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Exo- and endoglucanase production by *Curvularia affinis* using bean (*Phaseolus vulgaris* L.) waste biomass

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Abstract

Background: In many countries, agricultural residues are generated in large quantities, and most of these are underutilized and considered waste, especially in developing countries.

Result: In this study, *Curvularia affinis* was isolated from the leaves of *Phaseolus vulgaris* L. beans and identified using 18S rRNA sequencing. *C. affinis* was tested for exo- and endoglucanase production using biomass of bean waste compared with the use of microcrystalline cellulose (MCC) and carboxymethylcellulose (CMC) as its growth substrates. *C. affinis* was better able to produce exo- and endoglucanase enzymes on bean waste biomass than on MCC and CMC. The highest activities of exo- and endoglucanase were detected with substrate concentrations 2% using MCC or CMC and with 4% using bean waste. The optimum incubation period for enzymes activity was 6 days with MCC or CMC (activity was 5.90 and 2.99 U/g of exoglucanase and endoglucanase, respectively) and 8 days with bean waste where activity was 3.64 U/g and 0.92 U/g of exoglucanase and endoglucanase, respectively. Exo- and endoglucanase production showed the highest activity at pH 5–6. In process wherein surfactant (Tween 80) was used, the exoglucanase activity gradually increased from 5.92 U/g to 6.20 U/g and then decreased to 5.70 U/g at 0.50% compared with that using the MCC substrate. The exoglucanase activity gradually increased from 3.80 U/g at 0.0% to 4.12 U/g at 0.20% and then decreased to 3.01 U/g at 0.50% Tween 80 using bean waste. Pretreated bean biomass also yielded higher enzyme production than the non-pretreated biomass. Alkaline-pretreated biomass showed the highest enzyme production compared with acid-treated residues, followed by the H_2O_2-treated ones.

Conclusion: The study concluded that *C. affinis* produce exo- and endoglucanase enzymes using cheap and abundant biomass of beans. Moreover, optimization of enzymes indicated that pretreatment of biomass bean biomass is a good choice process for enhanced enzymes productivity.

Keywords: Exoglucanase, Endoglucanase, *Curvularia affinis*, Bean, Waste biomass

Introduction

The global food, agricultural, and forestry industries produce great amounts of wastes annually, which lead to several environmental problems (Rodríguez-Couto 2008). Agricultural residues represent substantial raw materials that can be used to produce value-added products. The major constituents of agricultural residues include cellulose (35%–50%), hemicellulose (20%–35%), and lignin (15%–25%) as well as a number of other components comprising the residues (Wyman 1994; Keegstra 2010). Cellulose consists mainly of long polymers of 1-4, linked glucose units (Somerville 2006). Current academic and applied studies are devoting increasing efforts to minimizing the quantity of these wastes by finding alternative uses. Their composition rich in sugars, which is due to their organic nature, is easily assimilated by microorganisms (Rodríguez-couto 2008; Abdel-Ghany

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et al. 2018a). Diverse waste bioresources are available in our planet for conversion into bioproducts (Verma et al. 2011) such as pea peel (Verma et al. 2011) sugarcane bagasse (Rocha et al. 2014), barley straw (Abdelghany et al. 2018a). Bean (*Phaseolus vulgaris* L.) is the most important food legume worldwide, and its suitability as fungus immobilization carrier for solid-state fermentation has been assessed in a past study (Orzua et al. 2009). Tunali et al. (2007) reported that the waste biomass of bean (*P. vulgaris* L.) has a potential to be utilized as an alternative for the removal of acid red 57 dye from aqueous solutions because of its availability, low cost, and reasonable biosorption capacity.

Cellulase enzymes have been applied in a number of industrial processes, including textile, paper; and pulp manufacturing; food and animal feed production; biofuel and chemical processes; waste management; pharmaceutical processes; and pollution control (Bhat 2000; Acharya et al. 2008; Makky and Abdel-Ghany 2009; Mohajershojaei et al. 2013; Rocha et al. 2014). Cellulases consist of three major enzyme components, which are endo-β-(1-4)-D-glucanase or carboxymethylcellulases (CMCases), exo-β-(1-4)-D-glucanase, and β-glucosidase that work synergically in complex cellulose degradation (Saha 2004 and Kim et al. 2008). β-Glucosidases, also named β-D-glucosideglucohydrolase, EC 3.2.1.21), catalyze the hydrolysis of β-glucosidic linkages, such as alkyl and aryl β-glucosides, β-linked oligosaccharides, and several oligosaccharides with the release of glucose (Béguin 1990; Lin et al. 1999). β-Glucosidases are a prominent class of enzymes that catalyze cellulose degradation acting synergistically with cellobiohydrolase and endoglucanase (Henrissat et al. 1985).

