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Agricultural residues for production of cellulase from *Sporotrichum thermophile* LAR5 and its application for saccharification of rice straw

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Abstract
Cellulases have got numerous industrial applications including saccharification of lignocellulosic biomass for environmentally-friendly production of bioethanol-biofuel and other platform chemicals. In the current study, fungal isolate *Sporotrichum thermophile* LAR5 successfully utilized low-cost agricultural residues as the substrates and produced considerable titre of cellulase. Wheat bran supported maximum cellulase production (2000 IU/L) and was followed by maize bran (1800 IU/L) and rice husk (1600 IU/L). Cellulase production was enhanced substantially by peptone (7900 IU/L), mustard cake (7000 IU/L) and soybean meal (6000 IU/L) as compared to control (2000 IU/L); cotton cake and casein too supported higher enzyme production (3900 IU/L and 3800 IU/L, respectively) than control. Though optimum temperature for cellulase activity was 60-70^{o}C but significant activity was observed even at higher temperatures (80-90^{o}C). Cellulase showed through thermostability at 50-60^{o}C for 30 min but stability deceased as the time and temperature increased further. Cellulase showed optimum pH of 5 but sufficient activity was observed over broad range of acidic and alkaline pH (3-10). Cellulase was thoroughly stable at pH 3-6, however, towards higher alkaline pH stability gradually decreased. Furthermore, cellulase showed good saccharification ability on acid-pretreated rice straw, and could have potential for lignocellulosic biomass transformation.

Key-words: Sporotrichum thermophile LAR5, Cellulase production, Agricultural residues, Rice straw, Saccharification

1. Introduction

Biotechnological potential of cellulases in various industries such as food, feed, biofuel, brewery, textile, pulp and paper, waste management, pharmaceutical and agriculture, has been the driving force for intense research focus on cellulases for past several decades [1]. However, application of cellulases for hydrolysis of lignocellulosic biomass (LB) for production of renewable biofuel (bioethanol) has attracted major research focus in recent years in view of rapidly diminishing fossil fuel reserves–world's major source of energy supply [2], coupled with serious environmental concerns including greenhouse gas emissions, and rapid fluctuations in market prices of petroleum products [3,4]. Among several energy resources, bioethanol generated from lignocellulosic biomass (LB) has emerged as one of the strongest contender for renewable transport fuel resource [3,4]. Abundant quantum of LB generated from agricultural and forestry represents an inexpensive, and underutilized renewable feedstock.

Rice straw represents one of the major cereal crops residues. Asia only accounts for production of 667.6 million tons of rice straw annually representing about 91% of the total world production [5]. Rice straw just like any LB material is a complex polymer composed of cellulose, hemicellulose and lignin, but has got significant amount of silica. Structure and biochemical composition of rice straw may differ depending upon climate, soil type, genetic and environmental variability [5]. Cellulose in rice straw is crystalline and complexed with lignin which makes it highly resistant to enzymatic action. Cellulose is a major polysaccharide constituent of plant cell walls, accounting for up to 40% of plant biomass, and is the most abundant biopolymer on the earth. It is a β-1,4-linked linear polymer of 8000–12,000 glucose units [6].

Exploitation of LB for the production of energy, specialty chemicals, nutrient-rich poultry/animal feed, biofuel and industrially important enzymes, would have long term economic, environmental and strategic advantages [6,7]. However, there are several limitations for successful utilization of LB such as energy-intensive and cost-intensive severe pretreatments for LB, dearth of suitable enzyme system (cellulases and accessory enzymes) for efficient LB hydrolysis; most of the available cellulases lack process-suitable

characteristics, and are highly expensive [5-8]. Cellulase industry is struggling with high cellulase production cost due to the expensive substrates [8,9]. Enzymatic saccharification of cellulose requires cellulase enzyme system comprising of three types of enzymes; endo-1,4-β-glucanase (CMCase), cellobiohydrolase or exoglucanases (Avicelase), and β-glucosidase (cellobiase) which act synergistically in the hydrolysis of cellulose [7]. Though fungi and bacteria have been exploited for production of a wide variety of cellulases and hemicellulases but special emphasis has been placed on the use of fungi because of their capability to produce substantial amounts of cellulases and hemicellulases which are secreted into the medium for easy extraction and purification, and their ability to use low-cost agricultural residues as substrates for growth [10]. In addition, the fungal enzymes are often less complex than bacterial glycoside hydrolases and can therefore be more readily be cloned and produced via recombination in a rapidly growing bacterial host such as *E. coli*.

