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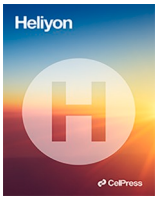

Research article

Cellulase production under solid-state fermentation by *Aspergillus* sp. IN5: Parameter optimization and application

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ABSTRACT

Microbial cellulases are highly versatile catalysts with significant potential in various industries, including pulp and paper, textile manufacturing, laundry, biofuel production, food and animal feed, brewing, and agriculture. Cellulases have attracted considerable attention from the scientific community owing to their broad industrial applications and the complex nature of enzymatic systems. In the present study, a novel fungal isolate of *Aspergillus* sp. IN5 was used to produce cellulases. We optimized each parameter, including carbon source, incubation temperature, pH, and incubation time, for maximum cellulase production using isolate IN5 under solid-state fermentation conditions. The optimized parameters for cellulase production by isolate IN5 under solid-state fermentation were as follows: substrate, soybean residue; incubation temperature, 35 °C; pH, 7.0; and incubation duration, 5 days. These conditions resulted in the highest total cellulase activity (0.26 U/g substrate), and carboxymethyl cellulase and β-glucosidase activities of 3.32 and 196.09 U/g substrate, respectively. The obtained fungal cellulase was used for the enzymatic hydrolysis of acid- or alkali-pretreated rice straw, which served as a model substrate. Notably, compared with acid pretreatment, the pretreatment of rice straw with diluted alkali led to higher yields of reducing sugars. Maximum reducing sugar yield (286.06 ± 2.77 mg/g substrate) was obtained after 24-h incubation of diluted alkali-pretreated rice straw mixed with an enzyme loading of 15 U/g substrate. The findings of this study provide an alternative strategy for utilizing agricultural waste and an approach to efficiently produce cellulase for the degradation of lignocellulosic materials, with promising benefits for sustainable waste management.

1. Introduction

Global energy consumption is consistently increasing, and fossil fuels are the primary source of energy worldwide. However, the indiscriminate use of fossil fuels has led to numerous environmental issues, including energy shortages and escalation in global temperatures, because of the release of carbon dioxide and other waste gases during fuel combustion [1–3]. Substantial release of

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energy into the atmosphere contributes to the greenhouse effect and exacerbates global warming. Global heating can be mitigated by making a conscious and significant shift toward renewable and alternative energy sources, reducing reliance on fossil fuels [1,2,4–6]. The conversion of waste materials such as lignocellulosic biomass and cotton textiles into value-added compounds, including biofuels, sorbitol, lactic acid, terephthalic acid, and ethylene glycol, is a promising method for generating alternative energy with numerous advantages [7]. Cellulose, a major structural component of plant and bacterial cell walls, is a fundamental polysaccharide composed of glucose molecules linked via β (1–4) glycosidic bonds. Moreover, cellulose accounts for approximately 45% of all organic matter in the environment [8]. Endoglucanases and exoglucanases play important roles in cellulose degradation. Endoglucanases randomly cleave the β-1,4-D-glycosidic linkages to generate new reducing and non-reducing ends of cellulose molecules [9]. Exoglucanases further break down cellulose chains into cellobiose units at their ends [10]. Finally, β-glucosidases hydrolyze glucose dimers and oligomers into individual glucose molecules that can be utilized by microorganisms as sources of energy and carbon [9]. Cellulases are used in several industries such as food, paper and pulp, textiles, pharmaceuticals, alcoholic beverages, starch processing, and biofuel production [11,12]. Fungi, especially *Trichoderma* spp. and *Aspergillus* spp., are primary sources of commercial cellulases [13,14].

Currently, two technologies are used for enzyme production: traditional submerged fermentation (SmF), a liquid-based fermentation process, and solid-state fermentation (SSF), a solid-based fermentation process that does not involve dissolution in water [15]. Most enzyme-producing companies use SmF technology for production because of its ease of management and control over abiotic parameters, including temperature and pH. However, SSF technology may be an interesting alternative, as it can improve production yield and reduce production costs [16,17]. Another advantage of SSF technology is that direct raw materials such as lignocellulose can be used to stimulate enzyme production [17]. This technology has also been recognized for its reduced water usage and fewer adverse effects. The yield of products produced using SSF is higher than that obtained using SmF [17–19]. Filamentous fungi are the most commonly used microorganisms for enzyme production via SSF because they can thrive on solid materials with a low water content [20]. Several studies have reported the use of agro-industrial residues, such as rice bran, rice husk, and wheat bran, as substrates for cellulase production [18,21–23]. Other advantages of SSF technology include its simple procedure, low investment cost, low energy requirements, reduced water usage, improved yield [17,24,25], and enhanced product recovery rate [26].

