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Utilization of Mahua Cake for Cellulase Production by Using *Trichoderma harzianum* NAIMCC-F-02957 under Submerged Fermentation

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Abstract: Conditions were optimized for cellulase production by *Trichoderma harzianum* using mahua (*Bassia latifolia*) pomace as substrate under submerged fermentation conditions. Substrate concentration of 10% (w/v) was found optimum for CMCase and FPase whereas 5% (w/v) for β-glucosidase production. The optimum pH for production was found as 4 for CMCase and 5 for both β-glucosidase and FPase. The optimum temperature for CMCase and FPase was 30°C, while 40°C for β-glucosidase. Maximum production of CMCase and β-glucosidase was achieved on 7th day while of FPase on 5th day of incubation. Addition of mineral nutrient *viz.* Mo^{3+}, Mn^{2+}or Zn^{2+} individually into the growth medium increased CMCase production. While for β-glucosidase production, mineral ions, *viz.*, Mo^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+} or Zn^{2+} showed positive effect. Whereas, FPase production, only Mg^{2+}, Ca^{2+}, Fe^{2+} showed positive effect. Purification to the extent of 5.05 fold could be achieved by ammonium sulphate (50-70% saturated) precipitation. Maximum activity of CMCase, β-glucosidase and FPase forms of cellulase was observed at 40°C, pH 5.5; 30°C, pH 5.5 and 30°C, pH 4.5, respectively. K_m values for CMCase, β-glucosidase, and FPase were found to be 1.67, 0.31 and 66 mg g^{-1}, respectively. Similarly, V_{max} values were found to be 0.011, 0.16 and 0.177 μmole $g^{-1}min^{-1}$ respectively.

Key words CMCase · β-glucosidase · FPase · Mahua Pomace · *Trichoderma harzianum*

INTRODUCTION

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds [1]. The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1,4)-D-glucanase (endoglucanase, endocellulase, CMCase [EC 3.2.1.4], which cleaves linkages at random, commonly in the amorphous parts of cellulose, exo-(1,4)-D-glucanase (cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91]), which releases cellobiose from non reducing or reducing end, generally from the crystalline parts of cellulose and β-glucosidase (cellobiase [EC 3.2.1.21]). Cellulase degrades cellulose to yield glucose and other soluble sugars which can be used either in juice liquefaction or as fuel. Higher saccharification efficiency, mild operating conditions with respect to pH and temperature, absence of by products and avoidance of pollution makes enzymatic hydrolysis superior over chemical processes in industry. However, production of cellulase enzyme has been widely studied in submerged condition. Cost of enzyme production has restricted its application in large scale processes. Therefore, using cellulosic waste as substrate rather than expensive pure cellulase is better economically viable strategy. There are reports of using various agriculture waste including rice bran [2], wheat straw [3], banana waste [4] and food process waste like oil palm [5] and apple waste [6] as substrate for cellulase production.

Mahua (*Bassia latifolia*) is widely grown in tropical and subtropical climatic zones. Its flowers are rich in sugars and and are used for making syrup, wine, liquor [7]. Mahua pomace is generated as solid lignocellulosic waste which constitutes about 20% of total mass weight after processing mahua flowers and is generally used as cattle feed. Since mahua pomace is rich in fibrous material its use for cellulase production by *Trichoderma harzianum* was studied.

MATERIALS AND METHODS

Substrate: Dried and powdered mahua pomace was used as substrate for cellulase production.

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Screening of Isolates for Cellulolytic Activity: Preliminary screening was carried out for cellulase production by plate assay method as described by Carder [8]. Based on the size of clearance zone formed around the colonies using 1% Congo red dye staining for 1 h followed by detaining with 1M NaCl, the potency index of each isolate was calculated as the ratio of zone diameter to colony diameter. While, secondary screening was based on enzyme assay. CMCase activity was assayed as described by Wood and Bhat [9]. A fungal isolate, isolated from degrading bio-dynamic preparation identified as *Trichoderma harzianum* NAIMCC-F-02957 from National Bureau of Agriculturally Important Microorganism (NABIM) showed maximum enzyme activity and was taken up for further studies. The isolate was maintained as pure culture on potato dextrose agar (PDA) slants.

Culture Conditions and Enzyme Extraction: One ml of aqueous spore suspension (containing 10^6 spores (EUTECH Instruments pH 510) was inoculated into sterilized mahua pomace suspension in Erlenmeyer flasks. The flasks were incubated at 30±2°C for 5 days under stationary conditions. The culture was filtered using Whatman No.4 filter paper and the filtrate was centrifuged at 12,000 g at 4°C for 20 min (Eppendorf Centrifuge, 5417-C, USA) to remove the cell debris. The supernatant was assayed for cellulase activity.