Considering importance of cellulases for wide range industrial applications, attempts have been made by several researchers to isolate microbial cellulases with desirable industrial applications viz. high specific activity, long shelf life, thermostability, pH stability etc. [7,11-12]. Furthermore, to reduce the cost of enzyme production low-cost agro-residues have been explored as carbon and nitrogen sources for microbial production of cellulases [7,8, 13-15]. Current study reports production of cellulase equipped with industrially suitable properties from *Sporotrichum thermophile* LAR5 by using agroresidues as substrates, and its application assessment for hydrolysis of rice straw.

2. Materials and methods

2.1 Cellulolytic fungal isolates, cellulase production and assay

Samples from diverse sources such as compost, agricultural waste, degrading woody material, sawdust etc. were collected and used for isolation of cellulolytic fungi. Samples were suspended in saline (0.85% NaCl, w/v) and suitable dilutions were spread-plated on potato-dextrose-agar (PDA) and plates were incubated at 45^{o}C. Fungal colonies which appeared after 3–5 days of incubation at 45^{o}C were transferred from PDA to carboxymethyl cellulose agar plates (CMC medium) containing (g/L) KH_2PO_4 5, $(NH_4)_2SO_4$ 5, $CaCl_2$ 8, $MgSO_4.7H_2O$ 0.6, $FeSO_4.7H_2O$ 0.05, $MnSO_4.H_2O$ 0.16, $ZnSO_4.H_2O$ 0.16, $CoCl_2.6H_2O$ 0.2, carboxymethyl cellulose 2, agar 20; Tween 80 0.2ml, pH 5-10, and plates were incubated at 45^{o}C for 3–5 days. Colonies developed were assayed for cellulolytic activity by flooding the colonies with Congo red stain (0.1%, w/v in distilled water). Plates were allowed to stand for 15 min and destained repeatedly with 1N NaCl solution for 5-10 min. The colonies showing clear hallow around them were earmarked as presumptive cellulase producers. The cellulolytic fungal colonies were purified by repeated streaking, and finely maintained at 4^{o}C on CMC agar slants.

The cellulolytic fungal isolates were examined for cellulase producing ability under submerged fermentation. Isolates were grown on CMC agar for 6 days, and two discs (6 mm, each) were cut out from fungal colony and inoculated into cellulase production medium (same composition as CMC medium but minus agar). Submerged fermentation was executed at 45^{o}C under shaking at 180 rpm (Innova, New Brunswick, USA). Samples withdrawn periodically at different time intervals were centrifuged at 10,000 g for 10 min at 4^{o}C (Eppendorf, 5804R), and supernatant was considered to be enzyme equivalent and assayed for cellulase activity.

Cellulase (CMCase) activity was assayed at 45^{o}C by using CMC as substrate (1% w/v, in acetate buffer 0.05M, pH 5.6). The reducing sugars were assayed spectrophotometrically (UV-VIS 1800, Shimadzu, Japan) by dintrosalicylic acid (DNSA) method using glucose as standard [16]. One unit of cellulase activity is defined as the amount of enzyme required to release 1 µmol of reducing sugar equivalent per min under assay conditions. Protein content was estimated by Bradford method [17]. Among the fungal isolates examined the one LAR5, identified as *Sporotrichum thermophile* [18] was the most promising with regard to cellulase yield and enzyme properties, and was selected for detailed study.

