the resulting supernatant (crude enzyme extract) was mixed with 1 mL of 0.5% (w/v) CMC solution. The reaction mixture was incubated at 37 °C for 30 min. To terminate the reaction, 1 mL of dinitrosalicylic acid **(**DNS) reagent (sodium hydroxide – 16.0 g/L, DNS – 10.0 g/L, sodium potassium tartarate – 300.0 g/L) was added into the solution and boiled for 5 min. After cooling to room temperature, the absorbance was measured at 540 nm using a UV-Vis spectrophotometer. One unit (U) of cellulase activity was defined as the amount of enzyme that releases 1 µg of reducing sugars per minute under assay conditions. Total protein content was determined using the Bradford method (Bradford 1976), and specific enzyme activity was expressed as U/mg of protein.

Identification of Hyper-cellulase Producing Bacterium

Morphological (colony properties and Gram staining) and certain biochemical traits of hyper-cellulase producing bacterium were performed using standard Bergey's Manual of Systemic Bacteriology (Sneath 1994). Furthermore, genomic DNA isolation (using NucleoSpin® DNA isolation kit), PCR amplification, and 16S rRNA sequencing of the isolate were carried out using standard methodologies. 16S rRNA sequences of the bacterium were further submitted to GenBank, NCBI.

Cellulase Production (SSF)

Substrate used

Goat dung was collected locally from Guduvanchery, Chengalpattu, Tamil Nadu, India, and dried 5 to 7 days in sunlight. The dried goat dung was powdered, filtered, and stored in a screw capped bottle for further processing.

Solid state fermentation and cellulase activity

Ten g of goat dung was moistened to 100% using 0.1M Tris-HCl buffer (pH 8.0) and subsequently autoclaved. Once cooled, 5% (v/w) of bacterial inoculum (OD_{600} = 1.12) was aseptically inoculated into the UV-irradiated (20 min) and sterilized goat dung. The mixture was incubated at 37 °C under static conditions for 48 h. Following fermentation, 25 mL of sterile distilled water was added, and the mixture was incubated on an orbital shaker at 150 rpm for 30 min to extract cellulase. The resulting slurry was filtered and centrifuged at 6000 *g* for 15 min to obtain the supernatant, which was used as the crude cellulase extract. Cellulase activity was assessed using the modified method of Ghosh (1987), and total protein concentration was measured using the Bradford assay (Bradford 1976).

Optimization of Cellulase Production

One factor at a time method

A non-statistical method, *i.e.*, the OFAT method was followed to assess the impact of various variables, such as incubation period (12 to 96 h), pH (6.0 to 10.0), temperature (32 to 50 °C), moisture content (60 to 120%), carbon sources (1% w/w), and nitrogen sources (0.5% w/w) on cellulase activity in the presence of goat dung as per the methodology described above.

Response surface methodology

As per the results of OFAT method, factors showing maximum cellulase activity from bacterium were further selected for statistical optimization using the Box-Behnken Design (BBD) of RSM. Three variables (pH, temperature, and CMC amount) were optimized at three different levels (-1, 0, +1), with a central coded value of zero. As per the design, the total number of combinations is calculated as $2^k + 2k + n$, where "k" is the number of independent parameters and "n" is the number of repetition of experiments at the central point. The experimental design consisted of 20 runs of 3 variables for enhancing cellulase activities. Cellulase activity (Y) was analysed using a second-order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where Y is the dependent variable, β_0 is the intercept, β_1, β_2, and β_3 are the linear coefficients, β_{11}, β_{22}, and β_{33} are the squared coefficients, β_{12}, β_{13}, and β_{23} are the interaction coefficients, and A, B, C, A^2, B^2, C^2, AB, AC, and BC are the levels of independent variables. The coefficient of determination R^2 represents goodness of fit of the equation and statistical significance level was calculated by the F test by keeping the desirability at maximum. The accuracy and general ability of the above polynomial model could be evaluated by R^2. The inter-relationship between two factors influencing cellulase activity was observed as three-dimensional (3D) response graphs. Further, based on the optimized parameters determined through BBD for enhanced cellulase production, validation experiments were conducted under SSF conditions. Cellulase activity of the bacterial strain was quantified using the previously described method, and the experimental results were compared with the predicted response values to evaluate the accuracy and reliability of the model.

Saccharification of Aquatic Weed

Collection of aquatic weed

Para grass was collected from Loktak lake, Imphal West, Manipur, India. The aquatic weed was brought in polythene bags to the laboratory and dried for 7 to 10 days.

Pre-treatment of weed biomass

The dried weed plant was powdered using grinder, sieved to a particle size of less than 0.5 mm, and stored at room temperature for various pre-treatment processes. Para grass was pre-treated using 5 different methods: hydrothermal pre-treatment, alkaline pre-treatment, acid pre-treatment, hydrogen peroxide pre-treatment, and microwave heating pre-treatment.

The hydrothermal treatment was performed by mixing 10% (w/v) of para grass biomass with distilled water. The mixture was incubated at room temperature for 2 to 3 h

and autoclaved at 80, 90, 100, and 121 °C for 30 min. The sterilized mixture was washed with water until the neutral pH was obtained (da Silva *et al*. 2018). The untreated plant biomass was used as control.

The alkaline treatment was carried out by mixing 10% (w/v) of para grass biomass with NaOH solution (2, 4, 6, and 8% w/v). The mixture was incubated at room temperature for 2 to 3 h, autoclaved at 121 °C for 30 min, and then washed with water until neutral pH was achieved (da Silva *et al*. 2018). The untreated plant biomass was used as control.

For acid treatment, para grass biomass (10% w/v) was mixed with H_2SO_4 solution (1, 2, 3, and 4% v/v), incubated at room temperature for 2 to 3 h, and then autoclaved at 121 °C for 30 min, followed by washing with water in order to attain neutral pH (da Silva *et al*. 2018). The untreated plant biomass was used as control.

