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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

Production, Partial Purification and Characterization of Enzyme Cocktail from *Trichoderma citrinoviride* AUKAR04 Through Solid-State Fermentation

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Abstract A strain of *Trichoderma citrinoviride* AUKAR04 was identified on the basis of morphological and 5.8S ribosomal RNA sequencing [GenBank: KF698728]. It produces cocktail of enzymes such as xylanase (55,000 IU gds^{-1}), CMCase (375 IU gds^{-1}) and β-1,3-glucanase (695 IU gds^{-1}) after 72 h under solid-state fermentation. These enzymes were partially purified by a three-phase partitioning method, which recovered the maximum activities of xylanase (99.8 %) with 5.7-fold, CMCase (96.5 %) with 5.5-fold and β-1,3-glucanase (98.4 %) with 5.6-fold purification. The maximum activity of xylanase was observed at pH 5.0, CMCase at pH 5.0–6.0 and β-1,3-glucanase at pH 6.0. Optimum temperature of xylanase and β-1,3-glucanase was found to be at 50 °C, while for CMCase was at 60 °C. The activities of these enzymes were enhanced by Mg^{2+} and Mn^{2+} ions. Eucalyptus pulp fiber was incubated for 14 h with the enzyme cocktail. Xylanase hydrolyzed the pulp to yield arabinose (475 mg L^{-1}) and xylose (1795 mg L^{-1}), CMCase and β-1,3-glucanase released glucose (18763 mg L^{-1}). The length of fiber was reduced from 0.881 to 0.056 mm. This is indicative of the potential application on bioconversion of lignocellulosic biomass into fermentable sugars by the enzyme cocktail produced from *T. citrinoviride* AUKAR04 for sustainable production of bioethanol.

Keywords Xylanase · CMCase · β-1,3-Glucanase · Solid-state fermentation · Three-phase partitioning · *Trichoderma* sp.

1 Introduction

The sustainable production of bioethanol from lignocelluloses has become an active area of research over recent years because of a number of environmental and economic benefits. The depletion of the oil supply, its fluctuating price and the negative impingement of fossil fuels on the environment predominantly greenhouse-gas emissions lead to find out alternative fuel resources. The most common renewable fuel today is ethanol produced from sugar or starch. On the other hand, this raw material base will not be fully sustainable and will lead to food crisis [1,2]. Lignocellulosic biopolymers extracted from wood and plants, typically cellulose, hemicelluloses and lignin, have been well recognized as the most abundant and potential alternative resources toward the production of bioethanol and other value-added products [3,4]. Lignocellulosic biomasses like agricultural residues, wood and wood wastes are composed of cellulose (45–56 %), hemicelluloses (10–25 %) and lignin (18–30 %) [5–7]. The commercialization of cellulosic ethanol can be enhanced by the production of cocktail biocatalysts able to operate in a single fermentation process, inducing after suitable pretreatment followed by enzymatic hydrolysis. There is a complete breakdown of fermentable sugars from xylan and cellulose and further conversion of both C5 and C6 sugars into ethanol. This is fully necessary for an economically viable process [8,9].

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Xylanase (EC 3.2.1.8) hydrolyzes xylan by cleaving the β-1,4-glycosidic linkages randomly to releases xylose and xylobiose. Cellulases are a multienzyme system that hydrolyzes cellulose. The enzymatic hydrolysis of cellulose to glucose requires synergistic action of cellobiohydrolase or exo-glucanase (EC 3.2.1.91), endoglucanase or carboxymethyl cellulase (EC 3.2.1.4) and cellobiase or β-glucosidase (EC 3.2.1.21) [10]. β-1,3-Glucanases consist of two enzymes, namely exo-glucanase (EC 3.2.1.58) and endo glucanase (EC 3.2.1.39), that act on (1, 3) and (1, 6) positions of β-D-glucan to release glucose [11].

Trichoderma sp. is considered to be a good producer of cellulase and xylanase enzymes in solid-state fermentation (SSF) [12]. Development of an economic process for the bulk production of enzyme cocktail comprising CMCase, xylanase and β-1,3-glucanase through submerged fermentation (SmF) is hindered because of the high costs of media ingredients, chemicals, accumulation of secondary metabolites, operation and labor cost, etc. To overcome these bottlenecks, SSF is an attractive process to produce mixture of enzymes economically, with low capital investment and low operating cost due to utilization of agro-industrial wastes for the production of enzymes in concentrated solutions with higher yield compared to SmF [13].

Several methods have been reported on the purification of enzymes such as ammonium sulfate precipitation, ion-exchange or affinity chromatography, gel permeation, aqueous two-phase partitioning, hydrophobic interaction chromatography (HIC) and preparative electrophoresis [14]. Most of the techniques involve a number of steps, and the scale-up of these methods is difficult and also expensive. In this regard, three-phase partitioning (TPP) is a simple and often used one-step procedure, for effective separation and purification of enzymes and proteins. This emerging method of purification has been reported for exo-polygalacturonase [15], beta-galactosidase [16], proteases [17], laccase [18], invertase [19] and xylanase [20].

The main aim of the present work is to discuss the significance of SSF, employing the single culture of *Trichoderma* sp. to produce celluloytic, xylanolytic and β-glucan hydrolyzing enzymes in a single fermentation step and the importance of non-chromatographic separation of xylanase, CMCase and β-1,3-glucanase by three-phase partitioning method.

