Journal of Chemical Health Risks

www.jchr.org

JCHR (2025) 15(4), 712-716 | ISSN:2251-6727

Cellulase Production from *Bacillus Subtilis* and Its Role in Saccharification of Alkali Pre-Treated Para Grass Biomass

Pranam Sinha[1], Ameer Khusro[2*], and Dhanraj Ganapathy[3]

[1]*Dentistry Department, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai – 600077, India*

[2]*Department of Research Analytics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai – 600077, India*

[3]*Department of Prosthodontics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai – 600077, India*

(Received: 16 May 2025 Revised: 20 June 2025 Accepted: 02 July 2025)

KEYWORDS

B. subtilis, Cellulase, Lignocellulosic biomass, Para grass, Saccharification

ABSTRACT:

Production of second-generation bioethanol has created enormous interest globally as sustainable and ideal alternative source due to the gradual depletion of fossil fuels. In view of this, the present study was investigated to isolate cellulase producing bacterium from poultry soil sample and assess its role in the saccharification of *Brachiaria mutica* (Para grass). Initially, the cellulase producing bacterium was identified as *Bacillus subtilis* strain KPA based on biochemical and molecular characterization tools. Further, *B. mutica* was pre-treated using NaOH solution (4% w/v) and utilized for saccharification process as per the standard methodology. Results showed low impact of cellulase on untreated Para grass biomass with TRS yield of 2.24±0.18, 3.11±0.2, 4.1±0.17, 4.98±0.2, and 4.14±0.18 mg/g at 12, 24, 48, 72, and 96 h of treatment. The alkali (NaOH) pre-treated biomass exhibited maximum TRS yield of 3.23±0.2, 4.34±0.18, 5.25±0.17, 7.88±0.17, and 6.16±0.17 mg/g from 12-96 h. Similarly, the saccharification ability of untreated Para grass biomass was estimated lower than that of pre-treated one. The alkali pre-treated biomass showed maximum saccharification efficiency of 29.07±0.8, 39.06±0.8, 47.2±0.7, 70.9±0.7, and 55.4±0.8% in the presence of 4% (w/v) NaOH from 12-96 h. In conclusion, the findings suggested the prominent role of strain KPA-associated cellulase in the saccharification of *B. mutica* and its potential role in biofuel production in future.

1. Introduction

Lignocellulose is a vital renewal energy source composed of cellulose, hemicellulose, and recalcitrant lignin [1]. Among these, cellulose, is the most abundant natural homopolysaccharide in the biosphere [2]. The enzymatic hydrolysis of lignocellulosic biomass into fermentable sugars is a key step in bioethanol production, facilitated by cellulolytic enzymes that act synergistically [3].

Cellulase is a complex enzyme system comprising endo-1,4-beta-D-glucanase (EC 3.2.1.4), exo-1,4-beta-D-glucanase (EC 3.2.1.91), and beta-glucosidase (EC 3.2.1.21). Endoglucanase randomly cleaves internal beta-1,4 bonds in cellulose, generating oligosaccharides of varying lengths. Exoglucanase further degrades these oligosaccharides by removing cellobiose units from the chain ends. Beta-glucosidase hydrolyzes cellobiose into glucose, completing the saccharification process [2, 4]. Beyond bioethanol production, cellulases have broad industrial applications, including food processing, brewing, pulp and paper manufacturing, textiles, pharmaceuticals, detergents, livestock feed, and agriculture [5].

Various microorganisms, such as yeast, bacteria, and fungi serve as natural sources of cellulase. However, bacteria have gained considerable attention due to their rapid growth rates, thermal stability, alkali tolerance, and ability to produce multi-enzyme complexes [6]. Several novel cellulase-producing bacterial species have been identified in the past [2, 7, 8]. Nevertheless, the search for hyper-cellulase-producing bacteria remains ongoing. In this regard, a significant study was undertaken to saccharify *Brachiaria mutica* (Paragrass) for fermentable sugar production using crude cellulase from *Bacillus* sp., highlighting its potential for industrial applications.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15(4), 712-716 | ISSN:2251-6727

2. Methods

Isolation of cellulase producing bacteria

Bacteria were isolated from poultry soil sample using serial dilution technique. Soil sample (1 g) collected was mixed with 9 mL of sterile distilled water. The suspension was serially diluted (10^{-1}-10^{-6}) and 1 mL of the suspension was poured onto sterilized Nutrient agar medium (g/L: peptone 5.0, beef extract 3.0, yeast extract 2.0, sodium chloride 5.0, agar 18.0, and pH 7.0±0.2) plates containing 0.5% carboxy methyl cellulose (CMC). After spreading the suspension using L-rod, plates were incubated at 37°C in incubator for 24 h under aerobic condition. After required period of incubation, the appearance of different colonies on the agar plates was observed. A single colony was picked and purified. Bacterium was identified using molecular characterization and bioinformatics tool.