Hydrogen peroxide treatment was performed by mixing 10% (w/v) of para grass biomass with H_2O_2 solution (1, 2, 3, and 4% w/v). The mixture was incubated at room temperature for 2 to 3 h, followed by autoclaving at 121 °C for 30 min, and washing with water until achieving neutral pH (da Silva *et al*. 2018). The untreated plant biomass was used as control.

Microwave irradiation-based pre-treatment was carried out by immersing 10% (w/v) of para grass biomass into distilled water. The biomass was initially allowed to soak in water for 1 h and then the beaker containing the mixture was kept in the centre of the rotating ceramic plate inside the microwave oven. The mixture was heated for 1, 2, 5, and 8 min. Every 1 min, the microwave oven was stopped, the beaker was taken out, and it was stirred thoroughly for further heating process. After the pre-treatment process, the biomass was filtered using clean muslin cloth and washed with water (Agu *et al*. 2017). The untreated plant biomass was used as control.

Enzymatic hydrolysis

Enzymatic hydrolysis of untreated and pre-treated para grass biomass was performed following the method of Khobragade *et al*. (2004) with slight modifications. One gram of untreated or pre-treated biomass (pre-soaked in 10 mL of 0.1 M sodium citrate buffer, pH 5.0) was incubated with 50 mL of statistically optimized crude cellulase (1725.54±32.63 U/g). To prevent microbial contamination, streptomycin sulphate (0.01% w/v) was added to the mixture. Additionally, polyethylene glycol (0.4% w/v) was included to mitigate the inhibitory effects of any by-products (Ladeira-Ázar *et al*. 2019). The mixture was incubated at 37 °C under shaking conditions for 96 h. At designated time intervals, samples were centrifuged at 6000 *g* for 30 min to separate unhydrolyzed biomass, and the supernatant was collected for the estimation of total reducing sugars (TRS) and saccharification efficiency.

Quantification of TRS and saccharification efficiency

Total reducing sugars released during enzymatic hydrolysis were estimated using the DNS method (Miller 1959). One milliliter of the hydrolyzed sample was mixed with 1 mL of DNS reagent, boiled for 5 min, and then cooled to room temperature. The final volume was adjusted to 10 mL using sterile distilled water, and absorbance was measured at 540 nm using a UV-Vis spectrophotometer. TRS concentration (mg/g biomass) was calculated using a glucose standard curve. Saccharification efficiency (%) was determined using the following formula described by Mandels and Sternberg (1976):

$$\% \text{ Saccharification} = \frac{\text{Reducing sugars (mg/g)} \times 0.9}{\text{Initial substrate concentration (mg/g)}} \times 100 \quad (2)$$

Statistical Analyses

For optimization purpose, Design Expert Version 10.0.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) statistical software was used in this study. Statistical significance was conferred using ANOVA, and $P<0.05$ was considered significant. All experiments were performed in triplicate and results were expressed as mean±SD.

RESULTS AND DISCUSSION

Isolation of Cellulase Producing Bacteria

Bacteria are considered as efficient factories for the production of disparate hydrolytic enzymes. Compared to the other microorganisms, bacteria are cultured easily, have limited nutritional requirements, show higher growth rate, exhibit high rate of enzyme stability, and tolerate harsh conditions (Shyaula *et al*. 2023). Bacteria are prolific producers of cellulase, and these high-activity cellulases are considered to be ideal candidates for breaking down cellulose (Chukwuma *et al*. 2025). In this context, of 12 bacteria that were isolated from the soil sample, 11 bacteria were identified as cellulase producers. Among them, isolate BTH showed maximum zone of CMC hydrolysis of 22.3 ± 0.57 mm (Fig. 1) with cellulase activity of 1003.46 ± 26.6 U/mL. Other isolates showed comparatively lower cellulase activities in the order of isolate BTE (987.82 ± 26.3 U/mL) > isolate BTD (831.46 ± 22.3 U/mL) > isolate BTA (718.32 ± 25.6 U/mL) > isolate BTF (651.65 ± 27.5 U/mL) > isolate BTB (612.22 ± 25.5 U/mL) > isolate BTJ (598.67 ± 27.3 U/mL) > isolate BTC (518.75 ± 25.3 U/mL) > isolate BTG (502.18 ± 23.3 U/mL) > isolate BTK (413.56 ± 25.7 U/mL) > isolate BTI (325.21 ± 24.3 U/mL). Isolate BTL showed lack of cellulase production (Table 1). Total protein content and specific activity for each isolate is also shown in Table 1. Previous studies also reported the successful isolation of cellulolytic bacteria from soil samples (Lingouangou *et al.* 2022; Shyaula *et al*. 2023)

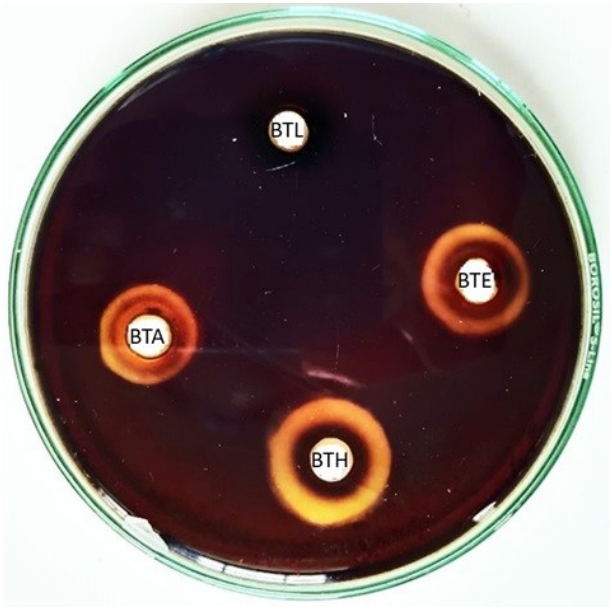

Fig. 1. Cellulase production (plate assay) from few bacterial cultures isolated from soil sample. Isolate BTH showed the highest zone of CMC hydrolysis (22.3 ± 0.57 mm).