2.2 *Cellulase production using various as carbon and nitrogen sources*

To test the effect of different carbon sources on CMCase production, CMC of the production medium was replaced with either of the different agriculture based substrates (10 g/L) such as wheat bran, rice husk, wood waste, maize bran or saw dust. To examine the effect of different nitrogen sources on CMCase production, the production media containing wheat bran (20 g/L) as carbon source was used with either of the following nitrogen sources (5 g/L): mustard cake, cotton cake, peptone, casein, yeast extract or soymeal. Six-day old fungal culture grown on CMC agar was used to seed the media containing different carbon and nitrogen sources, and fermentation conducted at 45°C with shaking (180 rpm). Samples withdrawn periodically after different time intervals were assayed for cellulase activity.

2.3 Effect of initial medium pH on cellulase production

To examine the effect of initial medium pH on cellulase production, fermentation media containing wheat bran as carbon source (20g/L), and peptone (5g/L) as nitrogen source, adjusted at pH 4 to 10 with NaOH (0.1M) or HCl (0.1M), was seeded with fungal biomass grown on CMC agar for six days, and fermentation was conducted at 45^{o}C under shaking (180 rpm).

2.4 Concentration of cellulase from cultural broth

Submerged fermentation was run and crude enzyme obtained after centrifugation was subjected to ammonium sulfate precipitation (90% saturation). Precipitated enzyme preparation was subjected to dialysis against acetate buffer (0.05M, pH 5.6). Cellulase activity and protein assay was executed. Dialysed enzyme preparation was used for studying some of the enzyme properties.

2.5 Some properties of cellulase

Effect of temperature on cellulase activity was determined by executing assay at different temperatures ranging from 50 to 100°C. For investigating heat stability of cellulase, enzyme was pre-incubated at 50°C, 60°C and 70°C for 30, 60 and 90 min, and then residual activity was assayed at 40°C.

Effect of pH on CMCase activity was deduced by employing buffers of different pH (citrate buffer pH 3, 4, 5 and 6; phosphate buffer pH 7; tris buffer pH 8 and 9; glycine-NaOH buffer pH 10). For determining pH-stability, the enzyme (without substrate) was pre-incubated at different pH (3-10) using appropriate buffers for 45 min, and then assayed for residual activity.

2.6 Pretreatment and enzymatic hydrolysis of rice straw

Rice straw procured locally, was chopped (2-3 cm) and subjected to acid and alkali treatment. For acid pretreatment chopped and dried rice straw was suspended at 5%, w/v in different concentrations of sulfuric acid (1-7%, v/v) and subjected to heat treatment in autoclave (121°C, 15 min). Similarly, alkali treatment was given to the chopped/dried rice straw at 5% (w/v) at varying concentration of NaOH (1-5%, w/v) at 121°C for 15 min. The solids and liquid were separated by pressing through cheese cloth. Liquid fractions were examined for the amount of reducing sugars, and solids were washed repeatedly with distilled water to attain neutral pH, and then dried in hot air oven, and used for enzymatic saccharification with *Sporotrichum* sp. LAR5 cellulase.

One gram of dried solids obtained after acid or alkali pretreatments was suspended in 100 ml of acetate buffer (0.05M, pH 5.6) and to this crude concentrated *Sporotrichum* sp. LAR5 cellulase was added (1ml, 7.88 IU), and contents were incubated at 55°C for 12 h in a water bath. The sugar content in the extract was measured by DNSA method.

3. Results and discussion

3.1 Celluloytic fungal isolates

Among a total of 19 fungal isolates examined, the isolate LAR5 showed appreciable cellulase producing ability (Figure 1). Time-profile for cellulase production by isolate LAR5 showed that maximum enzyme production occurred after 168 h (4.9 IU/ml). Isolate LAR5 was studied morphologically and microscopically and identified as *Sporotrichum thermophile* and designated as *Sporotrichum thermophile* LAR5 [18]. Nature is considered as the richest and most diverse source of industrially important microorganisms. Cellulolytic bacteria and fungi have been isolated from diverse natural sources, and their enzymes have been characterized for industrially desirable attributes [8, 13-15]. *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 isolates from rotten oil palm fruit bunches produced maximum cellulase after 7 days [8]. However, *Trichoderma inhamatum* KSJ1 [13] and *Aspergillus flavus* [12] produced maximum cellulase after 5 days and 3 days of fermentation, respectively. Fermentation time for maximum enzyme production varies among different microorganisms and depends upon cultural/environmental conditions during fermentation and genetic make-up of the organism [19].