Cellulase production-

Plate assay (Qualitative analysis)

The purified isolate was streaked on Nutrient agar medium plate containing 0.5% CMC and incubated at 37°C for 24 h. After 24 h, the agar plate was flooded with Lugol's iodine solution for 5 min. The plate was observed for the halo zone of CMC hydrolysis [2].

Cellulase activity (Quantitative analysis)

Cellulase activity was estimated using 0.5% CMC as the substrate. The isolate was inoculated in Nutrient broth containing 0.5% CMC and incubated at 37°C for 24 h with an agitation speed of 130 rpm. After required period of incubation, the culture was centrifuged and the collected supernatant was used as crude cellulase. The crude cellulase (1 mL) was mixed with 1 mL of 0.5% CMC and incubated at 37°C for 30 min. After incubation period, 1 mL of DNS reagent was added into the reaction mixture to stop the reaction, and then boiled in water bath for 5 min. The mixture was cooled, and the absorbance was read spectrophotometrically against the reagent blank at 540 nm. Cellulase activity (U) was defined as the amount of enzyme that liberated 1 µg of reducing sugars per minute under the standard conditions [2].

Para grass saccharification-

Collection of biomass

Brachiaria mutica (Para grass) was collected from Loktak lake, Imphal West, Manipur. It was brought in polythene bags to the laboratory and dried for 7-10 days. After complete drying, it was powdered and stored at room temperature.

Alkali pre-treatment of Para grass

The alkaline treatment was carried out by mixing 10% (w/v) of powdered Para grass biomass with NaOH solution (4% w/v). The mixture was incubated at room temperature for 2-3 h, autoclaved at 121°C for 30 min, and then washed with water until neutral pH was achieved.

Cellulase-based hydrolysis of Para grass

Crude cellulase (50 mL) was added into 10 g of pre-treated Para grass and incubated for 96 h. Every 24 h, the content was filtered and the supernatant was used to quantify total reducing sugar (TRS: mg/g) production.

TRS quantification

The production of TRS after the hydrolysis process was quantified using DNS method. One millilitre of the collected supernatant was incubated with 1 mL of DNS reagent, boiled for 5 min, and then cooled at room temperature. The volume of the solution was made up to 10 mL by adding sterile distilled water and the absorbance was read at 540 nm. The TRS production (mg/mL) was quantified using standard curve of glucose [3].

Saccharification efficiency

The saccharification rate (%) was calculated according to the equation as mentioned below [9]:

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

Statistical analysis

All the experiments were carried out in triplicate and data were represented as mean±standard deviation (mean±SD). ANOVA was implemented to analyse the significance ($P \leq 0.05$) level.

3. Results

Bacteria identification

Based on the morphological, biochemical, molecular characterization, and sequence analyses tools, the isolate was identified as *Bacillus subtilis* strain KPA (Accession no.: KC918878).

Cellulase assay

Strain KPA showed zone of hydrolysis on CMC agar medium plate, indicating the production of cellulase (Fig. 1). Further, as per quantitative analysis, cellulase activity was estimated as 265.34±13.42 U/mL.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15(4), 712-716 | ISSN:2251-6727