In this study, we investigated the optimal conditions for cellulase production using SSF technology and a fungal isolate from soil. The key factors that affect cellulase production include carbon source, temperature, pH, and incubation time. Additionally, the cellulase derived in our study demonstrated practical application by effectively degrading cellulose in rice straw, demonstrating its potential for eco-friendly and resourceful agricultural waste management.

2. Results and discussion

2.1. Isolation of cellulase-producing fungi and use of SSF for cellulase production

Forty-two fungal isolates were obtained from humus-rich surface soil samples collected from Nakhon Ratchasima Province in Thailand. Of the 42 isolates, five isolates—Ni1, DE8, DE9, IN3, and IN5—exhibited the maximum cellulolytic activity on Congo red agar plates based on their high ratios of clear-zone diameter to colony diameter, i.e., 1.46–2.21 (Table 1). These isolates, capable of producing cellulase, were cultured on minimal salt medium (MSM) 1 and 2, and SSF was performed using soybean residue as a substrate. After 7 days of incubation, the cellulase produced from the five fungal isolates was extracted using sodium citrate buffer, and the total cellulase activity was analyzed. Fungal isolate IN5 exhibited the highest filter paper cellulase (FPase) activity of 0.188 and 0.164 U/g substrate in MSM 1 and 2, respectively. Further, IN5 exhibited maximum CMCase and β-glucosidase activity of 5.30 and 842.4 U/g substrate, respectively, in MSM 1 (Table 2). The fungal isolate that exhibited the highest activity was selected for optimization of the SSF parameters for cellulase production. A contrasting fungal study revealed different enzyme activity results.

Fungal cellulase production through SSF has been reported previously by several researchers. Santa-Rosathe et al. [27] reported specific enzymatic activities of 24.170 U/mg for CMCase and 1.345 U/mg for β-glucosidase, both produced using CMC and cellobiose as substrates [27]. Mrudula and Murugammal [28] investigated cellulase production via SSF by *A. niger* and reported that the highest FPase and CMCase activities after 72 h of incubation were 8.89 and 3.56 U/g substrate, respectively. Darabzadeh et al. [29] reported the activities of cellulases from a new mutant strain of *T. reesei* using rice byproducts as the substrate via SSF. *T. reesei* exhibited the highest FPase activity at 1.317 U/gds with 74% moisture content after incubation for 4 days at 30 °C. Mohammadreza et al. [30] investigated the total and individual cellulase activities of *A. fumigatus* SK1 using empty fruit bunches of oil palms as substrates. The

Table 1
Dimensions of the clear zone representing cellulolytic activity of fungi isolated from a humus-rich soil sample.

Fungal isolates	Ratio of clear zone diameter and colony diameter (mm)
Ni1	$1.85^{b} \pm 0.11$
DE8	$1.66^{bc} \pm 0.25$
DE9	$1.46^{c} \pm 0.07$
IN3	$1.72^{b} \pm 0.11$
IN5	$2.21^{a} \pm 0.03$

[a,b,c] Mean values with different letters are significantly different at $p \leq 0.05$ based on the Duncan's multiple range test.

Table 2
Production of FPase, CMCase, and β-glucosidase (BG) by fungal isolates under solid-state fermentation in two types of minimal salt media (MSM 1 and MSM 2).

Isolate	Enzyme titer (U/g substrate)					
	MSM 1			MSM 2		
	FPase	CMCase	BG	FPase	CMCase	BG
NI1	$0.034^{c} \pm 0.0$	$2.25^{b} \pm 0.1$	$784.2^{b} \pm 48.7$	$0.058^{b} \pm 0.0$	$1.24^{c} \pm 0.0$	$751.36^{b} \pm 23.7$
DE8	$0.004^{d} \pm 0.00$	$0.40^{d} \pm 0.0$	$24.89^{d} \pm 1.9$	$0.013^{c} \pm 0.0$	$0.32^{d} \pm 0.0$	$229.64^{d} \pm 16.7$
DE9	$0.076^{b} \pm 0.0$	$0.32^{d} \pm 0.0$	$122.84^{c} \pm 11.2$	$0.012^{c} \pm 0.0$	$0.28^{d} \pm 0.0$	$413.60^{c} \pm 21.3$
NI3	$0.013^{d} \pm 0.0$	$1.84^{c} \pm 0.0$	$24.51^{d} \pm 2.4$	$0.011^{c} \pm 0.0$	$1.56^{b} \pm 0.2$	$15.34^{e} \pm 1.7$
IN5	$0.188^{a} \pm 0.0$	$5.30^{a} \pm 0.6$	$842.4^{a} \pm 26.3$	$0.164^{a} \pm 0.0$	$4.41^{a} \pm 0.3$	$831.46^{a} \pm 33.4$

[a,b,c,d] Mean values with different letters in the same column are significantly different at $p < 0.05$ based on the Duncan's multiple range test.

maximum FPase, CMCase, and β-glucosidase activities were 1.608, 21.1729, and 22.216 U/g, respectively.