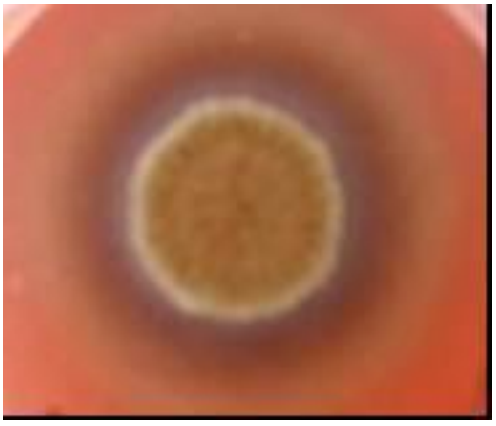


Figure 1: Cellulolytic activity of fungal isolate LAR5 on carboxymethyl cellulose (CMC) agar after congo red staining. Fungal colonies developed after 5 days of growth at 45°C were subjected to Congo red staining.

3.2 Effect of carbon source on cellulase production

Cost of the substrate constitutes one of the major factors for microbial production of industrial enzymes [19]. Exploitation of agroindustrial wastes as substrates may help reduce production-cost of microbial enzymes, and in addition assist valorization of agroresidues which are generated in abundance and are difficult to dispose-off [9, 20]. Among various agricultural residues examined in the current study, wheat bran supported highest cellulase production (2000 IU/L) from *S. thermophile* LAR5, and was followed by maize bran (1800 IU/L) and rice husk (1600 IU/L), as presented in Figure 2. Furthermore, it was observed that wheat bran at 2% (w/v) was the optimum for maximum cellulase production. Several cellulosic wastes have been explored as substrates for cellulase production. Gyalai-Korpos *et al.* [15] utilized detoxified liquid fraction from steam-exploded wheat straw as a carbon source for cellulase production by *Trichoderma reesei* RUT C30, and got enhanced cellulase yield. Similarly, Ibrahim *et al.* [8] utilized NaOH-pretreated oil palm empty fruit bunches as substrates for production of cellulase from *Trichoderma asperellum* UPM1 (FPase 0.8 U/ml, CMCase 24.7 U/ml) and *Aspergillus fumigatus* UPM2 (FPase 1.7 U/ml, CMCase 24.2 U/ml). Wheat bran (4%, w/v) was reported to be the best carbon source for cellulase production by *Aspergillus flavus* (CMCase 1.23 IU/ml) [12]. Pretreated sugarcane bagasse was used for production of cellulases (FPase 354 U/L and β-glucosidase 1,835 U/L) from *Penicillium funiculosum* [14]. Water hyacinth served as carbon source for cellulase production by *T. reesei* and a maximal cellulase activity of 0.22±0.04 IU/ml was obtained [21].