Most microbial cellulases are inducible enzymes; similar to other extracellular enzymes, they are secreted when microorganisms are grown on cellulose or cellulosic components present in raw materials (Jecu 2000). A number of fungi and plant pathogens play an important role in various industrial applications (Abdel-Razek et al. 2009; Abdel-Ghany 2013; Abdelghany et al. 2014; Abdel-Ghany and Masmali 2016; Abdelghany et al. 2018b; Abdel-Ghany et al. 2019) and can produce multiple groups of enzymes, called cellulases, which hydrolyze the β-1,4-D-glycosidic bonds within agricultural wastes (Moreira et al. 2005; Chen et al. 2018; Abdel-Ghany and Bakri 2019). Numerous studies reported that fungal cellulase production is regulated in response to carbon sources and metal ions (Mandels and Reese 1957; Chen et al. 2016 and 2018). Recently, Chen et al. (2018) found that Mn^{2+} induced cellulase production, and upregulated the cellulase genes in *Trichoderma reesei* through calcium channels and calcium signaling.

Curvularia is a species-rich genus of pathogens and saprobes associated with human, plant and animals worldwide (Madrid et al. 2014, Marin-Felix et al. 2017). Leaf blights, seed and root rot, seedling blights, grain discolouration have been recorded due to *Curvularia* species (Iftikhar et al. 2003). Numerous species of *Curvularia*, viz., *C. eragrostidis*, *C. geniculata*, *C. intermedia*, *C. pallescens*, *C. verruculosa*, *C. lunata* and *C. penniseti* are known to be pathogenic to crops and grasses in many countries of the world (Wilson 2000). *C. affinis* was recorded as phytopathogen (Huang et al. 2004; Sharma et al. 2012). Besides phytopathogenesis of *Curvularia* species, lignocellulolytic enzyme activities of *C. affinis* was detected on agricultural waste such as wheat bran (Anasontzis et al. 2017). Recently, the ability of *Curvularia verruculosa* to secrete hydrolytic enzymes viz., pectinase, xylanase, protease, cellulase and lipase was detected (Shirsath et al. 2018). The objectives of our study were to estimate the potential of opportunistic fungus *C. affinis* isolated from the leaves of *P. vulgaris* L. beans to produce exo- and endoglucanase enzymes using bean waste biomass, and to determine the different environmental factors that affect its secretion.

Materials and methods

Agricultural waste biomass used for *C. affinis* exo- and endoglucanase production

The agricultural wastes used in this study were provided in the form of biomass comprising leaves and stems of *P. vulgaris* L. beans. These were cultivated in Monufia Governorate fields (30° 31′ 12″ N, 30° 59′ 24″ E) located in Egypt.

Fungal isolate used for exo- and endoglucanase production

For isolation of fungal pathogen, small pieces (2–5 mm^2) of infected leaves of *P. vulgaris* L. with rust and spot diseases (Fig. 1) were surface-sterilized with hydrogen peroxide (50%) solution. The sterilized pieces were aseptically transferred to sterile Petri dishes containing potato dextrose agar (PDA) medium (dextrose 20 g, potato starch 4 g, agar 15 g/ L distilled water. A portion of mycelium growing on the PDA medium after 5 days was transferred to the PDA for purification, identification and storage for further examination. The morphological identification of *C. affinis* was conducted by observing the colony color, conidia shape, and colony growth using PDA. Molecular identification was then performed by 18S rRNA sequencing. For DNA extraction, 0.2 g of fungal mycelia were collected and placed in liquid nitrogen for 10 min, then

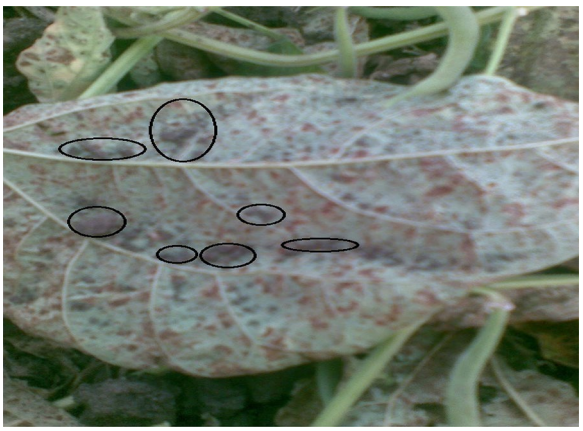

Fig. 1 *Curvularia affinis* spots inside black circle associated with rust spots (brown)