2 Materials and Methods

2.1 Screening of Xylanase-Producing Strain

One gram of soil sample mixed with 100 mL sterile distilled water was spread onto potato dextrose agar (PDA) plate containing beech wood xylan (0.5 % w/v) [17]. These plates were incubated at 30 °C for 48–64 h. The fungal colonies from the plates were transferred onto the fresh agar plate, which were again incubated at 30 °C for 48 h, and colonies developed were assayed for xylanase production by Congo red (0.1 %) for 15 min and then washed with 1M NaCl. The colonies showing zone of clearance around them were picked up and maintained on potato dextrose agar slants at 4 °C in a refrigerator.

2.2 Microorganism and Phenotypic Characteristics

Prominent isolate was identified on the basis of morphological, cultural and 5.8S rRNA partial sequencing, and the sequence was submitted in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/KF698728). The strain was cultivated in potato dextrose agar (PDA) at 28 °C for 72 h and subcultured at monthly intervals.

2.3 Effect of Seed Media pH and Morphology/Growth Relationship with Xylanase, CMCase and β-1,3-Glucanase Production

Previous studies have reported the use of conidial suspension as inoculums for SSF [22]. But for industrial-scale production, the development of large volume of spores is a complicated process. However, the current study deals with the influence of seed media and its initial pH on production. The composition of the seed media is ($g L^{-1}$); wheat bran(fine)-25; lactose-10; soya flour-20; corn Steep Liquor-20; KH_2PO_4-1; NaCl-0.1; Tween 20-0.1. The initial pH was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 by using 0.1N HCl and 0.1M NaOH. Fifty milliliters of the above-mentioned seed media was inoculated with loopful of 48- to 72-h-old *Trichoderma* sp. spores and incubated at 30 °C for 36 h with agitation of 180 rpm. These mycelial cells were used as a source of inoculum throughout the study.

2.4 Cultivation in Solid-State Fermentation (SSF)

The cultivation of *Trichoderma* sp. in SSF system was carried out in a shallow aluminum tray of 30 cm × 23 cm × 6 cm. Five hundred grams of wheat bran was moistened with 500 mL of 50 mM sodium acetate buffer (pH 5.0) autoclaved at 121 °C for 45 min. After cooling the tray at room temperature, it was inoculated with 300 mL of seed media with 40–50 % of packed mycelium volume. The inoculum and the wheat bran were mixed well by using a sterile spatula in order to ensure a uniform distribution. The trays were incubated at 30 °C for 72 h. The moisture content in this study was 56 % [23]. Experiments were carried out in triplicate, and the obtained results were reported as mean of the triplicate experiments.

2.5 Enzyme Extraction

Five grams of the fermented solid substrate from each tray was transferred into a 250-mL Erlenmeyer flask and mixed with 40 mL of 0.1 % (v/v) Tween-80 in 50 mM sodium acetate buffer (pH 5.0). The samples were then mixed using a rotary shaker (150 rpm) at 25 °C for 1 h. The suspension was centrifuged, and the supernatant was passed through Whatman No. 1 filter paper and the clear cell-free filtrate was used as the enzyme source [24].

2.6 Assay Methods

Xylanase activity was routinely measured in a reaction mixture (1.0 mL) containing 0.5 mL of 1 % (w/v), beech wood xylan (Sigma, #X4252-25G) in 50 mM sodium acetate buffer (pH 5.0) and 0.5 mL of each enzyme solution. The substrate and enzyme solution were preincubated separately at 50 °C for 5 min, and then the reaction was started by mixing the enzyme with the substrate. After 1-min incubation, the reaction was stopped by the addition of 1 mL of 3,5-dinitrosalicylic (DNS) acid reagent. The reaction terminated immediately was used as control. The absorbance (OD) of the reducing sugar was measured at 540 nm. The standard graph was prepared using 1–5 μmol ml^{-1} xylose in 50 mM sodium acetate buffer. One unit (U) of xylanase activity is defined as the amount of enzymes required to release 1 μmol of xylose per minute in the reaction mixture under the specified assay conditions.

CMCase activity was determined by measuring the amount of glucose released from CMC-Na by DNS method [25] with glucose as the standard. The reaction mixture contained 0.5 mL of 1 % CMC-Na (Sigma #C5678) in 50 mM citrate buffer (pH 5.0) and 0.5 mL of enzyme solution and was incubated at 50 °C for 10 min. After incubation, the reactions were terminated by the addition of 1 mL DNS. They were kept in the boiling water bath for 10 min and cooled down at room temperature. The absorbance of the reaction solutions were measured at 540 nm. One unit (U) of CMCase activity is defined as the amount of enzymes that liberates 1 μmol of glucose equivalents per minute under the assay conditions.

β-1,3-Glucanase activity was measured by mixing 200 μL of culture filtrates with 200 μL of 50 mM sodium acetate buffer (pH 5.0), containing 0.8 % β-glucan from barley (Sigma, #G6513) [26]. The enzyme assay was carried out at 50 °C for 10 min. The reducing sugar liberated was quantified by DNS method [27]. One unit (U) of activity is the amount of enzyme required to release 1 μmol of reducing sugar per minute under the above conditions. All assays were performed in triplicate.

2.7 Three-Phase Partitioning (TPP)

2.7.1 Effect of Crude Extract to t-Butanol Ratio on Partitioning of Xylanase, CMCase and β-1,3-Glucanase

The crude extract was saturated with 55 % (w/v) ammonium sulfate [28], and the *t*-butanol concentration was varied based on volume per volume in the range of 1:0.5, 1:0.75, 1:1, 1:1.25 and 1:1.5 with the constant stirring at 25 °C for 1 h. The mixtures were allowed to stand for 30 min at 25 °C and then subjected to centrifugation at 4000 rpm for 10 min to facilitate separation into three distinct phases (upper organic phase, middle precipitate and lower aqueous phase). The two phases were carefully separated from each other, and the middle precipitate was collected and dissolved in the sodium acetate buffer (10 mM; pH 5.0) and dialyzed against distilled water for 15 h at 4 °C. The water for the dialysis was changed twice. The ratio which achieved the maximum recovery of enzymes was chosen for further purification procedures.