Table 1. Cellulase Activity (Qualitative and Quantitative) of Bacterial Isolates

Isolates	Cellulase Assay		Protein Content (mg/mL)	Specific Activity (U/mg)
	Qualitative Method (mm)	Quantitative Method (U/mL)		
Isolate BTA	16.6±0.57	718.32±25.6	1.42±0.8	505.85
Isolate BTB	15.3±0.57	612.22±25.5	1.34±0.6	456.88
Isolate BTC	14.6±0.57	518.75±25.3	1.1±0.6	471.59
Isolate BTD	17±1	831.46±22.3	1.61±0.7	516.43
Isolate BTE	20.3±0.57	987.82±26.3	1.78±0.8	554.95
Isolate BTF	15.6±0.57	651.65±27.5	1.41±0.6	462.16
Isolate BTG	12.3±1.15	502.18±23.3	1.1±0.7	456.52
Isolate BTH	22.3±0.57	1003.46±26.6	1.88±0.7	533.75
Isolate BTI	9.5±1.15	325.21±24.3	0.73±0.8	445.49
Isolate BTJ	15±1	598.67±27.3	1.12±0.6	534.52
Isolate BTK	11±1	413.56±25.7	0.88±0.3	469.95
Isolate BTL	Nil	Nil	0.95±0.3	Nil

Table 2. Morphological and Biochemical Properties of Isolate BTH

Tests	**Results**
Colony colour	Cream white
Colony shape	Large, smooth, and round
Gram staining	Gram (+) rod-shaped
Endospore test	+
Indole	-
Methyl red	-
Voges-proskauer	+
Citrate utilization	+
Urease	-
Catalase	+
Oxidase	+

Note: '+' = Positive; '-' = Negative

Identification of Hyper-Cellulase Producing Bacterium

Based on cellulase activities of isolates, isolate BTH was considered to be a hyper-cellulase producing bacterium. Isolate BTH showed cream white, smooth, and round-shaped colonies appearance on nutrient agar medium. After performing gram staining technique, the isolate was observed as gram-positive bacteria. The isolate exhibited positive results for endospore, Voges-Proskauer, citrate utilization, catalase, and oxidase. On the other hand, negative results for indole, methyl red, and urease tests were observed (Table 2). The isolate was further identified as *Bacillus paramycoides* strain BTH after 16S rRNA sequencing, followed by BLAST, NCBI analysis and sequence deposition in GenBank, NCBI (Accession no.- PQ268930). In a recent investigation, *B. paramycoides* isolated from landfill leachate was also identified as cellulase producer (Chukwuma *et al*. 2025).

Cellulase Production under SSF

Production of enzymes from bacteria is one of the most prominent applications of SSF. Although several research activities have been conducted in the past to produce bacterial enzymes by the SSF technique, efforts are continued to explore the possibilities of isolating hyper-cellulase producing bacterial strains using less exploited solid wastes as feedstock at a more affordable cost. In this investigation, strain BTH showed cellulase

activity of 972.34 ± 24.3 U/g under SSF condition in the presence of goat dung as an ideal substrate. Total protein content and specific activity were estimated as 1.85 ± 0.6 mg/mL and 525.58 U/mg, respectively (figure not shown). In earlier reports, banana fruit stalk (Mussatto *et al*. 2012), maize bran (Sharma and Kumar Bajaj, 2017), and sugarcane bagasse (Tiwari *et al*. 2022) were used as solid substrate to produce cellulase from *Bacillus* sp. In a different study, goat dung was used as a promising substrate to produce cellulase from *Glutamicibacter arilaitensis* (Aarti *et al*. 2018).

OFAT-Based Optimization

Optimization of varied fermentation parameters plays a pivotal role at industrial scale in order to enhance the productivity of cellulase. The impact of incubation period on cellulase production from strain BTH is shown in Fig. 2a. The bacterium showed maximum cellulase activity of 1003.46±26.6 U/g at 48 h, followed by prominent reduction at 72 and 96 h. The investigation conducted by Shyaula *et al*. (2023) reported a similar trend in cellulase production (at 48 h) by *Bacillus* sp.