2.2. Identification of IN5 isolate using morphological characterization and molecular methods

Isolate IN5 was identified based on its morphological characteristics and the nuclear ribosomal internal transcribed spacer (ITS) sequence. The morphological features of the isolate were similar to those of *Aspergillus* sp. After 7 days of incubation on potato dextrose agar (PDA), IN5 produced creamy white substrate mycelia with black conidial areas (Fig. 1A). Scanning electron microscopy (SEM) analysis revealed that *Aspergillus* sp. IN5 formed conidia with spiny spore chains. The spores had a width of approximately 3 μm. (Fig. 1B). Therefore, this isolate was identified as a member of the genus *Aspergillus*. To confirm the identity of the isolate, we amplified the ITS region from its DNA using ITS5 and ITS4 primers and sequenced the amplicon. BLAST analysis of the ITS rDNA sequence of isolate IN5 revealed similarity with *A. piperis* CBS 112811 (99.83%), *A. tubingensis* NRRL 4875 (99.83%), and *A. tubingensis* CBS 134.48 (99.83%). Phylogenetic analysis was performed to determine the taxonomic position of isolate IN5 and to compare its ITS rDNA sequence with that of other species of *Aspergillus*. A phylogenetic tree constructed using the neighbor-joining method revealed that isolate IN5 belonged to the subclade *A. piperis* (Fig. 2). Notably, the phylogenetic analysis revealed that isolate IN5 was closely related to *A. piperis* CBS 112811 (99.83% similarity). Based on morphological and phylogenetic analyses, IN5 was closely related to *A. piperis* and its rDNA sequence was deposited in the GenBank database under accession number OR352510. In the molecular phylogeny of the *Aspergillus* group, 27 species were encompassed within section Nigri, which was further divided into seven clades: *A. tubingensis*, *A. niger*, *A. brasiliensis*, *A. carbonarius*, *A. heteromorphus*, *A. homomorphus*, and *A. aculeatus*. *A. piperis* is classified within the section Nigri, specifically within the *A. tubingensis* clade. Additionally, other species such as *A. costaricaensis*, *A. luchuensis*, *A. neoniger*, *A. tubingensis*, *A. eucalypticola*, and *A. vadensis* are also part of this section [31,32].

2.3. Effect of carbon source

Carbon sources are not only a source of energy for bacteria but also a crucial inducer of cellulase production [33]. In the present study, the effects of different carbon sources on the enzymatic activity of this strain were investigated. *Aspergillus* sp. IN5 was grown in MSM 1 containing urea (0.03%), KH_2PO_4 (0.2%), $(NH_4)_2SO_4$ (0.14%), $MgSO_4$ (0.03%), $CaCl_2$ (0.04%), $FeSO_4$ (0.0005%), $MnSO_4$ (0.00016%), $ZnSO_4$ (0.00014%), $CoCl_2$ (0.0002%), and yeast extract (2.5%) supplemented with combinations of different carbon sources, such as soybean residue, liquid hot water (LHW)-pretreated sugarcane bagasse, and alkali-pretreated sugarcane bagasse (each with 5% w/v concentration), to evaluate their effect on cellulase production. The highest cellulase production was observed when

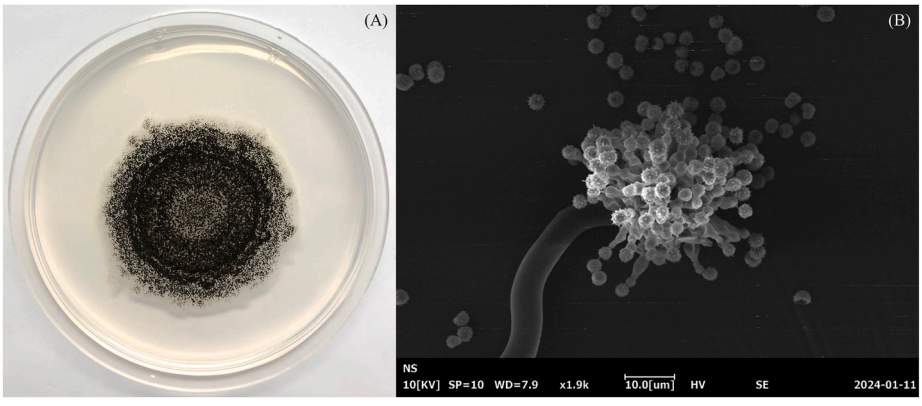

Fig. 1. Morphological features of fungal isolate IN5 cultured on a PDA plate (A) and as observed under a scanning electron microscope at a magnification of 1900× (B).