vigorously homogenized to extract its genomic DNA. DNA extract was mixed with 500 µL of DNA extraction buffer (200 mM Tris–HCl, 240 mM NaCl, 25 mM EDTA, and 1% SDS (pH 8.0), then shaken for 5 min and then centrifuged for 5 min at 10,000 rpm. Next, the supernatant was mixed with an equal volume of phenol: chloroform (1:1 v/v) for 30 min and again centrifuged for 5 min at 12,000 rpm, then the upper part was gently outgoing and mixed with an equal volume of 3 M sodium acetate buffer (pH 5.2) and 2 volumes of 96% ethanol for 1 h at − 20 °C. The collected DNA after centrifugation, was washed with 70% ethanol, followed by drying until removal of the ethanol, and resuspended in 100 µL of distilled water. PCR analysis was conducted according to Sambrook et al. (2001). Molecular Evolutionary Genetics Analysis software (Version 6; MEGA6) was used for the phylogenetic analyses (Tamura et al. 2013). DNA sequencing was performed by Macrogen Inc. (Seoul, South Korea). All intertranscribed spacer sequencing works were also completed by Macrogen on both strands of the submitted DNA fragments. The sequences were assembled, edited, and aligned using the DNA STAR SeqMan (DNASTAR Inc., Madison, Wisconsin USA) and the CLC sequence viewer. The *C. affinis* isolate was used for cellulase production assays.

Exo- and endoglucanase production by *C. affinis*

The submerged culture was used for the exo- and endoglucanase production by *Curvularia affinis,* using microcrystalline cellulose (MCC) and carboxymethylcellulose (CMC) as substrates and the agricultural waste of *P. vulgaris* L. bean for both enzymes at different incubation periods (2, 3, 4, 5, 6, 7, 8 and 9 days), and at different substrate concentrations of MCC, CMC, and bean substrates ranging from 1% to 5%.

Bean waste was washed in distilled water to remove any dust and then dried at 60 °C in an oven to obtain a constant weight. The dried waste was ground using a grinder (Molix, China) and sieved to select particle sizes of less than 0.001 mm. Spore suspension of *C. affinis* (5×10^6 spores/ml) was inoculated in sterile Czapek Dox broth containing MCC (2%) or CMC (2%) or bean waste (4%) instead of sucrose and then incubated in a rotary shaker at 120 rpm. After each incubation period, aliquots were centrifuged at 12,000 rpm to obtain the supernatant for the enzyme assay. The enzyme activity (unit) was measured as micromole sugar released per min. The exo- and endoglucanase activities were estimated using the MCC and CMC as substrates, respectively. The reaction mixtures contained 1 ml of culture supernatant having enzyme with 10 mg CMC or CMC suspended in 1 ml of 0.05 M sodium acetate buffer (pH 5.0). The mixtures were incubated for 30 min at 50 °C. For measuring the formed reducing sugar, 1 ml of dinitrosalicylic acid was added to 3 ml of the test sample, and the mixture was boiled in a water bath for 5 min. The developed color was measured at a wavelength of 540 nm using a spectrophotometer (Jenway Model 6300, EU). The reduction of sugar concentration was achieved via a standard glucose concentration curve (Zaldivar et al. 2001).

Effect of pH on exo- and endoglucanase production

In order to determine optimal pH, *C. affinis* was cultivated in a 250-ml conical flask containing 50 ml of optimized media with different pH ranging from 3 to 9. The pH of the media was adjusted by using buffers, including citrate, citrate phosphate, and Tris buffers with pH 3–6, 7, and 8–9, respectively.

Effect of polysorbate 80 on exo- and endoglucanase production

Polysorbate 80 (Tween 80) surfactant was added in concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mL/g of biomass. The same conditions for enzyme production (i.e., substrate concentration, incubation period of 8 days at 25 °C) were used, as described in the previous experimental procedures.

Pretreatment of bean substrate for exo- and endoglucanase production

The sun-dried bean substrate was ground to acquire its powdered form and then soaked in 1 N of H_2SO_4, NaOH, and H_2O_2 for the acid, alkali, and oxidative pretreatments, respectively, in the ratio of 1:10 (substrate: 1% solution)for 2 h at 25 °C. This procedure was performed

on the basis of a method described previously (Gharpuray et al. 1983; Singh and Bishnoi 2013) and with minimal modification. The treated biomass was filtered and washed repeatedly with distilled water to remove the solvents used for pretreatment and until the wash water became neutral. The resulting biomass was dried at 60 °C to a constant weight for enzyme production.

Statistical data analyses

The results are reported as mean ± standard error SE of three independent replicates. Statistical analyses of data were carried out by computer using SPSS ver. 22.0 software.

Results and discussion

Isolation and identification of the *C. affinis* from the infected *P. vulgaris* L.