2.7.2 Effect of pH of Crude Ammonium Sulfate Saturation to t-Butanol Ratio on Partitioning of Xylanase, CMCase and β-1,3-Glucanase

The pH of ammonium sulfate (55 %) saturation was varied between pH 3.0 and 7.0 with 0.1N HCl and 0.1M NaOH, respectively. After that the *t*-butanol (1:0.5) was added and the mixtures were incubated at 25 °C for 1 h under constant stirring and set aside to stand for 30 min. The mixtures were centrifuged (4000 rpm for 10 min) to facilitate the separation of phases then the interfacial precipitates were collected, dissolved in sodium acetate buffer (pH 5.0; 10 mM) and dialyzed against distilled water overnight at 4 °C.

2.8 SDS-PAGE Profile of Xylanase, CMCase and β-1,3-Glucanase

The molecular weight of xylanase, CMCase and β-1,3-glucanase was determined by SDS-PAGE as described by Laemmli. A discontinuous system made of 5 % stacking gel and 12 % separating gel was used. The molecular weight of the enzyme was estimated using the low molecular weight calibration kit containing the standard protein marker: phosphorylase b (97.0 kDa), albumin (67 kDa), ovalbumin (45.0 kDa), carbonic amylase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). To the TPP purified enzymes, equal volume of sample solubilizing buffer (10 % glycerol, 0.05 % bromophenol blue, 5 % β-mercaptoethanol, 2 % SDS and 0. 25M Tris-HCl buffer; pH 6.8) was added and boiled at 100 °C for 2 min. Then the samples were loaded in SDS-PAGE (acrylamide concentration in stacking gel and

resolving gel was 5 and 12 %, respectively). Electrophoresis was carried out at 25 °C with 100 V for 2 h [29].

2.9 Characterization of TPP Purified Xylanase, CMCase and β-1,3-Glucanase

2.9.1 Effect of pH on the Activity of xylanase, CMCase and β-1,3-Glucanase

In order to determine the pH optima for the activity of TPP purified xylanase, CMCase and β-1,3-glucanase, the enzyme solution was incubated in different buffers (50 mM) ranging between 3.0 and 11.0 (sodium acetate buffer, pH 3.0–6.5; phosphate buffer, pH 6.5–7.5; Tris buffer pH 7.5–9.0; glycine-NaOH, pH 9.0–10.0 and carbonate buffer, pH 9.0–11.0) at 30 °C. The experiments were performed in triplicate.

2.9.2 Effect of Temperature on the Activity of Xylanase, CMCase and β-1,3-Glucanase

The experiment was carried out to investigate the effect of different incubation temperature (30−70 °C) on the activity of enzyme. The TPP purified enzyme solution was incubated at different temperatures for 10 min. The relative activities (%) were expressed as the ratio of the activity obtained at certain temperature of each enzyme, to the maximum activity at the given temperature range.

2.9.3 Effect of Metal Ions on the Activity of Xylanase, CMCase and β-1,3-Glucanase

The effect of metal ions on the activity of TPP purified xylanase, CMCase and β-1,3-glucanase was analyzed by incubation of the enzyme solution in the presence of particular metal ion solution (10 mM) at 30 °C for 30 min. The residual activities (%) of the appropriate enzyme were analyzed by mentioned standard assay methods.

2.9.4 Determination of Kinetic Parameters of Xylanase, CMCase and β-1,3-Glucanase

The kinetic parameters K_m and V_{max} of xylanase, CMCase and β-1,3-glucanase were determined by using various concentrations (2–20 mg mL^{-1}) of beech wood xylan, CMC-Na and β-glucan from barley, respectively, at pH 5.0 and 50 °C [30]. The experimental data were fitted in Lineweaver–Burk plot by using HYPER32 enzyme kinetics software.

2.10 Productivity, Fiber-Length Analysis of Eucalyptus Fiber and Quantification of Monomers by HPLC

Alkali pretreated bleached eucalyptus pulp was supplied by FIBRIA-EUCA-BRESIL contained approximately 80 % cellulose, 18 % hemicelluloses and 2 % lignin, and it was used as a raw material for testing the efficiency of partially purified enzyme cocktail (xylanase, CMCase, and β-1,3-glucanase) for production of sugars. The pulp was soaked for 24 h in deionized water and subjected to disintegration for 10 min. Enzymatic hydrolysis of pulp [3 % (w/v)] was done on the dry weight basis in a total volume of 100 mL in 250-mL Erlenmeyer flask. The slurry was buffered at pH 5.0 ± 0.5 with 50 mM sodium acetate buffer. Hydrolysis began with the addition of TPP purified enzyme cocktail, and saccharification was carried out at 50 °C with the agitation of 150 rpm in a temperature-controlled shaker water bath for 24 h. Samples were drawn at 2, 4, 6, 8, 10 and 24 h and analyzed for reducing sugar by DNS method [27]. The length of fiber was measured by optical microscopy and quantified each monomer quantified by high-performance liquid chromatography (HPLC). Sugars were measured by pulsed amperometric electrochemical detection on a Dionex ICS 5000 HPLC. The method used a flow rate of 1 mL min^{-1} and mobile phase of 2 mM potassium hydroxide (KOH) solution for the first 30-min stabilization followed by 30 min of same KOH solution. Samples were diluted as required and adjusted the pH from 8.0 to 9.0 and filtered through 0.45-μm syringe filters. Samples (25 μL) was injected onto the column, a Dionex Carbopac PA10 fitted with guard column. Samples were measured against standards consisting of arabinose, galactose, glucose, xylose and mannose.