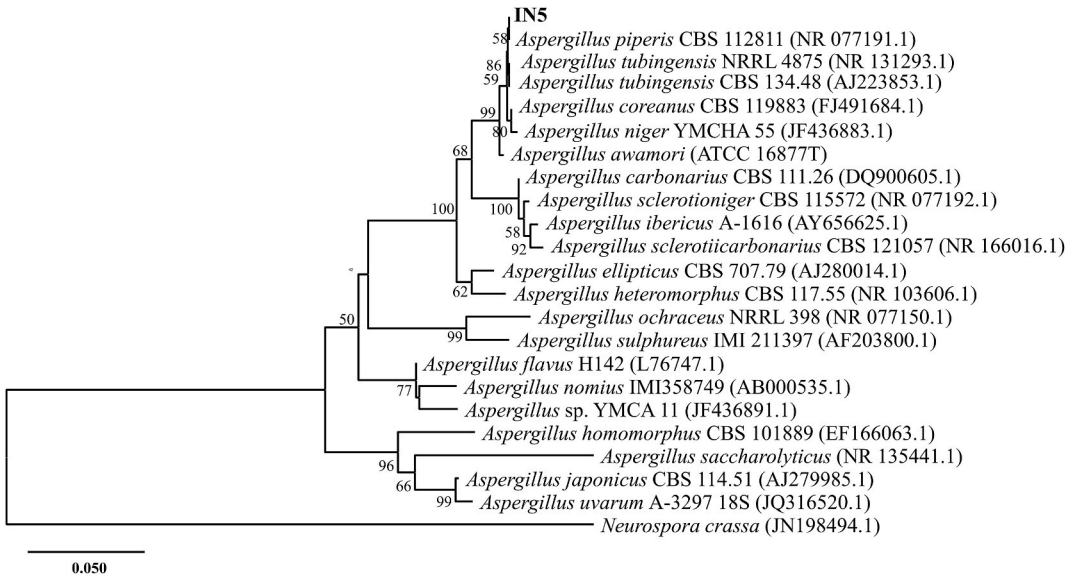

Fig. 2. Neighbor-joining phylogenetic tree generated using maximum likelihood analysis based on ITS rDNA sequences of isolate IN5 and related *Aspergillus* species. Bootstrap values ≥ 50% (1000 replicates) are shown at each branch. *Neurospora crassa* was used as the outgroup.

soybean residue was used as the carbon source after 7 days of incubation. The maximum FPase (0.25 ± 0.01 U/g substrate), CMCase (2.91 ± 0.03 U/g substrate), and β-glucosidase (183.43 ± 1.99 U/g substrate) activities were observed in MSM 1 supplemented with soybean residue as the carbon source. (Table 3). Ellilä et al. [34] reported similar cellulase production using soybean hulls as the substrate. Soybean hulls, as a substrate, can produce 23.5 g/L cellulase, which is similar to cellulase production by *T. reesei* in an optimized inducer medium (26.6 g/L) [34]. Salihu et al. [35] reported that maximum cellulase production by *A. niger* was observed when alkali-treated soybean hull was used as the substrate. Sethi et al. [36] reported the highest cellulase production using glucose as the carbon source. In this study, the highest cellulose production was achieved using soybean residue as the substrate. Soybean residue is rich in proteins, carbohydrates, and various minerals and sugars, including monosaccharides, oligosaccharides, and polysaccharides such as arabinose, glucose, galactose, fructose, stachyose, raffinose, sucrose, and starch [37]. Therefore, the sugar present in soybean residue may serve as a promising carbon source for cellulase production by *Aspergillus* sp. IN5.

2.4. Effect of temperature

We investigated the effect of different temperatures (30, 35, and 40 °C) on the production of cellulase by isolate IN5 in MSM 1 supplemented with soybean residue. Isolate IN5 exhibited maximum FPase (0.26 ± 0.01 U/g substrate) and CMCase (0.64 ± 0.40 U/g substrate) production at 35 °C after 7 days of fermentation (Table 3), whereas the highest β-glucosidase production (334.44 ± 16.24 U/g substrate) was observed at 40 °C (Table 3). Our results were consistent with those of Sohail et al. [38], who reported 35 °C as the

Table 3
Effect of each parameter on cellulase activity.