The identification of the *C. affinis* fungus was conducted morphologically (Fig. 2a–c), and the macroscopic examination of the isolated fungus on PDA indicated it to be wooly, white to gray at first. At growth maturation, the colony became black and appeared black on the reverse side of the agar plate. Identification was confirmed by molecular characterization, which was based on ITS rDNA. The 18S rRNA sequence of the *C. affinis* isolate was searched on a database (Basic Local Alignment Search Tool) using multiple sequence alignment (Fig. 3) with MEGA6 software. From the alignment profile results, the 18S ribosomal RNA gene amplicon of *C. affinis* strain 18 closely matched that of other *C. affinis* isolates (at > 89%). This method of the molecular identification of fungi at the species level is primarily based on

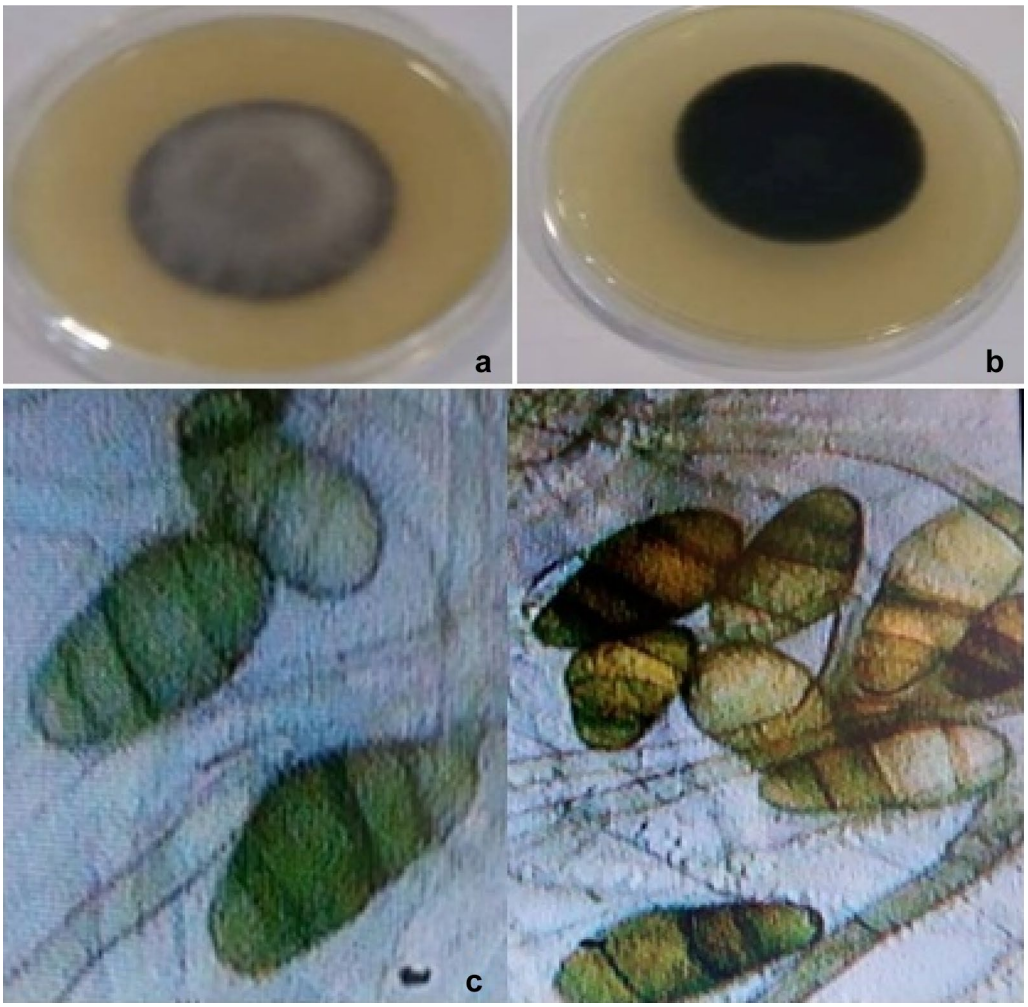

Fig. 2 Macroscopic (**a** colony color and **b** reverse side) and microscopic **c** diagnosis of *C. affinis*

Fig. 3 *Curvularia affinis* strain 18 18S ribosomal RNA gene and cluster analysis