3 Results and Discussion

3.1 Screening of Xylanase-Producing Strain

Out of the 19 fungal isolates, which were screened, the prominent higher zone of clearance producing isolate was confirmed as *Trichoderma citrinoviride* with the partial 5.8S rRNA sequencing having a length of 607-bp nucleotide. The sequence was deposited in GenBank (accession no. KF698728), and the blast search has shown 99 % homology with the *T. citrinoviride* strain H09-105 18S ribosomal RNA partial gene sequence; thus, it is designated as *T. citrinoviride* strain AUKAR04. The phylogenetic relation of this isolate is shown in Fig. 1.

3.2 Production of Xylanase, CMCase and β-1,3-Glucanase in SSF

Enzyme production from filamentous fungus in solid-state fermentation is also influenced by initial pH of seed media, growth and morphology. A filamentous fungus has the tendency to adhere to solid substrate surfaces, and the regulation of fungal physiology has not yet been thoroughly studied, especially when concerned with enzyme production [31]. In

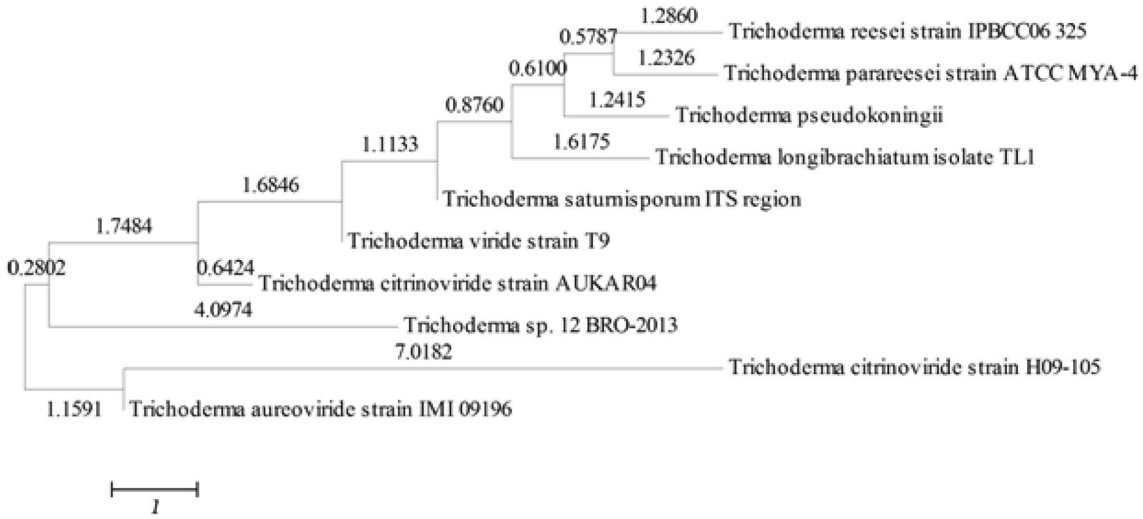

Fig. 1 Phylogenetic tree of *Trichoderma citrinoviride* strain AUKAR04 using neighbor-joining method (MEGA 6.0) based on the 5.8S rRNA sequence

Table 1 Effect of initial pH of seed media and the relationship with xylanase, CMCase and β-1,3-glucanase production

Initial pH of the seed media	Activity (U gds^{-1}[a])		
	Xylanase	CMCase	β-1,3-Glucanase
3 ± 0.1	9047 ± 15	204 ± 3	250 ± 4
4 ± 0.1	40,188 ± 16	287 ± 2	587 ± 3
5 ± 0.1	55,000 ± 20	385 ± 5	695 ± 5
6 ± 0.1	40,547 ± 19	375 ± 4	510 ± 4
7 ± 0.1	30,487 ± 20	150 ± 3	277 ± 3
8 ± 0.1	19,147 ± 18	89 ± 3	187 ± 2

[a] Units per gram dry substrate (wheat bran)

SSF, the production of enzymes is not only based on the parameters of temperature, moisture, size of the matrix, inoculum size, etc., but also correlated with the composition and initial pH of the seed media [5]. In order to evaluate the effect of initial pH of the seed media corresponding with the fungal morphology on the production of enzyme, the spore of *T. citrinoviride* strain AUKAR04 was inoculated in seed media with different pH ranging from 3.0 to 8.0. It was observed that the highest production of xylanase (55,000 IU gds^{-1}), CMCase (385 IU gds^{-1}) and β-1,3-glucanase (695 IU gds^{-1}) was obtained in SSF (Table 1) with the seed media which was developed in initial pH 5.0 ± 0.1 after 72-h incubation at 30 ± 1 °C.

3.3 Partial Purification of Xylanase, CMCase and β-1,3-Glucanase by Three-Phase Partitioning (TPP)

Protein purification by TPP is influenced by many factors such as ammonium sulfate concentration, *t*-butanol ratio, temperature and pH. An attempt using single-step partial purification of xylanase, CMCase and β-1,3-glucanase was made by TPP method. The effect of *t*-butanol ratio to crude ammonium sulfate saturation (55 %) was varied. In this regard, 1:0.5 ratio gave the highest xylanase yield (67.1 %) with 2.4-fold purification, CMCase yields (79.4 %) with 2.8-fold purification and β-1,3-glucanase yields (45.0 %) with 1.6-fold purification at the temperature of 25 °C. From this result, the 1:0.5 ratio of crude extracts to *t*-butanol was selected for investigating the importance of pH in TPP system. Protein concentration by salting out depends on the sulfate concentration and pH-dependent net charge of the proteins. The macromolecular contraction and conformational shrinkage are promoted by electrostatic forces and binding of sulfate ions to cationic protein molecules. Protein molecules have a tendency to precipitate near their *pI* (isoelectric point) [19]. Below its *pI*, proteins are positively charged and quantitatively precipitated out by TPP [32]. As a rule of thumb, the crude enzyme precipitate was adjusted to different pH. Among the various pHs (3.0–7.0) of TPP system, the maximum recovery of xylanase (99.8 %) with 5.7-fold, CMCase (96.5 %) with 5.5-fold and β-1,3-glucanase (98.4 %) with 5.6-fold purification was obtained in interface with the pH of 5.0 and 1:0.5 (crude extract to *t*-butanol) at 25 °C and the results are presented in Fig. 2 and Table 2.