Substrate	FPase (U/g substrate)	CMCase (U/g substrate)	β-Glucosidase (U/g substrate)
Soybean meal	$0.25^{a} \pm 0.01$	$2.91^{a} \pm 0.03$	$183.44^{a} \pm 1.99$
LHW-pretreated bagasse	$0.04^{b} \pm 0.00$	$0.21^{b} \pm 0.02$	$3.25^{c} \pm 0.4$
Alkali-pretreated bagasse	$0.02^{b} \pm 0.00$	$0.02^{c} \pm 0.02$	$2.59^{b} \pm 0.3$
Temperature (°C)			
30	$0.10^{c} \pm 0.00$	$2.98^{c} \pm 0.20$	$144.12^{b} \pm 3.62$
35	$0.26^{a} \pm 0.01$	$3.64^{a} \pm 0.40$	$166.30^{b} \pm 3.92$
40	$0.18^{b} \pm 0.00$	$3.23^{b} \pm 0.10$	$334.44^{a} \pm 16.24$
pH			
5	$0.22^{b} \pm 0.00$	$3.64^{ab} \pm 0.30$	$176.94^{ns} \pm 7.72$
6	$0.23^{b} \pm 0.00$	$3.80^{a} \pm 0.30$	$172.66^{ns} \pm 11.26$
7	$0.24^{a} \pm 0.00$	$3.53^{b} \pm 0.50$	$166.90^{ns} \pm 15.32$
Incubation time (d)			
3	$0.19^{c} \pm 0.00$	$1.45^{b} \pm 0.50$	$157.40^{b} \pm 2.96$
5	$0.25^{a} \pm 0.01$	$3.32^{a} \pm 0.30$	$196.09^{a} \pm 11.81$
7	$0.22^{b} \pm 0.00$	$3.40^{a} \pm 0.40$	$154.87^{b} \pm 16.87$

[a,b,c]Mean values with the different letters in the same column for each parameter are significantly different at $p \leq 0.05$.
[ns]In the same column for each parameter indicates that the value was not significant at $p > 0.05$.

optimum temperature for cellulase production by *A. terreus* MS105. Further, Darabzadeh et al. [29] reported the minimum cellulase yield by *T. reesei* at 20 °C and 40 °C and the maximum yield at 30 °C. Temperature plays a crucial role in the growth, development, and metabolic activities of organisms. At temperatures higher than the optimal temperature, enzyme activity decreases due to thermal denaturation of the enzyme, changes in the composition of membranes, and inhibition of fungal growth [39]. However, at lower temperatures, the substrate cannot pass through the cells, resulting in reduced cellulase production [40].

2.5. Effect of pH

We investigated the effect of different pH values (5.0, 6.0, and 7.0) on cellulase production by isolate IN5 after 7 days of incubation at 35 °C in MSM 1 supplemented with soybean residue (Table 3). The optimum pH for cellulase production was 7.0, with the highest FPase activity (0.24 ± 0.00 U/g substrate), whereas 6.0 was the optimum pH for CMCase activity (3.83.80 ± 0.30 U/g substrate), which was not significantly different from that at pH 5.0. Maximum β-glucosidase activity (176.94 ± 7.72 U/g substrate) was observed at pH 5.0, which was not significantly different from that at pH 6.0–7.0. Similar results were reported by Sakthi et al. [41], Dutt and Kumar [40], and Mai et al. [42], demonstrating the optimal pH of 5.0–7.0 for cellulase production by *Aspergillus niger*. The suitable pH value for other cellulolytic organisms varies depending on the acidic conditions, as reported by Akiba al [43]. A pH of 3.5 is most suitable for cellulase production by *T. reesei* [44]. Although *T. reesei* exhibited higher cellulase production at pH 7 [45], a pH range of 5–6 provides optimal cellulase activity [46]. Altering the pH of the medium affects the functionality of cellulase because bacteria require an acidic pH for growth and cellulase production [47].