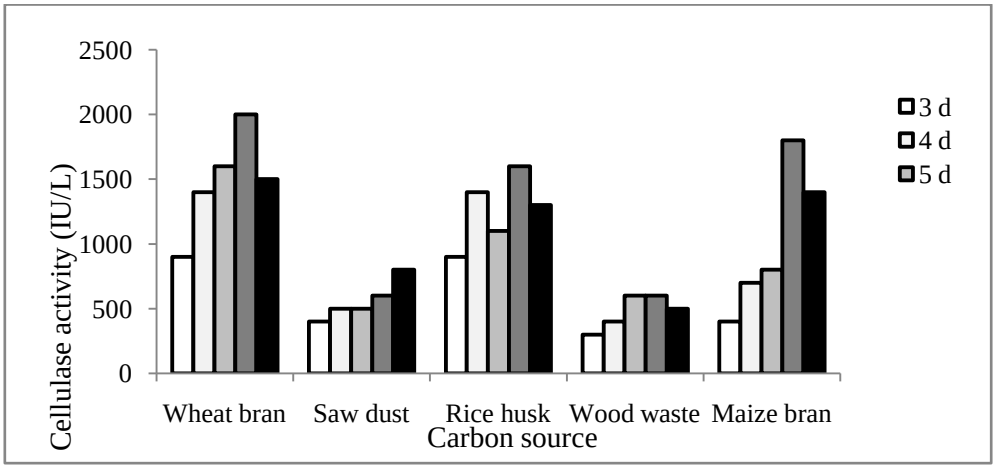


Figure 2: Cellulase production from *Sporotrichum thermophile* LAR5 using various agriculture based carbon sources. Carboxymethyl cellullose of the production medium was replaced with either of carbon source (10g/L), and fermentation was executed under shaking (180 rpm) at 45^{o}C.

3.3 Effect of nitrogen source on cellulase production

Nitrogen source may influence the enzyme production in several ways [10]. Replacement of inorganic nitrogen source (ammonium sulphate) of fermentation medium with either of the crude organic nitrogen sources like mustard cake, cotton cake, peptone, casein or soybean meal resulted in substantially enhanced enzyme yield. Maximum cellulase yield was supported by peptone (7900 IU/L vs 2000 IU/L in control) but comparable enzyme yield was obtained in media having mustard cake (7000 IU/L) or soybean (6000 IU/L) as nitrogen source (Figure 3). Cotton seed cake and casein also supported considerable enzyme yield that was higher than control. Yeast extract is generally recognized as good nitrogen source but proved to be poor for cellulase production from fungal isolate *S. thermophile* LAR5.

Requirement for specific nitrogen source for maximum enzyme production differs from organism to organism or even among the same species [22]. Peptone (1%) was found to be the best nitrogen source for cellulase production (1.634 IU/ml) by *A. flavus* [12]. Exploitation of soybean, waste yeast or Chunggookjang as the organic nitrogen source instead of peptone resulted in substantial cost-reduction (52.3%) for production of cellulolytic enzyme from *T. inhamatum* KSJ1 [13]. *T. reesei* Rut-C30 yielded higher cellulase when organic nitrogen source like soy peptone, glutamate, glycine and alanine were employed [23]. Peptone and ammonium salt enhance cellulolytic enzymes from some white-rot basidiomycetes but generally supplementation of media with additional nitrogen lower the fungi specific enzyme activities [9]. Casein hydrolysate enhanced production of FPase and β-glucosidase from mutant strain of *Hypocrea koningii* RSC1 while urea supported maximum CMCase production [5].