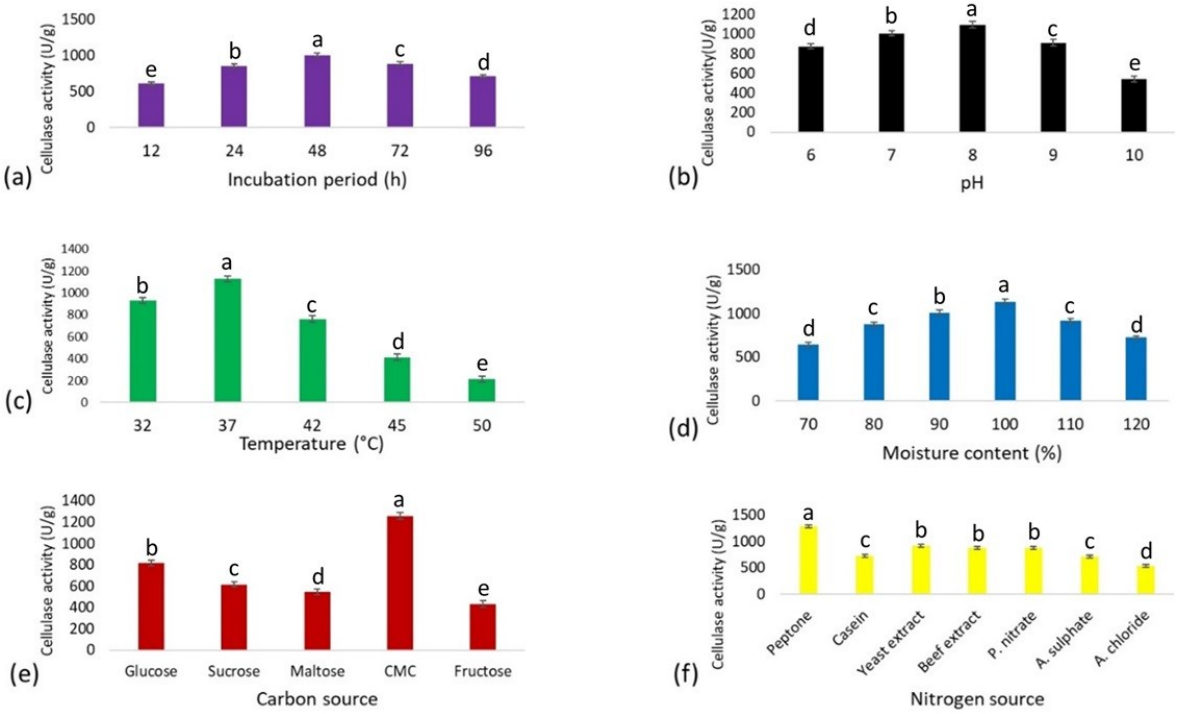

Fig. 2. Optimization of cellulase production at different parameters such as (a) incubation period, (b) pH, (c) temperature, (d) moisture content, (e) carbon source, and (f) nitrogen source using OFAT method. Values are represented as mean±SD of experiments carried out in triplicate (n = 3). [abcde] Values with different superscript letters are significantly (P<0.05) different.

Figure 2b illustrates prominent effect of pHs on cellulase production from the strain, which revealed maximum cellulase activity (1096.24 ± 31.3 U/g) at pH 8. Cellulase activity was reduced significantly (P<0.05) at pH lower and higher than 8. The findings agree with the outcomes of Lingouangou *et al.* (2022), who demonstrated maximum cellulase production from *Bacillus* sp. at pH 8. The impact of different incubation temperature on cellulase activity of strain BTH is depicted in Fig. 2c, which showed maximum cellulase yield of 1130.25±26.2 U/g at 37 °C. Higher temperature (42 to 50 °C) caused steep reduction in cellulase activity (P<0.05). Similar observation was recorded by Lingouangou *et al.* (2022) too.

Figure 2d depicts cellulase activity of strain in the presence of goat dung with varied moisture level. Maximum cellulase activity of 1134.56±32.3 U/g was obtained using goat dung with 100% moisture level. Further alterations in moisture level exhibited reduction in cellulase activity. Results agreed with the findings of Aarti *et al*. (2018), who reported maximum production of cellulase from bacterial strain with 100% moisture level. Among diversified carbon sources used, the strain showed maximum cellulase production (1258.25±28.4 U/g) in the presence of CMC. In contrast, cellulase production was significantly ($P<0.05$) reduced in the presence of other carbon sources, ranging from 432.23±28.4 to 814.34±26.3 U/g (Fig. 2e). Shajahan *et al.* (2017) also observed CMC as a prominent carbon source in inducing cellulase activity from *Bacillus* sp. Among diverse nitrogen sources used, peptone favoured the production of cellulase maximally (1280.32±27.3 U/g). Moreover, other nitrogen sources revealed comparatively lower cellulase activities (Fig. 2f). In contrast to these results, Pramanik *et al*. (2021) and Abada *et al*. (2021) demonstrated yeast extract as a potential nitrogen source towards the enhancement of cellulase activity of *Bacillus* sp.

Response Surface Methodology

Table 3 shows factors (pH, temperature, and CMC) at varied ranges used in BBD. Table 4 summarizes BBD of chosen parameters in coded units along with experimental and predicted cellulase activities.

Table 3. Experimental Range, Level, and Code of Independent Variables for BBD Design

Variables	Code	Unit	Range and Levels		
			-1	0	+1
pH	A	-	6	7	8
Temperature	B	ºC	30	35	40
CMC	C	% w/v	0.5%	1%	1.5%

Table 4. Box-Behnken Design for Optimizing Cellulase Production

Run order	A	B	C	Cellulase activity (U/g)	
				Experimental value	Predicted value
1	1	0	-1	1595.23	1592.72
2	-1	1	0	718.15	714.48
3	1	0	1	1725.54	1722.28
4	-1	-1	0	665.12	663.02
5	0	-1	1	1110.23	1109.82
6	0	0	0	1003.46	1007.11
7	1	-1	0	1010.17	1013.84
8	-1	0	1	1550.13	1552.64
9	0	0	0	1008.41	1007.11
10	0	-1	-1	845.34	844.18
11	1	1	0	1125.12	1127.22
12	0	1	1	1267.12	1268.29
13	0	0	0	1010.34	1007.11
14	0	0	0	1005.12	1007.11
15	0	0	0	1008.21	1007.11
16	0	1	-1	850.12	850.53
17	-1	0	-1	995.54	998.80

The model equation for the optimization of cellulase activity through BBD is shown below:

$$\text{Cellulase activity (U/g)} = 1007.11 + 190.89A + 41.21B + 170.85C + 15.48AB - 106.07AC + 38.03BC + 160.47A^2 - 287.94B^2 + 299.03C^2 \quad (3)$$

A total of 17 experiments were carried out using varied combinations of chosen factors. Highest cellulase activity of 1725.54±32.63 U/g was recorded from Run No. 3. The combination with goat dung medium of pH 8.0, supplementation with 1.5% (w/w) CMC, and incubation at 37 °C was found to be the best optimized conditions for the improved cellulase activity of strain BTH. The BBD-based optimization exhibited about 1.3-fold enhancement in cellulase activity as compared to OFAT technique and was recorded to be close to the predicted cellulase activity value (1722.28±34.31 U/g).

Table 5 presents the ANOVA results for the quadratic model of cellulase activity. The model showed a highly significant F-value of 10461.62, with only a 0.01% probability that such a high value could be attributed to random noise. A P-value (Prob > F) less than 0.05 confirmed the significance of the model terms.