2.6. Effect of incubation time

We determined the effect of incubation time on cellulase production by culturing isolate IN5 in MSM 1 supplemented with soybean residue under optimized pH and temperature conditions for different durations (3, 5, and 7 days). We observed that incubation time affected cellulase activity. After 3 days of incubation, FPase, CMCase, and β-glucosidase activities were 0.19 ± 0.00, 1.45 ± 0.50, and 157.40 ± 2.96 U/g substrate, respectively. These values increased to 0.25 ± 0.02, 3.32 ± 0.30, and 196.09 ± 11.81 U/g substrate for FPase, CMCase, and β-glucosidase, respectively, after 5 days of incubation, which was the optimum time for cellulase production (Table 3). After 5 days of fermentation, a significant decrease in cellulase activity (FPase and β-glucosidase) was observed, which could be because of nutrient deficiency or the accumulation of other byproducts in the fermentation medium. However, we did not observe a significant change in CMCase activity after 7 days of incubation. Therefore, to make the process more economical and efficient, an incubation period of 5 days was selected as the optimal time. Dutt and Kumar [40] reported the optimal incubation time for maximum cellulase production by *A. niger* and *A. flavus* to be 5 days. The cellulase activity of both strains decreased after 5 days of incubation, whereas the protein biomass increased until day 6 and then remained constant. However, these results do not agree with the findings of Duenas et al. [48] and Khan et al. [49], who reported the highest cellulase production after 4 days of incubation. Fadel [50] reported maximum cellulase production after 3 days of incubation. In this study, the highest level of cellulase production was observed after 5 days of incubation, which may be due to the cessation of growth and release of cellulase into the medium during the later growth phase of *Aspergillus* sp. IN5. The decreased enzyme activity observed after prolonged incubation may have resulted from irreversible absorption onto the substrate, feedback inhibition, or degradation of the enzyme. This was attributed to changes in pH and cellular metabolism during fermentation [51]. However, after 3 days of fermentation, maximum activity was achieved because of nutrient consumption and the production of other components in the fermentation medium [52]. Therefore, enzyme production depends on biomass during the exponential phase of growth. As cellulase is a primary metabolite, it is produced during the exponential growth phase, and its production begins to decrease as cells enter the death phase [40].

2.7. Application of fungal cellulase

In the present study, cellulases produced using soybean residues were further investigated for the enzymatic hydrolysis of lignocellulosic biomass to produce sugars using rice straw as a model substrate. Table 4 presents the reducing sugar yields from feedstock resources with different enzyme loadings. Pretreatment affected the yield of reducing sugars from rice straw. Alkali treatment of rice

Table 4
Reducing sugar yield obtained from enzymatic hydrolysis of pretreated-rice straw with different enzyme loadings.

Substrate	Fungal cellulase loading (U/g substrate)	Reducing sugar yield (mg/g substrate)		
		0	12	24
Alkali-pretreated rice straw	5	0	51.70 ± 5.18	186.71 ± 6.38
	10	0	71.96 ± 4.25	249.75 ± 14.20
	15	0	100.54 ± 5.21	286.06 ± 2.77
	25	0	36.05 ± 1.11	177.33 ± 3.87
Acid-pretreated rice straw	5	0	33.52 ± 4.48	64.10 ± 9.91
	10	0	57.95 ± 0.47	87.47 ± 13.30
	15	0	59.97 ± 6.94	108.64 ± 1.98
	25	0	27.59 ± 2.76	92.21 ± 2.50

straw resulted in a better yield of reducing sugars than acid treatment. The maximum yield of reducing sugar (286.06 ± 2.77 mg/g substrate) was obtained from the alkali-pretreated rice straw with an enzyme loading of 15 U/g substrate after 24 h of incubation. Although acid and alkali treatments can eliminate lignin from biomass to obtain purer cellulose, acid treatment results in the hydrolysis and removal of hemicellulose sugars. Conversely, alkali treatment preserves the hemicellulose polymers [53]. As crude enzyme extracts are used for hydrolysis, hemicellulase may be present in the extract, resulting in a higher yield of reductive sugars, as observed in the hydrolysis of hemicellulose and cellulose [54]. This was in accordance with the results obtained by Jankovičová et al. [55], who investigated a pretreatment method promoting the degradability of lignocellulosic biomass for biogas production. Effects of acid and alkali pretreatments were investigated on the lignocellulosic composition of maize waste (maize stalks, leaves, and cobs), rapeseed straw, and wheat straw. NaOH pretreatment increased biogas production by 159%, 240%, and 59% from rapeseed straw, wheat straw, and maize waste, respectively, and increased the degree of solubilization. Similarly, Sukumaran et al. [54] reported fermentable sugar production from water hyacinth and rice straw after pretreatment with alkalis or acids. Notably, the highest yield of fermentable sugars was obtained from alkali pretreatment of both types of biomass.

This study demonstrates that cellulase produced by filamentous fungi through SSF can efficiently degrade biomass and can be used for the conversion of different types of biomass as substrates. The amount of fungal cellulase obtained in this study was lower than that reported in other studies. However, fungal enzymes have relatively low production costs. Therefore, upscaling enzyme production and hydrolysis should be further investigated to design processes that are technically and economically feasible for industrial applications.

Furthermore, the pretreatment method influences the efficiency of enzymatic hydrolysis. This study showed that alkali pretreatment resulted in higher fermentable sugar production during the enzymatic degradation of rice straw. However, the appropriate pretreatment process depends on the type and composition of the raw material.