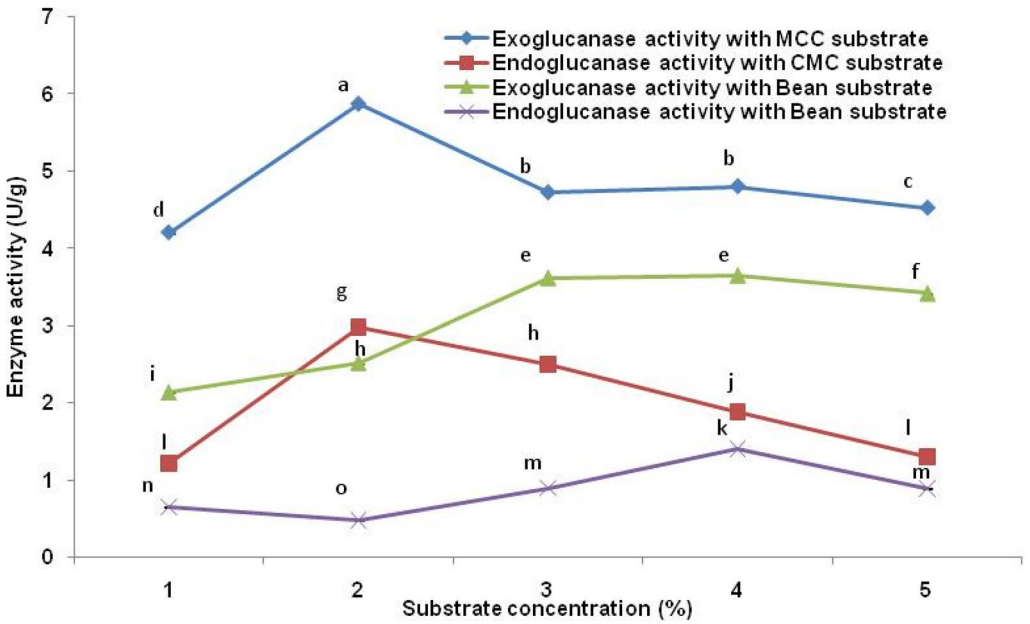

Fig. 4 Effect of different substrate concentrations (%) on exoglucanase and endoglucanase activities using MCC, CMC and bean substrates

the variable nature of the ITS regions of DNA (Romanelli et al. 2010; Delgado-Serrano et al. 2016).

C. affinis was isolated as opportunistic fungus from the infected *P. vulgaris* L. with rust diseases (Fig. 1). Some *Curvularia* species from different parts of the world have been reported as plant pathogens that cause leaf blight, sheath rot, black kernel, sheath blight, leaf spot, and grain discoloration of rice (Kamaluddeen et al. 2013; Krishnan et al. 2014; Madhusree and Surekha 2017). *Curvularia* spp., including *C. eragrostidis*, *C. geniculata*, *C. intermedia*, *C. inaequalis*, *C. lunata*, *C. pallescens*, *C. protuberata*, and *C. trifolii*, affects many species of grasses worldwide (Smith et al. 1989). Weng et al. (1997) found that the most frequent disease of warm season grasses in southern China was caused by *C. lunata*. Meanwhile, *C. affinis* has been found on *Festuca arundinacea* (Huang et al. 2004). Solehudin et al. (2012) also reported that *C. affinis* widely attacks oil palm leaves (*Elaeis guineensis* Jacq.).

Exo- and endoglucanase production from *C. affinis*

The current result showed that the *C. affinis* strain exhibited exo- and endoglucanase activities during the fermentation period in submerged cultures. The enzyme production was at the maximum level at 2% using MCC or CMC and at 4% concentrations of bean waste biomass (Fig. 4). However, the enzyme activity on bean waste biomass was lower than that produced in MCC or CMC at all substrate concentrations ranged from 1 to 5%. The results indicated that MCC or CMC easily produced carbon in enzyme induction. From the current study, *C. affinis* was recorded as plant pathogen; previously Lozovaya et al. (2007) reported that phytopathogenic fungi are able to produce laccase and lignin peroxidase required for lignin degradation in soybean. According to Male (1981), bean biomass was used as a cheap compost for mushroom cultivation and hydrolytic enzymes production (Male 1981). Several studies have shown that *Curvularia* spp. (Banerjee and Chakrabarti 1992; Okunowo et al. 2010) can produce cellulase, β-glucosidase, and xylanase enzymes in submerged cultures of lignocellulosic materials. Neoh et al. (2015) explored the capability of *C. clavata* to produce lignocellulolytic enzymes, such as carboxymethylcellulase, xylanase, manganese peroxidase, laccase, and lignin peroxidase from agro-industrial residues in the palm oil industry. With using bean waste, the enzymes activity was less than using MCC or CMC, which may be due to existence of inhibitors. This result is supported by the findings of Gbekeloluwa and Moo-Young (1991), who reported the inhibitory effects of accumulated cellobiose and cellodextrin with low degree of polymerization. Zhang et al. (2013) found that the activity of *Bursaphelenchus xylophilus* endoglucanase towards MCC and filter paper was less than towards oat gum.

Optimization of exo- and endoglucanase production

The current study focused on the optimization of enzyme production at different incubation periods in consideration of the myriad and continued demand for