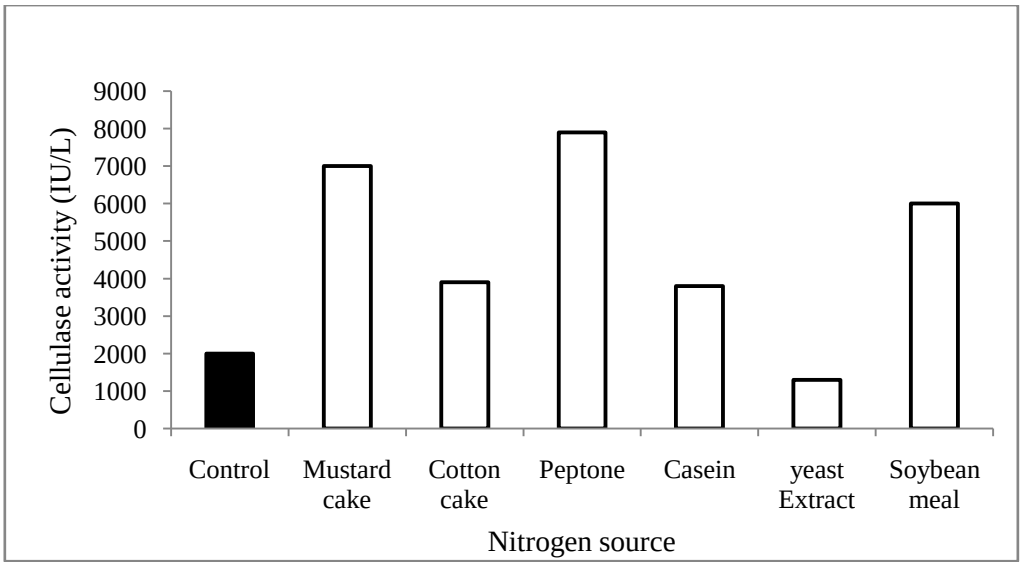


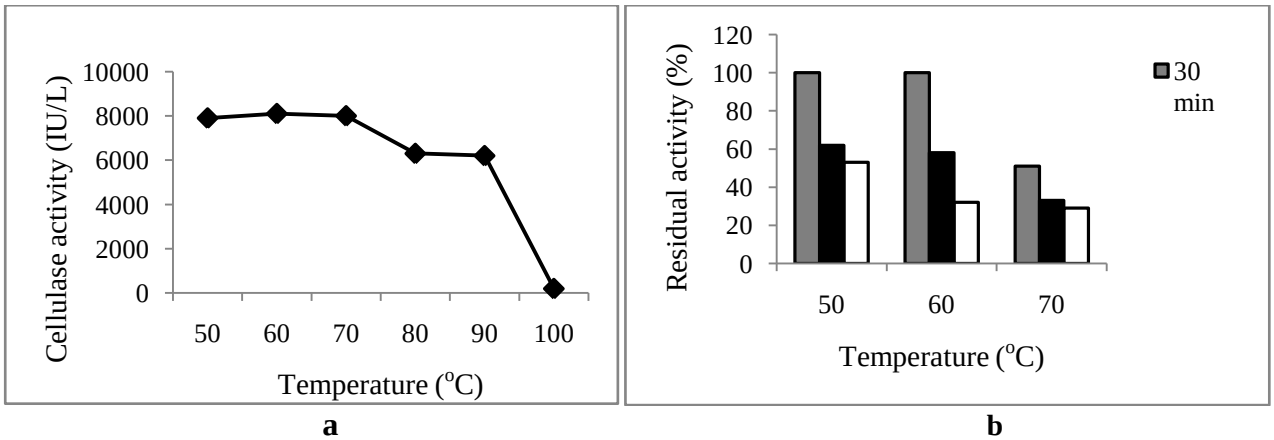
Figure 3: Cellulase production from *Sporotrichum thermophile* LAR5 using various agriculture based nitrogen sources. Ammonium sulphate of the production medium was replaced with either of nitrogen source (5g/L), and fermentation was executed under shaking (180 rpm) at 45^{o}C.

3.4 Effect of initial medium pH on cellulase production

Cellulase production was attempted from *S. thermophile* LAR5 in media with wheat bran as carbon source and peptone as nitrogen source, adjusted at different pH. It was observed that maximum cellulase production occurred at pH 3-5. Optimum medium pH of 4.5-5 has been reported for production of cellulase from various fungi [8]. Maximum cellulase production by *A. fumigatus* (0.292 IU/ml) and *A. niger* (0.262 IU/ml) was reported at pH 7 and pH 6, respectively [24]. However *H. koningii* RSC1 produced maximum cellulase at pH 5-7 [5].