3. Conclusions

We employed a novel fungal isolate, *Aspergillus* sp. IN5, which was found to be a highly efficient cellulase producer. We optimized various parameters, including carbon source, incubation temperature, pH, and incubation time, to maximize cellulase production. After optimization, the best settings were determined to be soybean residue as the substrate, an incubation temperature of 35 °C, a pH of 7.0, and an incubation duration of 5 days. These settings yielded the highest total cellulase activity of the 0.26 U/g substrate. In addition, carboxymethyl cellulase and β-glucosidase activities reached 3.32 and 196.09 U/g of substrate, respectively.

Fungal cellulases derived from *Aspergillus* sp. IN5 have practical applications in the enzymatic hydrolysis of acid- or alkali-pretreated rice straw and serve as a representative substrate model. Our results showed that diluted alkali pretreatment led to superior reducing sugar yields compared with acid pretreatment. The highest reducing sugar yield of 286.06 ± 2.77 mg/g substrate was achieved after a 24-h incubation with diluted alkali-pretreated rice straw and an enzyme loading of 15 U/g substrate. This study introduces an alternative approach for utilizing agricultural waste and establishes an efficient strategy for cellulase production, thereby contributing to the degradation of lignocellulosic materials and presenting promising implications for sustainable agricultural feedstock management.

4. Materials and methods

4.1. Isolation of cellulase-producing fungi

Humus-rich surface soil samples were collected from Nakhon Ratchasima Province, Thailand, in clean plastic bags and stored in ice boxes during transportation. Fungi were isolated using the serial dilution method. Each soil sample (10 g) was suspended in sterile 0.85% normal saline and serially diluted. Appropriate dilutions were spread in triplicate on PDA plates and incubated at 30 °C for 3–5 days. The fungal isolates were selected based on distinct morphological features, subcultured using PDA slants, and maintained at 4 °C. They were inoculated on CMC agar medium, which had the following composition: 0.2 g/L $FeSO_4 \cdot H_2O$, 4 g/L K_2HPO_4, 0.2 g/L KCl, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L yeast extract, 5 g/L polypeptone, 10 g/L CMC, and 15 g/L agar. They were then incubated at 30 °C for 7 days. We selected fungal isolates exhibiting a clear zone around the colony after flooding with 1% Congo red for 20 min and destaining with a 1 M NaCl solution for 15–20 min [56]. The clear zone ratio was determined as the ratio of the diameter of the clear zone to that of the fungal colony [57].

4.2. SSF parameters

Cellulase-producing fungal isolates were selected based on the diameter of the cellulose hydrolysis zone around the colonies. Cellulase production was induced using SSF. The fungal isolates were cultured on PDA plates at 30 °C for 7 days. A suspension of 1×10^7 spores/mL was prepared in distilled water. Soybean residue stored at −4 °C was used as the substrate. The assay was conducted in plastic bags containing 5 g of dried soybean residue adjusted to 70% moisture (wet basis) with Mendel's and Weber's media to obtain MSM 1, comprising 0.03% urea, 0.2% KH_2PO_4, 0.14% $(NH_4)_2SO_4$, 0.03% $MgSO_4$, 0.04% $CaCl_2$, 0.0005% $FeSO_4$, 0.00016% $MnSO_4$, 0.00014% $ZnSO_4$, 0.0002% $CoCl_2$, and 2.5% yeast extract [58]. MSM 2 was prepared by supplementing MSM 1 with 1% CMC and 2 mL of Tween 80. The cultures were added as spore suspensions (10^7 spores/mL) at a loading of 1 mL/g of dry substrate and incubated for 5 days at 35 °C. At the end of the fermentation period, 30 mL of sodium citrate buffer (50 mM, pH 4.8) was added to the fermented substrate and mixed for 1 h on a rotary shaker. The suspension was filtered and centrifuged, and the supernatant was used for crude enzyme assays.

4.3. Morphological and molecular identification

The fungal isolate with the highest cellulase activity was identified based on colony morphology on PDA and microscopic features. The isolate was inoculated on a PDA plate and incubated at 28 °C for 7 days. Morphological characteristics were examined using a scanning electron microscope (JSM 5600 LV; JEOL, Tokyo, Japan). Identification was confirmed by sequencing the ITS region. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide method [59]. The partial sequence of the ITS region was amplified using fungus-specific primers (ITS5 and ITS4), and the PCR product was sequenced in both directions using an automated DNA sequencer (Macrogen Inc., Korea). The similarity of the ITS partial sequences of the isolate with those of the sequences in the GenBank database was determined using the contig assembly program, an accessory application of BioEdit software version 7.0.