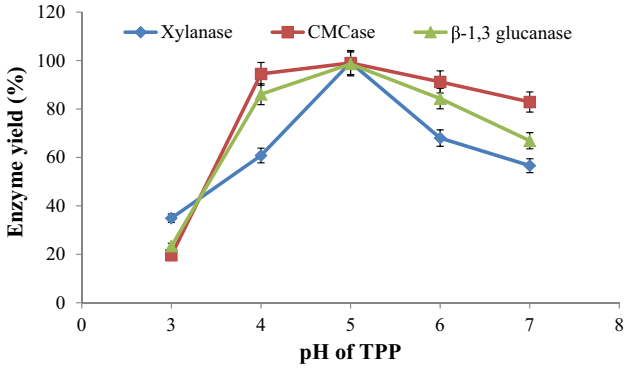

Fig. 2 Effect of pH of crude ammonium sulfate saturation to *t*-butanol ratio on partitioning of xylanase, CMCase and β-1,3-glucanase. The crude enzyme precipitate was adjusted to different pH from 3.0 to 7.0, and the ratio of crude ammonium sulfate saturation to *t*-butanol was 1:0.5

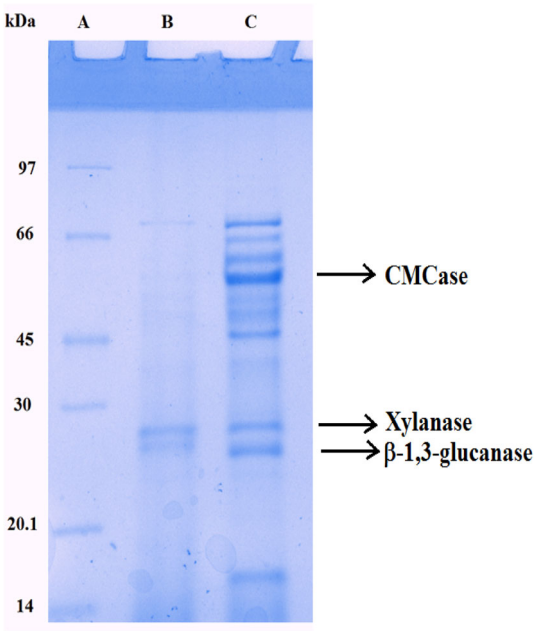

Fig. 3 SDS-PAGE analysis of TPP purified enzyme cocktail [*lane A* molecular weight marker (14–97 kDa) (20 μg), *lane B* TPP bottom phase and *lane C* TPP purified enzyme cocktail (middle phase)]

3.4 SDS-PAGE Profile of Xylanase, CMCase and β-1,3-Glucanase

SDS-PAGE analysis of the TPP purified enzymes (Fig. 3) showed that the molecular weight of xylanase (~29.8 kDa) was comparable with those reported by different organisms like *Bacillus* sp., and fungal genus is in the range of 22–45 kDa [33,34]. The molecular weight of CMCase is ~58 kDa [29] and β-1,3-glucanase ~27 kDa [35] as shown in lane C.

3.5 Characterization of Xylanase, CMCase and β-1,3-Glucanase

3.5.1 Effect of pH on the Activity of Xylanase, CMCase and β-1,3-Glucanase

The effect of pH for the TPP purified xylanase, CMCase and β-1,3-glucanase was investigated in the range of pH 3.0–10.0, and the results are shown in Fig. 4. The xylanase activity toward the beech wood xylan was maximum at pH 5.0 [36], and at least 85 % of the maximum activity was found from pH 4.0 to pH 6.0. CMCase hydrolyzed CMC in the pH range of 3.0–10.0 and exhibited 100 % activity at pH 5.0 and 6.0. The β-1,3-glucanase attained maximal activity in the pH range of 4.0–6.0, and the highest activity found at pH 6.0. Substantial activities of the appropriate enzymes were also exhibited on either side of this optimum point, which indicated that xylanase, CMCase and β-1,3-glucanase has the characteristic broad range of pH activity.

Table 2 Purification profile of xylanase, CMCase and β-1,3-glucanase

Steps	Total protein (mg)	Total activity ($U mL^{-1}$)		Specific activity ($U mg^{-1}$)	Yield (%)	Purification fold
Crude extract	9.2	Xylanase	35,014	2202	100	1
		CMCase	201	12	100	1
		β-1,3-Glucanase	513	32	100	1
TPP purified enzyme (pH not adjusted)	4.5	Xylanase	23,494	5221	67.1	2.4
		CMCase	159	35	79.4	2.8
		β-1,3-Glucanase	230	51	45.0	1.6
TPP purified enzyme (pH adjusted to 5.0)	1.6	Xylanase	34,944	12,480	99.8	5.7
		CMCase	194	69	96.5	5.5
		β-1,3-Glucanase	504	180	98.4	5.6