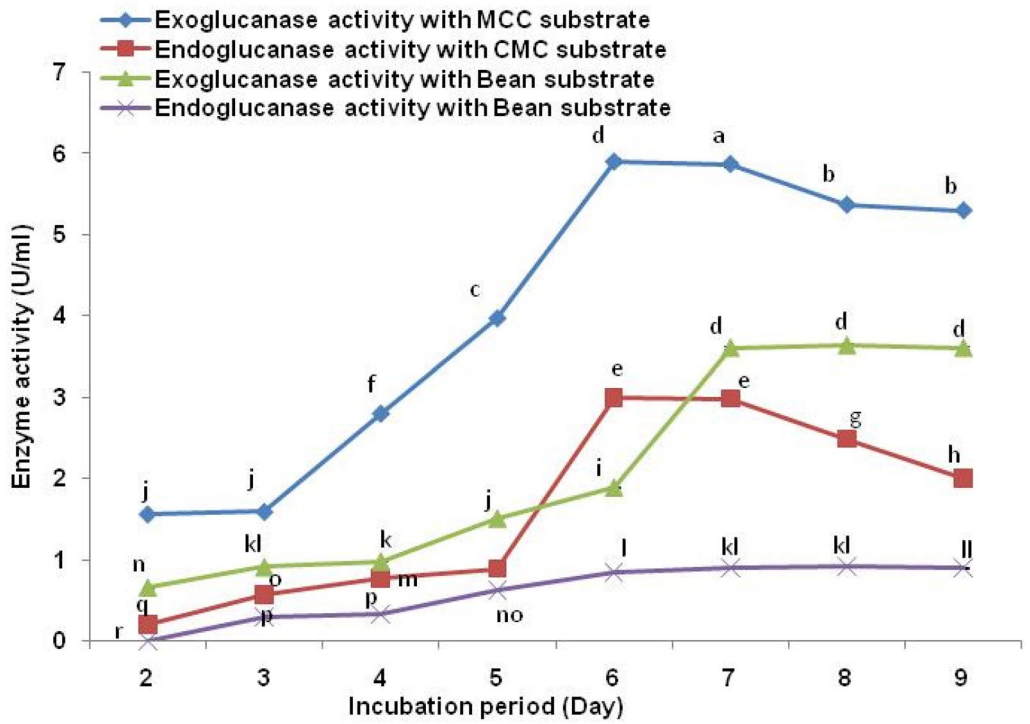

Fig. 5 Effect of different incubation periods on exoglucanase and endoglucanase activities using MCC, CMC and bean substrates

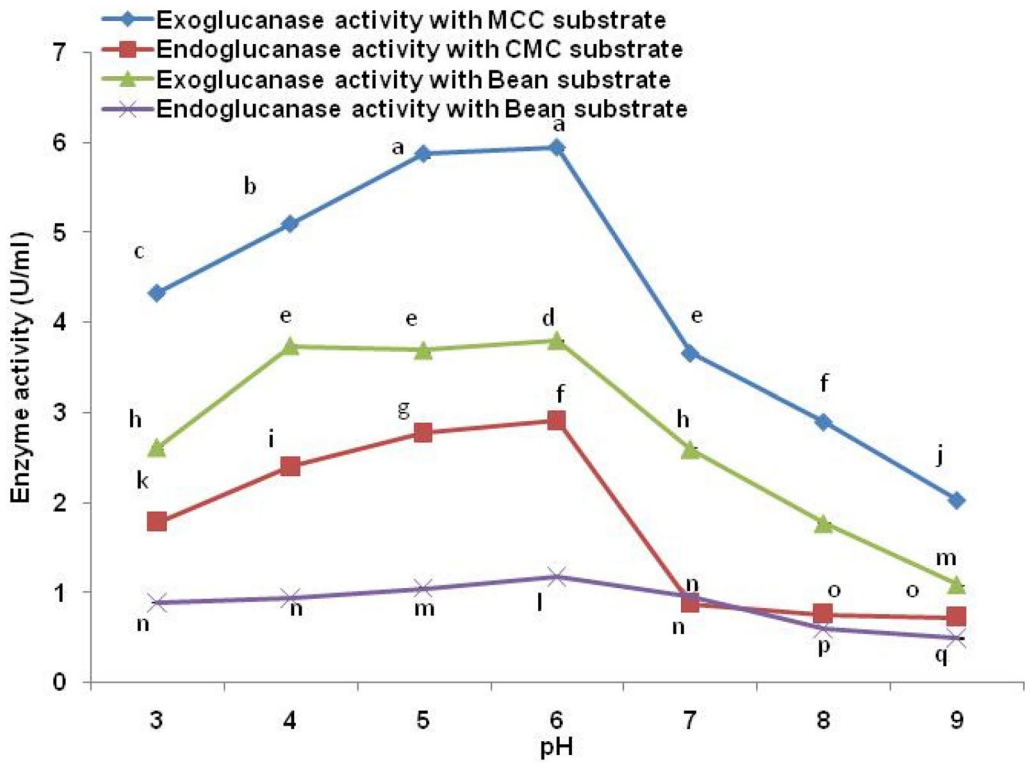

Fig. 6 Effect of different pH on exoglucanase and endoglucanase activities using MCC, CMC and bean substrates

biotechnological and industrial applications of enzymes (Fig. 5). The optimum incubation periods were identified to be 6 and 8 days for enzyme activity (5.90 and 3.64 U/g of exoglucanase; 2.99 and 0.92 U/g of endoglucanase) using MCC or CMC as substrates and bean waste biomass, respectively. The MCC and CMC both induced enzyme activity with 6 days of incubation, implying that they are good carbon sources for enzyme induction. However, they may be uneconomical at a large scale. The available agricultural lignocellulosic waste of bean used in this study also induced a favorable amount of enzymes using *C. affinis* after 8 days. Endoglucanase activity was undetectable at 2 days of incubation, whereas exoglucanase activity was detected using bean waste. This result indicated that exoglucanase was synthesized and excreted earlier than other enzymes, as reported by Li et al. (2013). El-Said et al. (2014) recorded the highest production of exo- and endo-β-1,4-glucanases by phytopathogenic fungi including *Alternaria citri, A. alternata* and *A. citri* and *Cochliobolus spicifer* isolated from broad bean diseased leaves after 6 and 8 days of incubation period, respectively.