In this analysis, factors A, B, and C, as well as their interactions (AB, AC, and BC) and quadratic terms (A^2, B^2, and C^2), were found to be significant contributors. The "Lack of Fit F-value" of 3.17 indicated that lack of fit was not significant compared to the pure error, supporting a good model fit. The coefficient of determination (R^2 = 0.9999) demonstrated excellent correlation between experimental and predicted values, reflecting high model accuracy. A low C.V. (0.3531%) highlighted experimental reliability. Moreover, the "Predicted R^2" (0.9991) closely matched the "Adjusted R^2" (0.9998), confirming model consistency. The Adeq Precision ratio of 359.5019 indicated a strong signal-to-noise ratio, validating the model's suitability for exploring the design space.

Table 5. Analysis of Variance for Optimized Cellulase Activity using BBD

Source	Sum of Squares	df	Mean Square	F-value	*P*-value	
Model	1.390E+06	9	1.544E+05	10461.62	< 0.0001	Significant
A-A	2.915E+05	1	2.915E+05	19751.89	< 0.0001	
B-B	13583.64	1	13583.64	920.38	< 0.0001	
C-C	2.335E+05	1	2.335E+05	15822.16	< 0.0001	
AB	958.52	1	958.52	64.95	< 0.0001	
AC	45003.38	1	45003.38	3049.28	< 0.0001	
BC	5784.36	1	5784.36	391.93	< 0.0001	
A^2	1.084E+05	1	1.084E+05	7346.41	< 0.0001	
B^2	3.491E+05	1	3.491E+05	23652.98	< 0.0001	
C^2	3.765E+05	1	3.765E+05	25510.84	< 0.0001	
Residual	103.31	7	14.76			
Lack of Fit	72.70	3	24.23	3.17	0.1474	Not significant
Pure Error	30.62	4	7.65			
Cor Total	1.390E+06	16				

R^2 = 0.9999; Adjusted R^2 = 0.9998; Predicted R^2 = 0.9991; C.V.% = 0.3531; Adeq precision = 359.5019; df = degree of freedom; Highly significant = P≤0.0001; Significant = P≤0.05; Non-significant = P>0.05

The interactions between factors towards cellulase activity is illustrated as 3D plots (Fig. 3a-c). The plot was obtained by interacting pH and temperature (Fig. 3a), pH and cellulose (Fig. 3b), and temperature and cellulose (Fig. 3c).

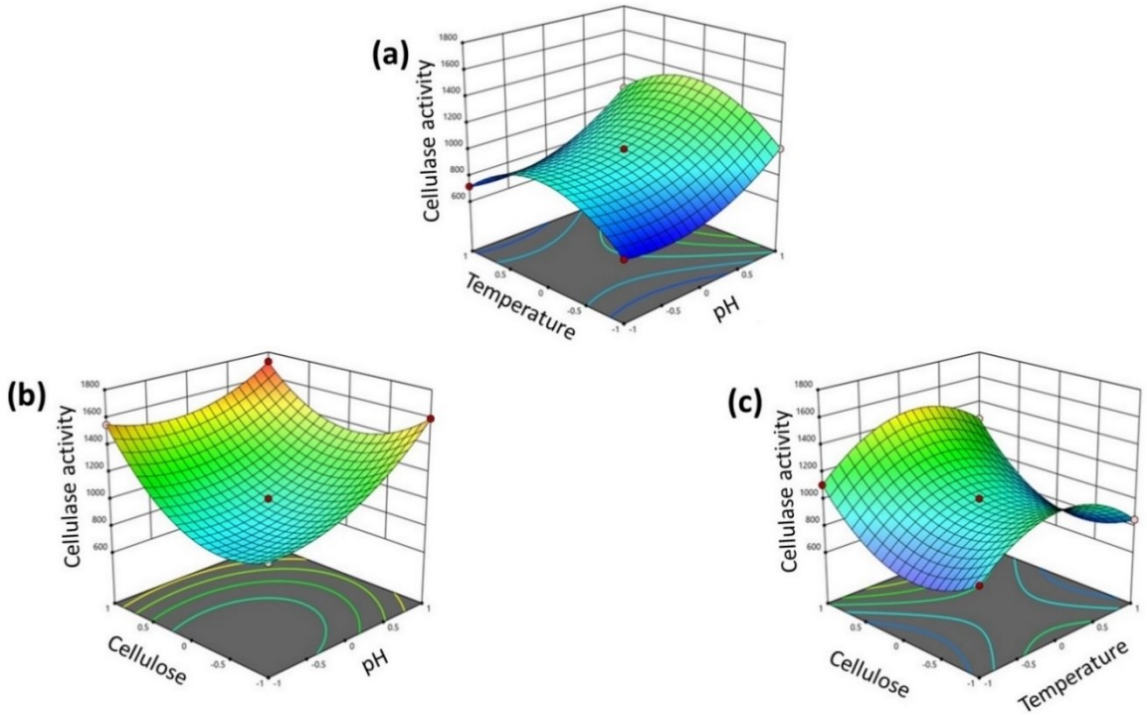

Fig. 3. 3D response plot showing interaction between **(a)** pH and temperature, **(b)** pH and cellulose, and **(c)** temperature and cellulose for enhanced cellulase production.

The validation of design was inferred under optimized parameters obtained through BBD. The experimental cellulase activities of strain BTH was recorded close to the predicted responses (Fig. 4), signifying the validation of model.

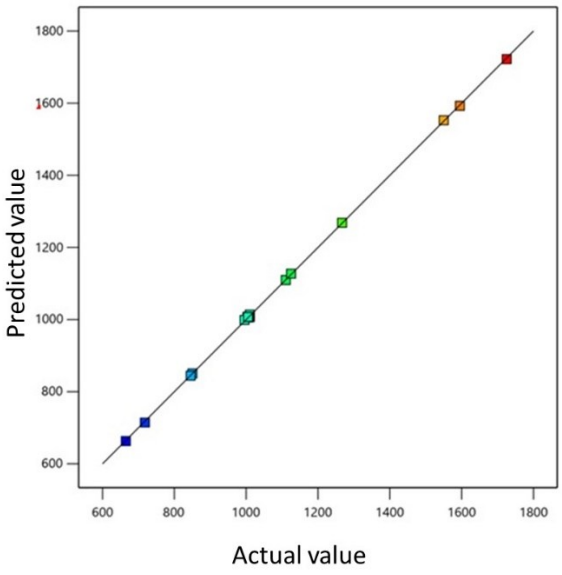

Fig. 4. Actual *vs.* predicted values of cellulase activity after BBD optimization

A cubic plot representing the interaction between pH, temperature, and cellulose for enhanced cellulase production is illustrated in Fig. 5.