3.5 *Effect of temperature on activity and stability of cellulase*

Crude cellulase preparation was concentrated by ammonium sulfate precipitation (90% saturation level) by 2.5-fold and used for studying some properties. Activity assay at different temperatures (50-100°C) showed that CMCase had activity over a broad range of temperatures (50-90°C) with maximum at 50-70°C (Figure 4a).



a **b**

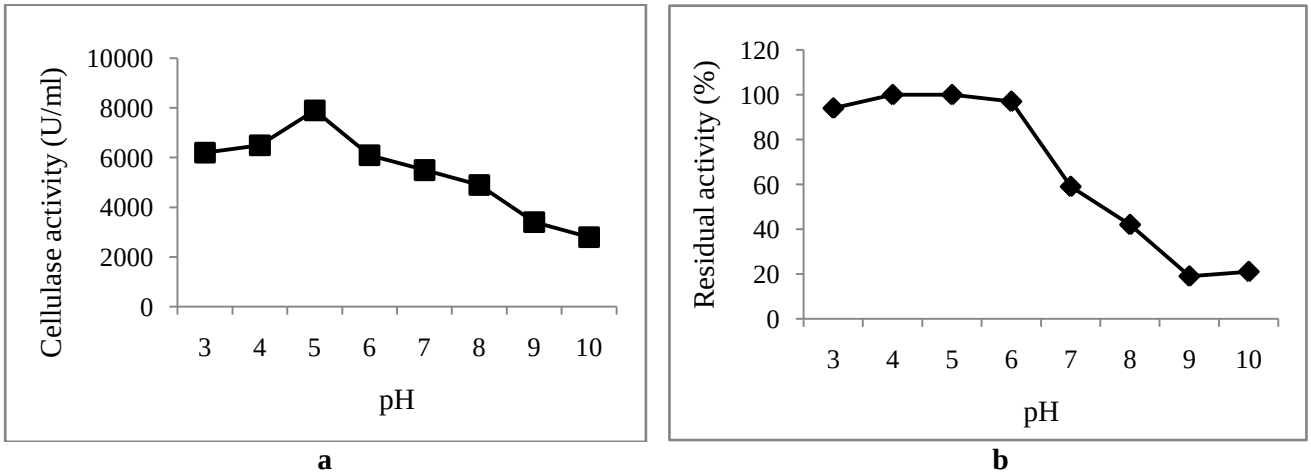
Figure 4: Effect of temperature (a) and thermostability (b) of *Sporotrichum thermophile* LAR5 cellulase. Assay was conducted at different temperatures (50-100°C) for elucidating effect of temperature, and heat stability was examined by pre-incubating the enzyme at different temperatures (50-70°C) for varying time periods (30-90 min), and then assaying the residual activity.

Though cellulase activity decreased at elevated temperatures but still substantial activity (78% of the maximum) was maintained at 80-90°C. Thermostability profile of cellulase showed that enzyme was thoroughly stable at 50-60^{o}C for 30 min (Figure 4b), however, stability decreased at elevated temperatures (60-70°C) when time of pre-incubation (60-90 min) was prolonged. Generally, fungal cellulases have the temperature optima and

thermostability at about 50°C [14, 24]. CMCase from *Sporotrichum* sp. HG-I had optimum at 70°C with considerable heat stability [25]. Recombinant *S. thermophile* cellulase showed optimum at 65°C, and fair stability at 50°C for 30 min [26]. In contrast, endoglucanase and β-glucosidase from *Aspergillus terreus* M11 had maximum activity at 70° C and maintained about 65% and 53% of their original activities for 6 h at 70° C [27]. Considering high temperature execution of most of the industrial processes it is highly desirable that cellulases intended for process applications must have high temperature optima and thermostability.