Exoglucanase by *C. affinis* was also analyzed in the current study to investigate the effect of pH on the activities of endoglucanase (Fig. 6). Exo- and endoglucanase production showed similar profiles, with maximum activity in cultures at pH 5–6 using MCC or CMC or bean waste. This result was similar to the results of Prasetyo et al. (2010), who studied the cellulase production in pH-controlled cultures. Exo- and endoglucanase production showed maximum activities in cultures at pH 5.5–6.0. In the current study, acidic pH (3 and 4) was more effective than alkaline pH (7 and 8) in enzyme production. This result is in agreement with the work of Ali et al. (1991), who reported those pH levels of 3 and 4 lead to high yields of cellulase enzyme. In other studies, the highest ligninolytic enzyme production has been reported under optimal conditions of pH 5.5 to 6.0 (Rosales et al. 2007; Patrick et al. 2011). Also, El-Said et al. (2014) observed that maximum production of exo- and endo-β-1,4-glucanases was at pH 6.

Surfactants, especially Tween-80, can increase the bioavailability of less soluble substrates for the fungi and stimulate growth of the fungal spores as well as hydrolytic enzymes (Zheng and Obbard 2001); therefore in

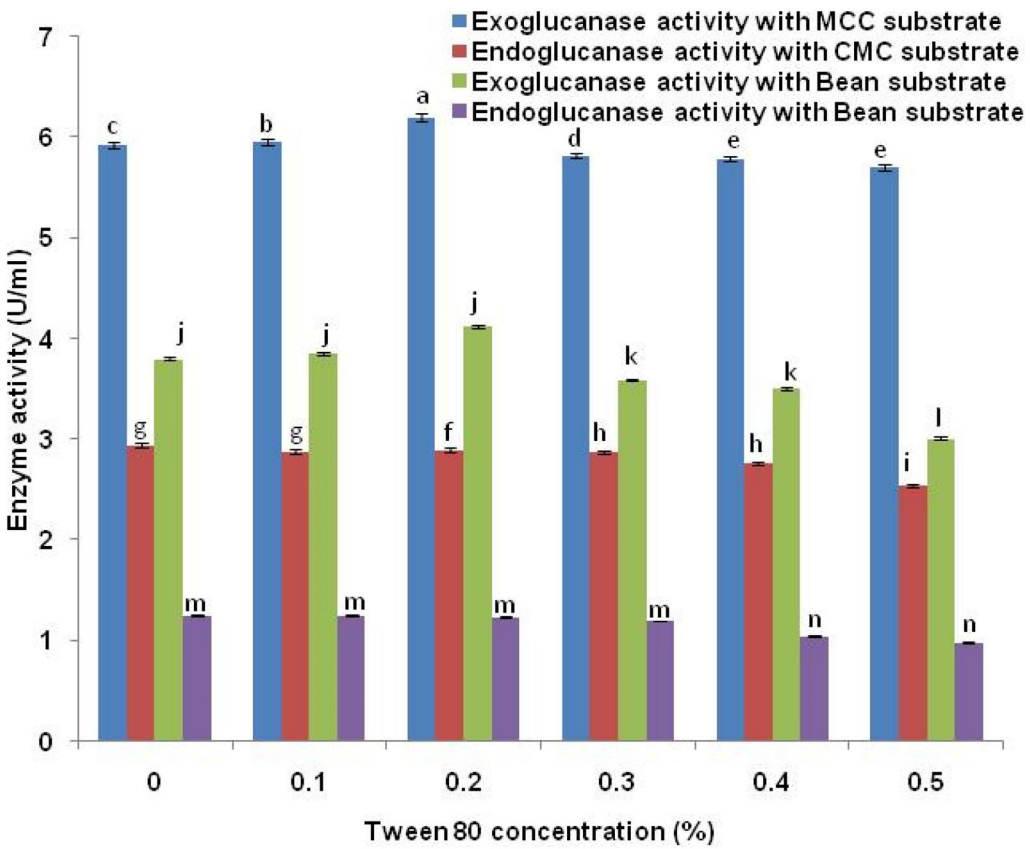

Fig. 7 Effect of different concentrations of Tween 80 on exoglucanase and endoglucanase activities using MCC, CMC and bean substrates