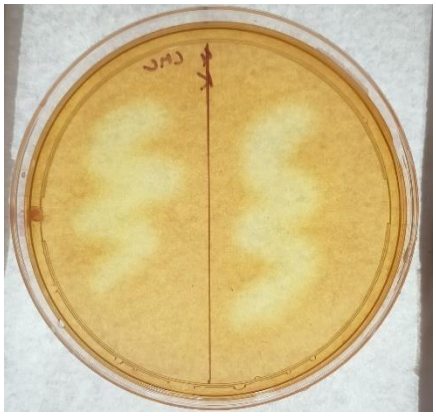

Fig. 1: Cellulase assay

TRS production and saccharification efficiency

Table 1A illustrates the effect of cellulase on TRS yield from untreated and pre-treated *B. mutica* biomass. Cellulase exhibited low impact on untreated biomass with TRS yield of 2.24±0.18, 3.11±0.2, 4.1±0.17, 4.98±0.2, and 4.14±0.18 mg/g at 12, 24, 48, 72, and 96 h of treatment. The alkali (NaOH) pre-treated biomass exhibited maximum TRS yield of 3.23±0.2, 4.34±0.18, 5.25±0.17, 7.88±0.17, and 6.16±0.17 mg/g from 12-96 h. The saccharification degree of untreated and pre-treated *B. mutica* biomass is shown in Table 1B. The saccharification ability of untreated biomass was estimated as 20.1±0.6, 27.9±0.7, 36.9±0.6, 44.8±0.8, and 37.2±0.8% at 12, 24, 48, 72, and 96 h of treatment. The alkali pre-treated biomass showed maximum saccharification efficiency of 29.07±0.8, 39.06±0.8, 47.2±0.7, 70.9±0.7, and 55.4±0.8% in the presence of 4% (w/v) NaOH from 12-96 h.

Table 1: TRS production (mg/g) from untreated and NaOH pre-treated *B. mutica* biomass.

Incubation period	TRS (mg/g)	
	Untreated	NaOH-treated
12	2.24±0.18^{e}	3.23±0.2^{e}
24	3.11±0.2^{d}	4.34±0.18^{d}
48	4.1±0.17^{c}	5.25±0.17^{b}
72	4.98±0.2^{a}	7.88±0.17^{a}
96	4.14±0.18^{b}	6.16±0.17^{c}

^{abcde}Values with different superscript letters within the same column are significantly (P<0.05) different.

Table 2: Saccharification efficiency of untreated and NaOH pre-treated *B. mutica* biomass.

Incubation period	Saccharification (%)	
	Untreated	NaOH-treated
12	20.1±0.6^{e}	29.07±0.8^{e}
24	27.9±0.7^{d}	39.06±0.8^{d}
48	36.9±0.6^{c}	47.2±0.7^{b}
72	44.8±0.8^{a}	70.9±0.7^{a}
96	37.2±0.8^{b}	55.4±0.8^{c}

^{abcde}Values with different superscript letters within the same column are significantly (P<0.05) different.

4. Discussion

To address the growing global energy demand, various plant biomasses are being pretreated and converted into biofuels through the fermentation of reducing sugars derived from saccharification [10-12]. In recent years, aquatic weeds have emerged as promising next-generation bioresources for sustainable value-added products through saccharification [13, 14].

Enzyme-based hydrolysis plays a crucial role in breaking down pretreated lignocellulosic biomass into reducing sugars, thereby determining saccharification efficiency [3]. This study focuses on the saccharification of *B. mutica* using cellulase derived from *Bacillus subtilis*. Previous studies have reported total reducing sugar (TRS) production and saccharification of various aquatic weeds, including *Alternanthera sessilis, Typha latifolia, Eichhornia crassipes, Bacopa monnieri, B. mutica*, *Ipomoea aquatica*, and *Pistia stratiotes*, utilizing both commercial and bacterial cellulases [14-16].

In this study, the saccharification efficiency and TRS production from untreated aquatic weed biomass were found to be lower than those of pretreated biomass. This difference is likely due to the high lignin content in untreated biomass, which reinforces the plant cell wall structure and restricts cellulase accessibility. However, NaOH-pretreated weed biomass exhibited the highest TRS production and saccharification efficiency. The superior efficiency of alkaline pretreatment can be attributed to its ability to increase the surface area and reduce the crystalline nature of cellulose, facilitating better enzyme penetration. This method effectively removes lignin and depolymerizes cellulose, thereby enhancing the overall saccharification process [3, 14]. The findings of this study underscore the significance of pretreatment strategies in optimizing biofuel production from aquatic weed biomass and highlight the potential of *B. subtilis*-derived cellulase as a cost-effective enzymatic tool for bioethanol generation.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2025) 15(4), 712-716 | ISSN:2251-6727

5. Conclusions

In summary, this study represented the isolation of cellulase producing bacterium from poultry soil sample and was identified as *B. subtilis* strain KPA. Further, strain KPA-associated cellulase exhibited its potentialities to hydrolyze pre-treated *B. mutica* and produced TRS with high saccharification efficiency. In a nutshell, cellulase produced from strain KPA can be employed as a crucial agent to saccharify aquatic weeds efficiently towards the production of next generation biofuel in future.