3.6 Effect of pH on cellulase activity and stability

Effect of pH on cellulase activity showed that enzyme expressed activity over a broad range of acidic (3-6) and alkaline pH (8-10). Optimum pH of cellulase was 5 but substantial activity was observed over highly acidic pH 3 (78%) and pH 4 (82%) as shown in Figure 5a. However, increase of pH above 5 lead to gradual reduction in the activity. Presence of moderate activity at highly alkaline pH (8-10) reflects potential industrial significance of enzyme. pH-stability analysis of cellulase for 45 min at different pH showed that cellulase was thoroughly stable at pH 3-6 (residual activity, 95-100%) , however, activity decreased considerably at pH 7-8 (59 and 42%, respectively), and at pH 9 only basal activity of 19-21% was observed (Figure 5b). Optimum pH of 4.5-7.5 was reported for maximum enzyme activity of *A. niger* and *A. fumigatus* cellulases [24]. CMCase from *Sporotrichum* sp. HG-I expressed maximum activity at pH 4.5-5.0 [25], however, *A. niger* cellulase had a broad range stability at pH 3-9 [28]. *S. thermophile* cellulase expressed in *Pichia pastoris* showed optimal activity at pH 8 [26], however, endoglucanase and β-glucosidase from *A. terreus* M11 exhibited maximum activity and stability in acidic pH of 2-5 [27].



a **b**

Figure 5: Effect of pH (a) and pH-stability (b) of cellulase from *Sporotrichum thermophile* LAR5. Assay was conducted at different pH (3-10) by employing apt buffers for determining effect of pH, and pH-stability was studied by pre-incubating the enzyme at different pH (3-10) for 45 min, and then assaying the residual activity.

3.7 Pretreatment of rice straw and saccharification by S. thermophile LAR5 cellulase

Pretreatment of rice straw biomass was attempted by employing sulphuric acid and sodium hydroxide. Following pretreatment liquid and solid fractions were separated and examined. Negligible sugar release was observed in the liquid fraction obtained on alkaline pretreatment (0.05 micromoles/ml) but considerable sugars were released in liquid fraction obtained after acid pretreatment (3.0-4.37 micromoles/ml). Furthermore, sugar-release was increased with increasing acid concentration. Reducing sugar yield in acid-treated liquid fractions was 3.1, 4.2, 4.3 and 4.37 micromoles/ml after pretreatment with 1%, 3%, 5% and 7% sulphuric acid, respectively. The solid fractions of acid and alkali pretreated rice straw biomasses were subjected to enzymatic saccharification using the *S. thermophile* LAR5 cellulase. Considerable sugars were produced by enzymatic hydrolysis of acid-pretreated solids (3.5, 5.7, 7.9, 7.7 micromoles/ml from 1, 3, 5 and 7% acid-pretreated solids, respectively) while negligible sugars were released from alkali pretreated solids (0.81 micromoles/ml).

Tsiegie *et al.* [4] reported that maximum saccharification of yeast *Yarrowia lipolytica* biomass occurred by pretreatment with 6% H_2SO_4 at 120°C for 1 h, and resulted in glucose yield of 35.89 g/L. Though acid-treatment results in substantial sugar-loss and production of inhibitors but nonetheless it is very effective for enzymatic saccharification. In contract to our results, recombinant JN11 cellulase from *T. reesei* caused higher

saccharification of NaOH-pretreated cellulosic biomasses, however, substantial saccharification also resulted with other pretreatments like sulfuric acid, hydrothermal and steam explosion [29]. Similarly, *H. koningii* RSC1 cellulases yielded higher reducing sugars (34g/L) from NaOH-treated rice straw than that treated with sulphuric acid [5]. Cellulases of *P. funiculosum* showed higher saccharification rate (470 mg/L/h) from corn cob when compared with commercial enzymes like Celluclast, GC 220 and Spezyme [14]. Recombinant *S. thermophile* cellulase showed potential to hydrolyze variety of cellulosic substrates with a peculiarity that presence of lignin in the various substrates enhanced the degree of saccharification [26].

Conclusion

Present investigation concludes that the fungal isolate *S. thermophile* LAR5 has excellent ability to utilize crude agriculture based materials as carbon and nitrogen sources to produce significant cellulase titre. Cellulase possessed desirable properties from industrial application view point such as activity and stability over broad pH range and high temperatures, and good saccharification ability on acid-pretreated rice straw. Further study on scale-up of enzyme production, and its purification and characterization must be accomplished to fully claim the potential of *S. thermophile* LAR5 cellulase for lignocellulose biotransformation for production of bioethanol-biofuel and other platform chemicals.

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