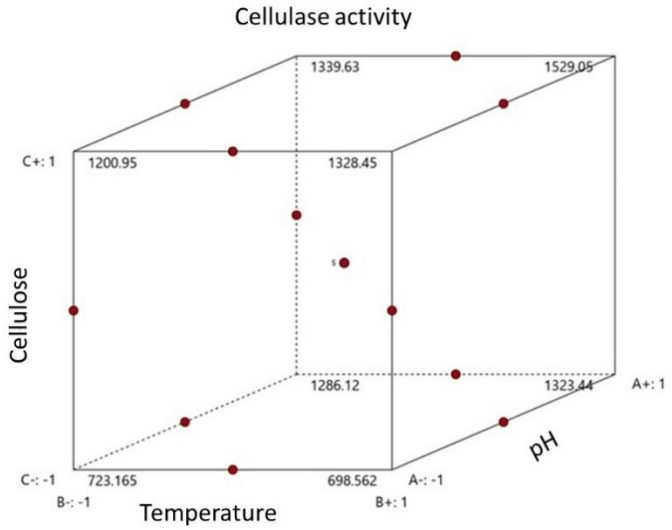

Fig. 5. Cubic plot showing interaction between pH, temperature, and cellulose for enhanced cellulase production

RSM is a statistical method-based tool, which examines varied parameters concomitantly. This multivariate approach shows multiple advantages *viz.* reduction in total number of trials, enhanced statistical justification ability, and detailed interaction of variables (Khusro *et al*. 2024). Previous studies depicted successful enhancement of cellulase production from bacteria using RSM tool (Shajahan *et al*. 2017; Aarti *et al*. 2018; Afzal *et al*. 2019; Wang *et al.* 2024; Cai *et al.* 2024; Chukwuma *et al.* 2025).

TRS Yield and Saccharification Rate

Pre-treatment of lignocellulosic biomass is an important step to cause structural variations in cellulosic biomasses in order to make cellulose more available to the hydrolytic enzymes that change the complex carbohydrate into simple sugars (Das *et al*. 2023). In the present study, para grass was pre-treated *via* hydrothermal, alkali, acid, hydrogen peroxide, and microwave heating method. Prior reports represented the promising role of pre-treatment methods in producing bioethanol from lignocellulosic biomasses (Tiwari *et al*. 2022; Chettri and Verma 2024).

Tables 6 through 9 illustrate the effect of cellulase on TRS yield from pre-treated para grass biomass. Cellulase exhibited low impact on untreated biomasses with TRS yield of 4.07±0.18, 5.04±0.17, 6.53±0.2, 8.43±0.2, and 6.8±0.2 mg/g at 12, 24, 48, 72, and 96 h of treatment. The hydrothermal pre-treated biomass showed maximum TRS production of 4.82±0.2, 5.91±0.18, 7.83±0.17, 9.89±0.18, and 8.1±0.2 mg/g at 121°C from 12 to 96 h. The TRS productions were observed comparatively and significantly ($P<0.05$) low for 80, 90, and 100 °C pre-treated biomass (Table 6). The alkali (NaOH) pre-treated biomass displayed maximum TRS production of 6.73±0.2, 9.25±0.16, 11.6±0.17, 14.11±0.16, and 11.54±0.16 mg/g in the presence of 4% (w/v) NaOH from 12 to 96 h. The TRS productions were significantly ($P<0.05$) reduced for 2, 6, and 8% (w/v) NaOH pre-treated biomass (Table 7). The acid (H_2SO_4) pre-treated biomass revealed maximum TRS production of 6.1±0.16, 8.3±0.17, 9.84±0.17, 11.15±0.18, and 9.25±0.17 mg/g in the presence of 2% (v/v) H_2SO_4 from 12 to 96 h.

Table 6. Effect of Cellulase on TRS Production (mg/g) from Hydrothermal Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	Hydrothermal (°C)			
		80	90	100	121
12	4.07±0.18^{e}	4.21±0.2^{e}	4.35±0.18^{e}	4.5±0.2^{e}	4.82±0.2^{e}
24	5.04±0.17^{d}	5.35±0.16^{d}	5.42±0.2^{d}	5.58±0.16^{d}	5.91±0.18^{d}
48	6.53±0.2^{c}	6.82±0.17^{c}	7.2±0.2^{c}	7.4±0.17^{c}	7.83±0.17^{c}
72	8.4±0.2^{a}	8.82±0.16^{a}	8.95±0.18^{a}	9.1±0.18^{a}	9.89±0.18^{a}
96	6.8±0.2^{b}	7.3±0.16^{b}	7.51±0.17^{b}	7.78±0.16^{b}	8.1±0.2^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 7. Effect of Cellulase on TRS Production (mg/g) from NaOH Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	NaOH (% w/v)			
		2	4	6	8
12	4.07±0.18^{e}	5.81±0.2^{e}	6.73±0.2^{d}	6.51±0.18^{e}	6.21±0.16^{e}
24	5.04±0.17^{d}	7.31±0.18^{d}	9.25±0.16^{c}	7.96±0.16^{d}	7.7±0.2^{d}
48	6.53±0.2^{c}	8.91±0.17^{c}	11.6±0.17^{b}	9.3±0.18^{c}	8.21±0.17^{c}
72	8.4±0.2^{a}	10.51±0.17^{a}	14.11±0.16^{a}	11.6±0.18^{a}	9.6±0.16^{a}
96	6.8±0.2^{b}	9.81±0.18^{b}	11.54±0.16^{b}	9.71±0.16^{b}	8.72±0.17^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

The TRS productions were significantly (P<0.05) decreased for 1, 3, and 4% (v/v) H_2SO_4 pre-treated biomass (Table 8). Hydrogen peroxide pre-treated biomass showed maximum TRS production of 6.12±0.2, 8.22±0.16, 9.73±0.17, 10.92±0.2, and 9.15±0.16 mg/g in the presence of 3% (v/v) H_2O_2 from 12-96 h. The TRS yields were significantly (P<0.05) reduced for 1, 2, and 4% (v/v) H_2O_2 pre-treated biomass (Table 9). Microwave irradiation of biomass for 5 min exposure showed maximum TRS production of 4.63±0.2, 5.62±0.16, 7.42±0.2, 9.2±0.17, and 7.75±0.2 mg/g from 12-96 h. The TRS productions were decreased significantly (P<0.05) from biomass during the microwave exposure of 1, 2, and 8 min (Table 10).