4.4. Optimization of culture conditions for maximum cellulase production using the one factor-at-a-time method

In this study, media and culture conditions, including the substrate/carbon source, temperature, pH, and incubation time, were optimized for maximum cellulase production with SSF technology using a one-factor-at-a-time method at three levels [29]. Optimization of culture conditions is important for the improvement of fermentation technology to reduce the cost of enzymes.

Various carbon sources, such as soybean residue and sugarcane bagasse, subjected to LHW and alkali (NaOH) pretreatment were examined for their effects on cellulase production. In all experiments, we weighed 5 g of the substrate, added it to a plastic bag with 70% moisture, and incubated the bag at 35 °C for 168 h under static conditions.

To identify the optimum temperature, the SSF setup containing the optimum concentration of substrate was incubated at 30, 35, or 40 °C. To study the effect of pH on enzyme activity, the medium containing the optimum substrate concentration was adjusted to pH 5.0, 6.0, or 7.0, and incubated at the optimal temperature. To analyze the effect of incubation time, SSF was performed at the optimum substrate concentration, temperature, and pH. Samples were collected at 24, 72, 120, and 168 h, and enzyme activities were measured as described above.

4.5. Application of fungal cellulase on cellulose materials

Rice straw obtained from a local field in the Nakhon Sawan Province of Thailand was used as the substrate for enzymatic hydrolysis using the fungal cellulase obtained in this study. Rice straw was cut into small pieces (1–2 inches) and dried at 60 °C for 24 h. Sulfuric acid (1 N H_2SO4) and sodium hydroxide (1 N NaOH) were used for pretreatments. The straw (5 g) was mixed with the pretreatment liquid (100 mL) in a 500-mL Erlenmeyer flask and autoclaved at 121 °C for 15 min. After pretreatment, the solid phase was separated through filtration and washed several times with tap water until the pH reached 7. The neutralized solid was dried at 30 °C for 48 h and stored in plastic bags. Pretreated rice straw was subjected to enzymatic hydrolysis. The fungal cellulase produced by IN5 was used in this experiment. Each substrate (1 g) was added to a citric acid buffer (100 mL, 50 mM, pH 4.8) containing enzyme at different concentrations (5, 10, 15, and 25 U/g substrate). The mixture was incubated at 50 °C and 200 rpm for 48 h, and the samples were periodically collected to determine the concentration of reducing sugars using the dinitro salicylic acid (DNS) method [60].

4.6. Enzyme assay

The cellulase activity was measured using the filter paper method. We used a Whatman No. 1 filter paper strip (dimension: 1 × 6.0 cm, equivalent to 50 mg of the substrate) to perform the assay, according to the method reported by Ghose [38]. Sodium citrate (1.0 mL, 0.05 M, and 5.0 mL), filter paper strips, and crude enzymes (0.5 mL) were mixed and incubated at 50 °C for 1 h. The concentration of the released reducing sugars was measured by adding 3,5-dinitrosalicylic acid to the reaction mixture, which was boiled in a water bath for 15 min; glucose was used as the standard. Absorbance was measured at 540 nm using a spectrophotometer.

The endoglucanase or CMCase activity was measured using CMC as a specific substrate. The appropriately diluted crude enzyme (0.5 mL) was mixed with 1% CMC (5 mL) in sodium acetate buffer (0.05 M, pH 5.0) and incubated at 50 °C for 30 min. The amount of reducing sugars released was estimated using the DNS method [60].

β-Glucosidase activity was determined by measuring the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma). The crude enzyme (20 μL) was mixed with *p*NPG (180 μL, 5 mM) in sodium citrate buffer (50 mM, pH 7.0). After incubation at 50 °C for 10 min, the reaction was stopped by adding ice-cold Na_2CO_3 (100 μL, 0.5 M). The amount of *p*-nitrophenol was determined using a UV spectrophotometer at 405 nm [61].

In both the endoglucanase and exoglucanase assays, one unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmole of glucose in 1 min expressed as U/g substrate under the aforementioned assay conditions. One unit of β-glucosidase activity was defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol (*p*NP) per minute from the substrate.

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Data availability

The data generated and/or analyzed in the current study are available from the corresponding author, N.N., upon reasonable request.

Additional information

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CRediT authorship contribution statement

Antika Boondaeng: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Methodology. **Jureeporn Keabpimai:** Formal analysis. **Chanaporn Trakunjae:** Project administration. **Pilanee Vaithanomsat:** Project administration. **Preeyanuch Srichola:** Formal analysis. **Nanthavut Niyomvong:** Conceptualization, Methodology, Supervision, Writing – review & editing, Resources.

Declaration of competing interest

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

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