Conflict of interest

None declared.

References

1. Ashokkumar V, Chandramughi VP, Kumar G, Ngamcharussrivichai C, Piechota G, Igliński B, *et al*. Advancements in lignocellulosic biomass: A critical appraisal of fourth-generation biofuels and value-added bioproduct. *Fuel* 2024, 365, 130751.
2. Aarti C, Khusro A, Agastian P. Carboxymethyl cellulase production optimization from *Glutamicibacter arilaitensis* strain ALA4 and its application in lignocellulosic waste biomass saccharification. *Preparative Biochemistry and Biotechnology* 2018, 48, 853-66
3. Aarti C, Khusro A, Agastian P. Saccharification of alkali pre-treated aquatic weeds biomass using partially purified cellulase immobilized on different matrices. *Biocatalysis and Agricultural Biotechnology* 2022, 39, 102283, doi.org/10.1016/j.bcab.2022.102283
4. Sukumaran S, Raguvaran K, Nagarajan K, Chinnaperumal K, Maheswaran R, Chinnasamy R. Isolation, molecular identification, and characterization of cellulolytic microbes from the gut of grasshopper *Eyprepocnemis alacris alacris* (Serv. 1838). *Biomass Conversion and Biorefinery* 2024, 14, 21121-33.
5. Sadhu S, Ghosh PK, Aditya G, Maiti TK. Optimization and strain improvement by mutation for enhanced cellulase production by *Bacillus* sp. (MTCC10046) isolated from cow dung. *Journal of King Saud University – Science* 2014, 26, 323–32.
6. Khusro A, Aarti C. Molecular identification of newly isolated *Bacillus* strains from poultry farm and optimization of process parameters for enhanced production of extracellular amylase using OFAT method. *Research Journal of Microbiology* 2015, 10, 393-420.
7. Fuji K, Satomi M, Fukui Y, Matsunobu S, Morifuku Y, Enokida Y. *Streptomyces abietis* sp. nov., a cellulolytic bacterium isolated from soil of a pine forest. *International Journal of Systematic Evolutionary Microbiology* 2013, 63, 4754–9.
8. Podosokorskaya OA, Bonch-Osmolovskaya EA, Novikov AA, Kolganova TV, Kublanov IV. *Ornatilinea apprima* Gen. nov., sp. nov., a cellulolytic representative of the class Anaerolineae. *International Journal of Systematic Evolutionary Microbiology* 2013, 63, 86–92.
9. Mandels M, Sternberg D. Recent advances in cellulase technology. *Journal of Fermentation Technology* 1976, 54, 267–86.
10. Iyyappan J, Pravin R, Al-Ghanim KA, Govindarajan M, Nicoletti M, Baskar G. Dual strategy for bioconversion of elephant grass biomass into fermentable sugars using *Trichoderma reesei* towards bioethanol production. *Bioresource Technology*. 2023, 374, 128804.
11. Manikandan S, Subbaiya R, Biruntha M, Krishnan RY, Muthusamy G, Karmegam N. Recent development patterns, utilization and prospective of biofuel production: Emerging nanotechnological intervention for environmental sustainability–A review. *Fuel* 2022, 314, 122757.
12. Sandhya CH, Sashirekha K. Experimental investigation on comparison of production of biofuel from fried *Arachis hypogaea* oil using sodium hydroxide and ethanol catalysts. InAIP Conference Proceedings 2024, 3193.
13. Kaur M, Kumar M, Sachdeva S, Puri SK. Aquatic weeds as the next generation feedstock for sustainable bioenergy production. *Bioresource Technology* 2018, 251, 390-402.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2025) 15(4), 712-716 | ISSN:2251-6727

14. Aarti C, Khusro A, Agastian P, Kuppusamy P, Al Farraj DA. Synthesis of gold nanoparticles using bacterial cellulase and its role in saccharification and bioethanol production from aquatic weeds. *Journal of King Saud University – Science*, 2022, 34, 101974, doi.org/10.1016/j.jksus.2022.101974
15. Sunil KR, John M, Girish V, Girisha ST. A comparative study of bioethanol production from aquatic weeds. *International Journal of Applied Science and Biotechnology* 2015, 3, 446-51.
16. Sahoo D, Ummalyma SB, Okram AK, Sukumaran RK, George E, Pandey A. Potential of *Brachiaria mutica* (Para grass) for bioethanol production from Loktak Lake. *Bioresource Technology* 2017, 242, 133-8.