Table 8. Effect of Cellulase on TRS Production (mg/g) from H_2SO_4 Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	H_2SO_4 (% v/v)			
		1	2	3	4
12	4.07±0.18^{e}	4.31±0.2^{e}	6.1±0.16^{e}	5.9±0.17^{e}	5.51±0.2^{e}
24	5.04±0.17^{d}	5.4±0.17^{d}	8.3±0.17^{d}	7.21±0.16^{d}	6.52±0.2^{d}
48	6.53±0.2^{c}	6.63±0.17^{c}	9.84±0.17^{b}	8.82±0.18^{b}	8.33±0.17^{b}
72	8.4±0.2^{a}	9.32±0.16^{a}	11.15±0.18^{a}	10.21±0.2^{a}	9.93±0.18^{a}
96	6.8±0.2^{b}	7.4±0.18^{b}	9.25±0.17^{c}	8.52±0.16^{c}	8.18±0.17^{c}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 9. Effect of Cellulase on TRS Production (mg/g) from H_2O_2 Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	H_2O_2 (% v/v)			
		1	2	3	4
12	4.07±0.18^{e}	4.22±0.2^{e}	5.82±0.16^{e}	6.12±0.2^{e}	5.7±0.16^{d}
24	5.04±0.17^{d}	5.33±0.16^{d}	7.11±0.2^{d}	8.22±0.16^{d}	6.5±0.2^{c}
48	6.53±0.2^{c}	6.62±0.17^{c}	8.8±0.17^{b}	9.73±0.17^{b}	8.21±0.17^{b}
72	8.4±0.2^{a}	9.22±0.16^{a}	9.95±0.16^{a}	10.92±0.2^{a}	9.83±0.18^{a}
96	6.8±0.2^{b}	7.3±0.16^{b}	8.42±0.17^{c}	9.15±0.16^{c}	8.22±0.16^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 10. Effect of Cellulase on TRS Production (mg/g) from Microwave Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	Microwave irradiation (min)			
		1	2	5	8
12	4.07±0.18^{e}	4.21±0.2^{e}	4.4±0.18^{e}	4.63±0.2^{e}	4.53±0.2^{e}
24	5.04±0.17^{d}	5.35±0.17^{d}	5.49±0.2^{d}	5.62±0.16^{d}	5.32±0.2^{d}
48	6.53±0.2^{c}	6.84±0.16^{c}	7.18±0.17^{c}	7.42±0.2^{c}	7.25±0.17^{c}
72	8.4±0.2^{a}	8.6±0.16^{a}	8.93±0.16^{a}	9.2±0.17^{a}	8.74±0.16^{a}
96	6.8±0.2^{b}	7.32±0.18^{b}	7.61±0.2^{b}	7.75±0.2^{b}	7.42±0.17^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

The saccharification percentage of pre-treated para grass biomass is shown in Tables 11 through 15. The saccharification of untreated biomasses was recorded as 18.31±0.7, 22.68±0.7, 29.38±0.7, 37.8±0.8, and 30.6±0.7% at 12, 24, 48, 72, and 96 h of treatment. The hydrothermal treated biomasses showed a maximum saccharification of 21.69±0.7, 26.59±0.8, 35.23±0.7, 44.5±0.7, and 36.45±0.8% at 121 °C from 12 to 96 h. The saccharification efficiency was significantly (P<0.05) reduced for 80, 90, and 100 °C pre-treated biomass (Table 11). The alkali-pretreated biomass showed maximum saccharification efficiency of 30.28±0.8, 41.62±0.6, 52.2±0.7, 63.49±0.6, and 51.93±0.8% in the presence of 4% (w/v) NaOH from 12 to 96 h. The saccharification ability was significantly (P<0.05) reduced for 2, 6, and 8% (w/v) NaOH pre-treated biomass (Table 12). Maximum saccharification degree of 27.45±0.8, 37.33±0.7, 44.28±0.6, 50.17±0.7, and 41.62±0.8% was estimated from 2% (v/v) H_2SO_4 pre-treated biomass for 12 to 96 h. The efficiency was reduced significantly (P<0.05) for 1, 3, and 4% (v/v) H_2SO_4 pre-treated biomass (Table 13). Hydrogen peroxide treated biomasses showed maximum saccharification efficiency of 27.54±0.7, 36.99±0.6, 43.78±0.7, 49.14±0.8, and 41.17±0.6% in the presence of 3% (v/v) H_2O_2 from 12 to 96 h. The efficiency was significantly (P<0.05) reduced for 1, 2, and 4% (v/v) H_2O_2 pre-treated biomass (Table 14). Microwave irradiation of biomass for 5 min exposure revealed maximum saccharification efficiency of 20.83±0.8, 25.29±0.6, 33.39±0.7, 41.4±0.8, and 34.87±0.6% from 12 to 96 h. The efficiency was reduced significantly (P<0.05) from biomass during the microwave exposure of 1, 2, and 8 min (Table 15). These findings supported the outcomes of Tiwari *et al*. (2022), Aarti *et al*. (2022b), and Chettri and Verma (2024), who demonstrated promising levels of TRS production, followed by saccharification of alkali-pretreated lignocellulosic biomasses.