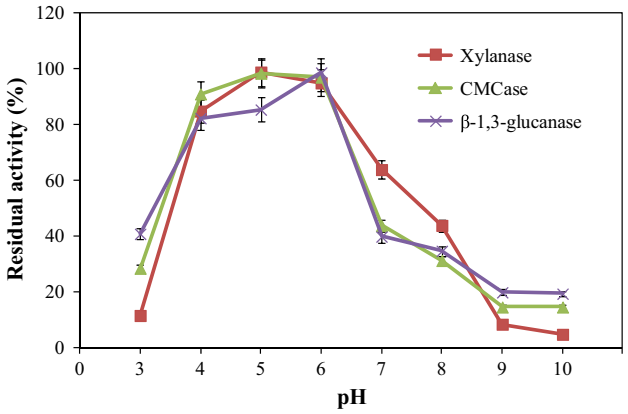

Fig. 4 Effect of pH on the activity of xylanase, CMCase and β-1,3-glucanase

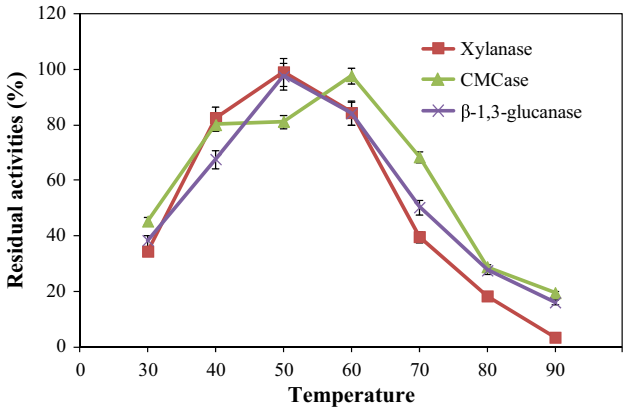

Fig. 5 Effect of temperature on the activity of xylanase, CMCase and β-1,3-glucanase

3.5.2 Effect of Temperature on the Activity of Xylanase, CMCase and β-1,3-Glucanase

Temperature is one of the crucial parameters to characterize the enzyme. The temperature optima of TPP purified xylanase, CMCase and β-1,3-glucanase were analyzed (Fig. 5). The optimum temperature for xylanase and β-1,3-glucanase was found to be 50 °C. The optimum temperature of xylanase from *Aspergillus fumigatus* MA28 exhibited at 50 °C [30], temperature optima of β-1,3-glucanase from *A. fumigates* was at 55 °C and 65 °C [37], whereas the maximum CMCase activity was observed at 60 °C. Sadhu et al. [25] reported that the optimum temperature of CMCase was at 50 °C. However, all the three enzymes showed 80 % activity from 40 to 60 °C which significantly declined at 90 °C.

3.5.3 Effect of Metal Ions and Metal Chelator on the Activity of Xylanase, CMCase and β-1,3-Glucanase

The effect of different metal ions and the metal chelator on the activity of TPP purified xylanase, CMCase and β-1,3-glucanase was measured at final concentration of 10 mM, with the results shown in Table 3. Xylanase activity was very much enhanced by Mg^{2+}, Mn^{2+} and Zn^{2+}, whereas Cu^{2+} and Fe^{3+} inhibited activity up to 60 %. CMCase activity was inhibited by Cu^{2+} and significantly increased up to 111.1 and 154.5 % by Mg^{2+} and Mn^{2+}, respectively, and similarly, Yin et al. [10] reported that CMCase from *Bacillus subtilis* YJ1 was greatly activated by Mg^{2+} and Mn^{2+}. β-1,3-Glucanase activity was strongly inhibited by Cu^{2+} and Zn^{2+} and raised up to 104 and 141 % by Mg^{2+} and Mn^{2+}, respectively. Complete inhibition of all the three-enzyme activity were observed in the presence of Hg^{2+}, and Sandrim et al. [14] reported similar effects of Hg^{2+} on the activity xylanase I from *A. caespitosus.* On the other hand, the addition of EDTA had no remarkable effect on the activity of all the three enzymes, indicating that metal ions were not present in the active sites of the respective enzymes.

3.5.4 Determination of Kinetic Parameters of Xylanase, CMCase and β-1,3-Glucanase

The kinetic parameters such as K_m and V_{max} of TPP partial purified xylanase, CMCase and β-1,3-glucanase were calculated from Lineweaver–Burk double reciprocal plots (Fig. 6) at 50 °C under appropriate enzyme standard assay methods, as mentioned in the experimental section. Xylanase had the lower K_m value of 0.713 mg mL^{-1} and highest V_{max} value of 24.28 mmol min^{-1} mL^{-1}. Similarly, the K_m value of CMCase and β-1,3-glucanase was found to be 0.811 and 1.31 mg mL^{-1}, respectively. The V_{max} values of CMCase and β-1,3-glucanase were found to be 630.9 and 329.6 μ mol min^{-1} mL^{-1}, respectively. In the literature, different ranges of K_m and V_{max} for different fungal and bacterial species have been reported. According to Sandrim et al. [14], K_m and V_{max} values of xylanase from *A. caespitosus* were 2.5 mg mL^{-1} and 1679 U, 11 mg^{-1} protein, respectively. Iqbal [29] reported that the K_m and V_{max} values of CMCase from *T. viride* were 68 μmol and 148 U mL^{-1}, respectively. K_m value of β-1,3-glucanase was 0.9 mg ml−1, and V_{max} of 0.11 U from *B. subtilis* NSRS 89-24 was reported by Leelasuphakul et al. [39].