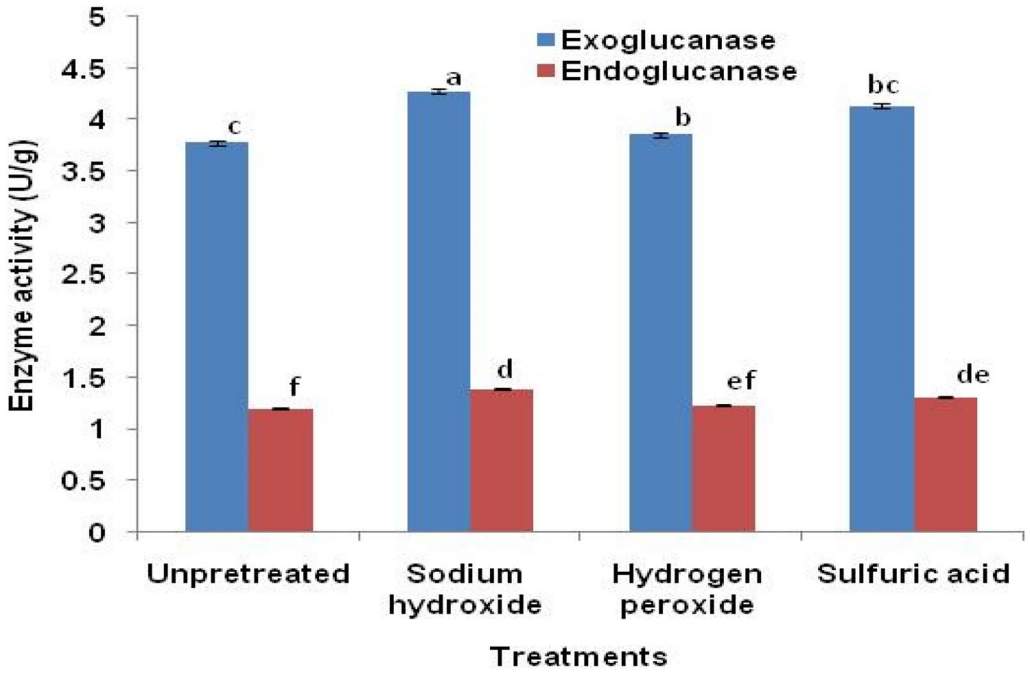

Fig. 8 Effect of pretreatments on exoglucanase and endoglucanase activities using bean substrates

the current study, addition of 0.20% Tween 80 stimulates the activity of exoglucanase and then decreased with increasing Tween 80 concentration (Fig. 7). The exoglucanase activity gradually increased from 5.92 U/g at 0.0% to 6.20 U/g with 0.20% of Tween 80 and then decreased to 5.70 U/g at 0.50% Tween 80 using the MCC substrate. The activity gradually increased from 3.80 U/g at 0.0% to 4.12 U/g at 0.20% and then decreased to 3.01 U/g at 0.50% Tween 80 using bean waste substrate. On the contrary, the endoglucanase activity was negatively influenced by Tween 80. Although the mechanism by which the surfactants enhance extracellular enzyme production in filamentous fungi has not been elucidated (Wang et al. 2006), the obtained results may be due to the surfactant affecting the permeability of fungal cell membrane, thus contributing to enzyme secretion into the media and lowering the concentration of intracellular enzymes. Our results agree with those reported by Guoweia et al. (2011).

Effect of pretreatments of bean substrates for exo- and endoglucanase production

Bean biomass pretreated with any treatments yielded higher enzyme production than that of unpretreated substrates (Fig. 8). Sun and Cheng (2002) stated that alkaline pretreatment increased internal surface area and fiber distension in agricultural biomass; therefore, the released cellulose and hemicellulose induce *C. affinis* growth and its hydrolytic enzymes. The results indicated that alkaline-pretreated biomass showed the highest enzyme production compared with acid-treated residues, followed by H_2O_2-treated ones. The findings in this study agree with those reported (Zhang et al. 2012; Salihu et al. 2015) using acid- and alkali-treated agricultural wastes. Enzymes yielded from biomass pretreated with H_2O_2 showed lower activities than those with alkaline or acid pretreatments, which might be due to the inhibitory effects induced by the hydrolysates from bean biomass.

Conclusion

Results confirm the suitability of using low-cost and abundant bean waste as solid substrate for exo- and endoglucanase production. *C. affinis* is a relatively good producer of exo- and endoglucanase under optimum conditions of pH, incubation period, substrate concentration, and surfactant.

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Authors' contributions
AMM designed the experiments, wrote the paper, is the corresponding author for journal, analyzed and interpreted the data. AAA contributed to fungal identification, analyzed and interpreted the data, performed the experiments; contributed reagents, materials and analysis tools or data, analyzed and

interpreted the data; wrote and English edited the paper. All authors read and approved the final manuscript.

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Availability of data and materials
The data set (table and graphs) supporting this article's conclusion is available.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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