Table 11. Effect of Cellulase on Saccharification Efficiency (%) of Hydrothermal Pre-Treated Para Grass Biomass

Incubation period (h)	Untreated	Hydrothermal (°C)			
		80	90	100	121
12	18.31±0.7^{e}	18.94±0.7^{e}	19.57±0.7^{e}	20.25±0.7^{e}	21.69±0.7^{e}
24	22.68±0.7^{d}	24.07±0.6^{d}	24.39±0.7^{d}	25.11±0.5^{d}	26.59±0.8^{d}
48	29.38±0.7^{c}	30.69±0.8^{c}	32.4±0.8^{c}	33.3±0.8^{c}	35.23±0.7^{c}
72	37.8±0.8^{a}	39.69±0.8^{a}	40.27±0.6^{a}	40.95±0.7^{a}	44.5±0.7^{a}
96	30.6±0.7^{b}	32.85±0.7^{b}	33.79±0.7^{b}	35.01±0.7^{b}	36.45±0.8^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 12. Effect of Cellulase on Saccharification Efficiency (%) of NaOH Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	NaOH (% w/v)			
		2	4	6	8
12	18.31±0.7^{e}	26.14±0.7^{e}	30.28±0.8^{e}	29.29±0.8^{e}	27.94±0.8^{e}
24	22.68±0.7^{d}	32.89±0.6^{d}	41.62±0.6^{d}	35.82±0.5^{d}	34.65±0.6^{d}
48	29.38±0.7^{c}	40.09±0.8^{c}	52.2±0.7^{b}	41.85±0.8^{c}	36.94±0.8^{c}
72	37.8±0.8^{a}	47.29±0.7^{a}	63.49±0.6^{a}	52.2±0.7^{a}	43.2±0.6^{a}
96	30.6±0.7^{b}	44.14±0.7^{b}	51.93±0.8^{c}	43.69±0.7^{b}	39.24±0.7^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 13. Effect of Cellulase on Saccharification Efficiency (%) of H_2SO_4 Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	H_2SO_4 (% v/v)			
		1	2	3	4
12	18.31±0.7^{e}	19.39±0.6^{e}	27.45±0.8^{e}	26.55±0.7^{e}	24.79±0.8^{e}
24	22.68±0.7^{d}	24.3±0.6^{d}	37.35±0.7^{d}	32.44±0.8^{d}	29.34±0.6^{d}
48	29.38±0.7^{c}	29.83±0.8^{c}	44.28±0.6^{b}	39.69±0.8^{b}	37.48±0.8^{b}
72	37.8±0.8^{a}	41.94±0.7^{a}	50.17±0.7^{a}	45.94±0.7^{a}	44.68±0.7^{a}
96	30.6±0.7^{b}	33.3±0.8^{b}	41.62±0.8^{c}	38.34±0.8^{c}	36.81±0.8^{c}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 14. Effect of Cellulase on Saccharification Efficiency (%) of H_2O_2 Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	H_2O_2 (% v/v)			
		1	2	3	4
12	18.31±0.7^{e}	18.99±0.7^{e}	26.19±0.7^{e}	27.54±0.7^{e}	25.65±0.8^{d}
24	22.68±0.7^{d}	23.98±0.7^{d}	31.99±0.7^{d}	36.99±0.6^{d}	29.25±0.6^{c}
48	29.38±0.7^{c}	29.79±0.8^{c}	39.6±0.8^{b}	43.78±0.7^{b}	36.94±0.8^{b}
72	37.8±0.8^{a}	41.49±0.8^{a}	44.77±0.6^{a}	49.14±0.8^{a}	44.23±0.7^{a}
96	30.6±0.7^{b}	32.85±0.7^{b}	37.89±0.7^{c}	41.17±0.6^{c}	36.99±0.8^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 15. Effect of Cellulase on Saccharification Efficiency (%) of Microwave Pre-treated Para Grass Biomass

Incubation period (h)	Untreated	Microwave irradiation (min)			
		1	2	5	8
12	18.31±0.7^{e}	18.94±0.7^{e}	19.8±0.7^{e}	20.83±0.8^{e}	20.38±0.7^{e}
24	22.68±0.7^{d}	24.07±0.7^{d}	24.7±0.7^{d}	25.29±0.6^{d}	23.94±0.7^{d}
48	29.38±0.7^{c}	30.78±0.8^{c}	32.31±0.8^{c}	33.39±0.7^{c}	32.62±0.8^{c}
72	37.8±0.8^{a}	38.7±0.7^{a}	40.18±0.6^{a}	41.4±0.8^{a}	39.33±0.6^{a}
96	30.6±0.7^{b}	32.94±0.8^{b}	34.24±0.7^{b}	34.87±0.6^{b}	33.39±0.8^{b}

Values are represented as mean±SD of experiments carried out in triplicate (n = 3). ^{abcde} Values with different superscript letters within the same column are significantly (P<0.05) different.

CONCLUSIONS

1. *B. paramycoides* strain BTH was identified as a hyper-cellulase producing bacterium after molecular characterization and 16S rRNA sequencing.
2. The OFAT-based non-statistical method of optimization improved the activity of this cellulase up to 1280.32±27.3 U/g.
3. RSM-based optimization enhanced the above-mentioned cellulase activity to 1725.54±32.63 U/g in the presence of goat dung medium (pH 8.0), supplemented with 1.5% (w/w) CMC and incubated at 37 °C.
4. Alkali [4% (w/v) NaOH] pre-treated para grass biomass exhibited maximum TRS yield of 6.73±0.2, 9.25±0.16, 11.6±0.17, 14.11±0.16, and 11.54±0.16 mg/g from 12-96 h. Likewise, 4% (w/v) NaOH pre-treated para grass biomass showed maximum saccharification efficiency of 30.28±0.8, 41.62±0.6, 52.2±0.7, 63.49±0.6, and 51.93±0.8% from 12 to 96 h.
5. Results of this study represented the promising role of *B. paramycoides*-associated cellulase in the saccharification of para grass for the production of bioethanol in future.

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