3.6 Productivity, Fiber-Length Analysis of Eucalyptus Fiber and Quantification of Monomers by HPLC

The sugar productivity of eucalyptus pulp hydrolyzate by enzymatic treatment was carried out by HPLC method. Arabinose, glucose and xylose produced during hydrolysis were analyzed at different intervals of time (Fig. 7). The maximum concentration of arabinose (475 mg L^{-1}), glucose (18,763 mg L^{-1}) and xylose (1795 mg L^{-1}) was attained at 14 h, and prolonged hydrolysis beyond 14 h resulted in min-

Table 3 Effect of various metal ions and metal chelator on the activity of xylanase, CMCase and β-1,3-glucanase

Metal ions	Concentration (mM)	Relative activity (%)		
		Xylanase	CMCase	β-1,3-Glucanase
Control	(none)	100.0	100.0	100.0
Ca^{2+}	10	105.4	103.1	101.0
Cu^{2+}	10	46.2	56.7	33.0
EDTA	10	93.9	88.9	94.0
Fe^{3+}	10	41.6	88.2	106.0
Hg^{2+}	10	ND	ND	ND
K^{+}	10	97.7	97.5	93.0
Mg^{2+}	10	108.5	111.1	104.0
Mn^{2+}	10	126.9	154.5	141.0
Na^{+}	10	96.9	92.6	94.0
Zn^{2+}	10	112.7	82.7	33.0

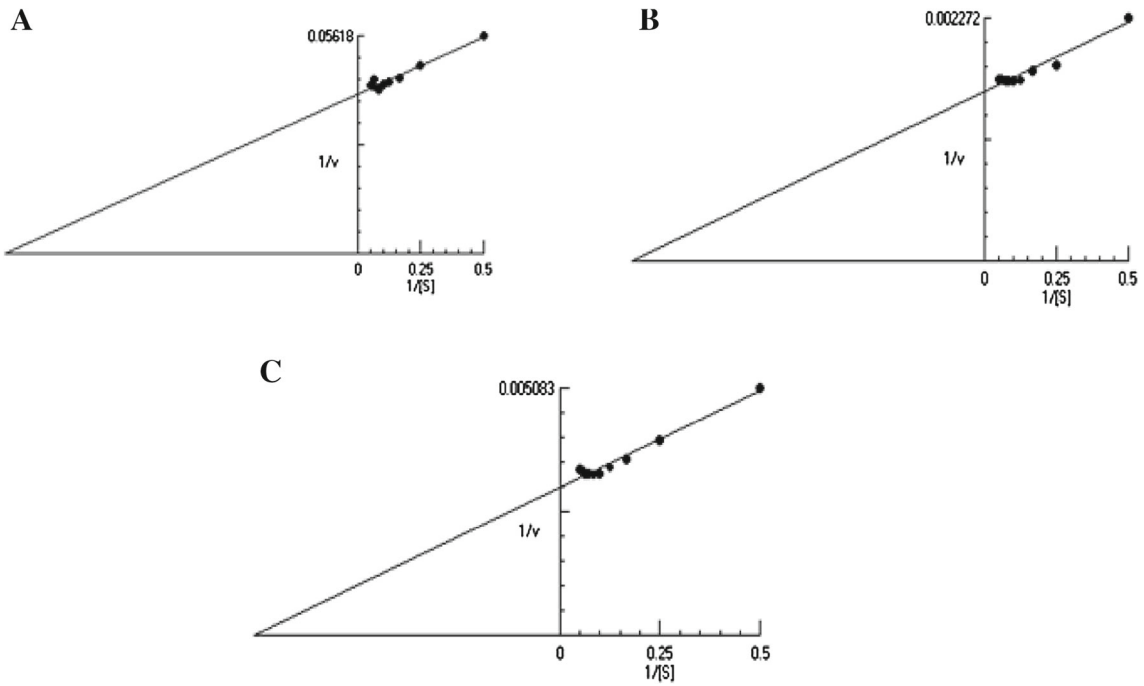

Fig. 6 Lineweaver–Burk double reciprocal plots for the determination of K_m and V_{max} value of TPP partially purified xylanase (**a**), CMCase (**b**) and β-1,3-glucanase (**c**) from *T. citrinoviride* strain AUKAR04

imal increase in the hydrolysis yield. The length of the fiber was observed and measured under optical microscope as shown in Fig 8. To obtain an average fiber size, the pulp was subjected to a refining mill process and passed through 14-mesh screen. The resulting substrate consisted predominantly of fibers between 0.5–1.0 mm in length. The length of fiber was about 0.881 mm at 0 h. After 20 h of enzymatic hydrolysis, the fiber length significantly reduced to about 0.05 mm as shown in Fig. 9. Simultaneously, the amount of reducing sugar released by the enzyme cocktail was also measured by DNS method and the results are also shown in Fig. 9.

4 Conclusion

The outstanding feature of *T. citrinoviride* strain AUKAR04 was that it produces a beneficial amount of extracellular enzymes like xylanase, CMCase and β-1,3-glucanase in SSF with wheat bran as sole carbon and nitrogen sources, and the non-chromatographic, cost-effective single-step purification

processes by TPP are the major advantages in the industrial sector, particularly in the perspective of efficient depolymerization of lignocelluloses to simple sugars to bioethanol production. These industries can reduce the import cost of the enzymes by producing in solid-state fermentation in large scale and make the whole process at competitive cost.

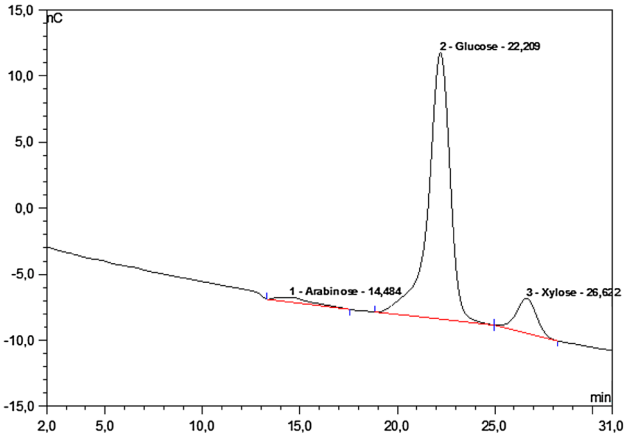

Fig. 7 Enzymatic hydrolysis and quantification of sugars by HPLC

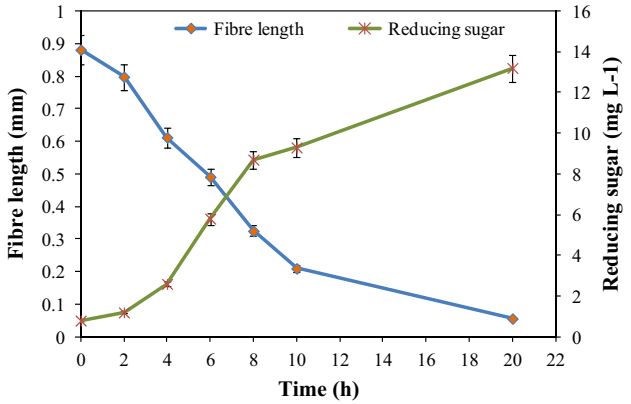

Fig. 9 Measurement of fiber length using optical microscope and reducing sugar analysis by DNS method at 0–20 h

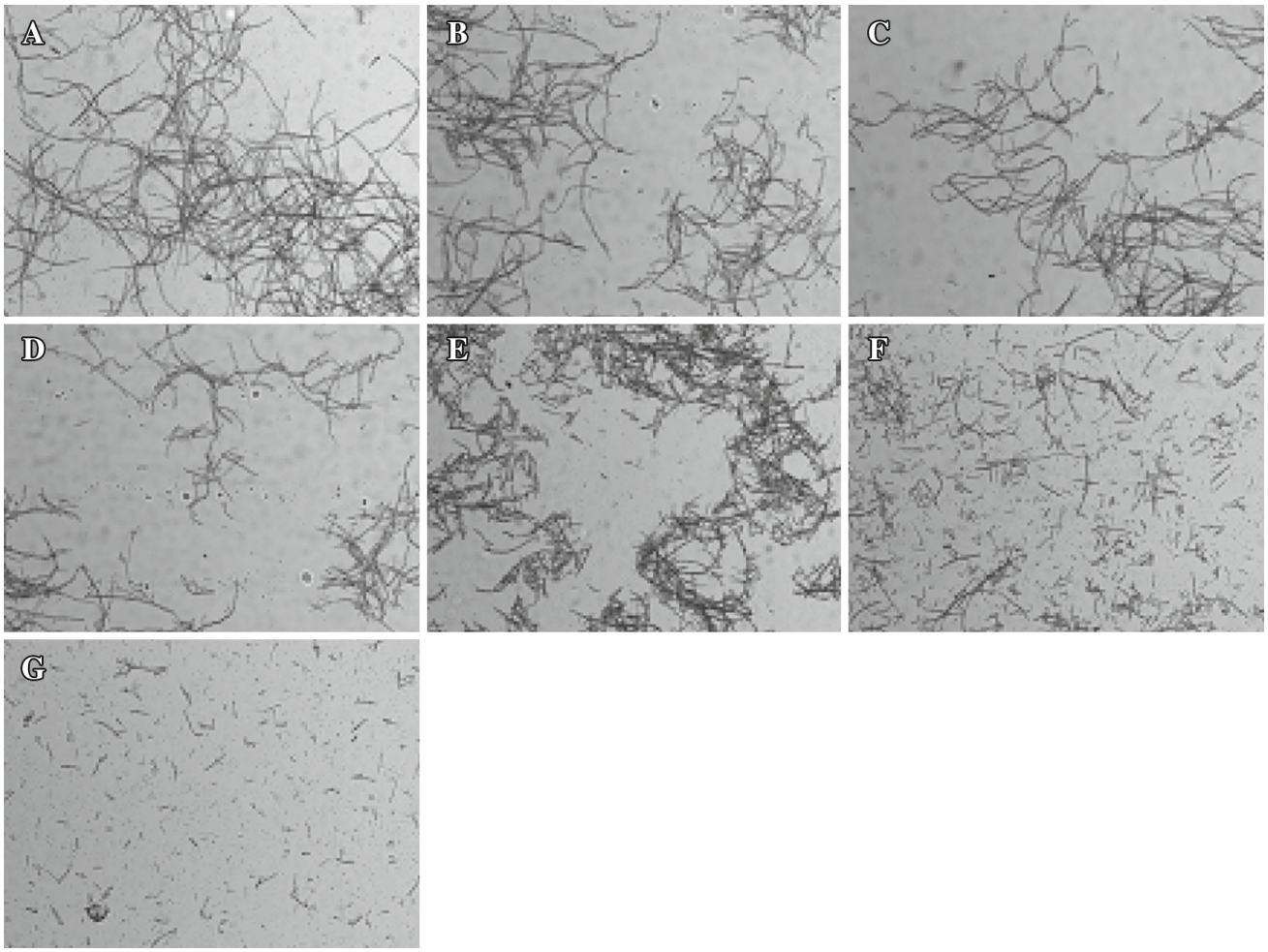

Fig. 8 Optical microscope image of enzymatic hydrolysis of fiber by xylanase, CMCase and β-1,3-glucanase produced by *T. citrinoviride* AUKAR04 at **a** 0 h, **b** 2 h, **c** 4 h, **d** 6 h, **e** 8 h, **f** 10 h, **g** 20 h

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