Analytical Methods

Carboxymethyl-Cellulase (CMCase) Activity: CMCase (1,4, β-D-Glucanhydrolase) activity was assayed as per method of Wood and Bhat [9] using carboxymethyl cellulose as standard. To 0.4 mL of 1% CMC in 0.05M acetate buffer (pH 5.0) taken in a test tube, 0.2 mL of culture filtrate was added and incubated at 30°C for 60 minutes. The reaction was terminated by adding 1.0 mL of dinitrosalicylic acid (DNS) reagent and subsequently incubating the tubes in water bath at 100°C for 10 min [10]. The absorbance was recorded at 550 nm against the blank (of 0.05M acetate citrate buffer). (EC-Double Beam UV-VIS Spectrophotometer UV 5704SS, India; Merck, Fixed Beam Spectroquant NOVO 60, Ireland). One unit of CMCase activity was expressed as 1 μmole of glucose released per mL enzyme per minute.

β-Glucosidase activity: Cellobioase (1, 4-β-glucosidase) was measured by a modified method [11], where 0.5 mL of enzyme solution was incubated with 0.5% cellobiose in 0.05M acetate buffer at pH 5.0 for 60 min at 30°C. The reaction was stopped by heating in a boiling water bath (Water Bath, Model No: LE110, India) for 10 min. The enzyme activity was determined by measuring the concentration of the glucose released in the medium. One enzyme unit was considered as the amount of enzyme necessary to release one μmole of the reducing sugar under assays condition.

Filter-Paperase (FPase) activity: Filter paper activity (FPA) for total cellulase activity in the cultural filtrate was determined according to the method of Mandels *et al* [12]. Mandels *et al.* [12] using Whatman no. 1 filter paper disc @ 50 mg soaked in 400 μL 0.05M acetate buffer having (pH5.0), as substrate with 0.5 mL enzyme solution. The samples were incubated at 30°C for 60 minutes. The reaction was terminated by adding 1.0mL of dinitrosalicylic acid (DNS) reagent and then incubating in water bath at 100°C for 10min [10]. The absorbance was recorded at 550 nm against the blank (0.05M acetate buffer). One unit of Fpase activity was determined as 1 μmole of glucose liberated per mL enzyme per min.

Optimization of fermentation conditions: The effect of substrates concentration [by varying the concentration between 1-20% (w/v)] and temperature (20-70°C) with increments of 10°C and pH (4-8) with increments of 1 each were examined for cellulase activity. All the experiments were conducted in triplicate and the results show the mean values of the activities.

Partial Purification: The proteins in the crude preparation were precipitated using ammonium sulphate (60-70% saturation) at 4°C for 24 h, and then collected by centrifugation at 12,000 g in a cold centrifuge at 4°C for 30 min. The precipitate was re-dissolved in 0.05 M acetate buffer, pH 5.0 and dialyzed against 50mM acetate buffer (pH 5) twice to remove the excess salts.

Determination of Kinetic Constants: Kinetic parameters of the cellulase for substrate standards of three cellulase forms were determined at specific optimized pH and temperature. The values of Michaelis constants (K_m) and the maximum velocity (V_{max}) were determined from Lineweaver-Burk plot.

RESULTS AND DISCUSSION

Cellulosic biomass to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by bacteria [13-16]. Though the growth period of bacteria is shorter than that of fungi, their half-backed cellulase

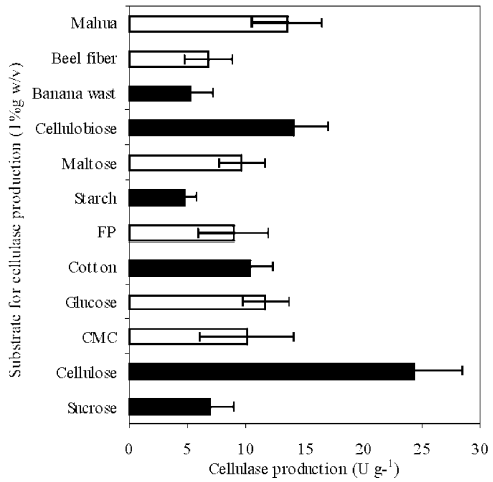

Fig. 1: Optimization of potential substrate for CMCase production using *Trichoderma harzianum* NAIMCC-F-02957 under submerse fermentation

system makes them less useful in the industrial production of cellulase. However, high cost of cellulases production hindered use of this enzyme in industry.

Screening for Most Potential Cellulolytic Isolate: Primary and Secondary screening of microorganism tested revealed that *Trichoderma harzianum* of BD-501 origin had highest cellulolytic activity. The fungi *Trichoderma sp*. is mostly reported to degrade cellulose and other compounds in production of cellulase enzyme [17]. For the utilization of cellulosic biomass like agro-waste wheat straw [3], banana waste [4] and food process wastes like oil palm [5] and apple waste [6] was utilized for cellulase production. It is necessary step to enhance the cellulase production and reduce its production cost. The use of pure cellulose substrate is uneconomical for large scale production of cellulases. Therefore, a cheap and easily available agricultural cellulosic waste, mahua pomace, was tested to find out whether it could support the production of cellulases by *T. harzianum* under submerged fermentation.

The fungal extracellular cellulolytic enzyme complex leads to release of sugar. The degree of saccharification was assayed on the basis of release of reducing sugar. Saccharification is affected by a number of factors such as pH, temperature and incubation time. Among the various substrates tested, mahua pomace was found (Fig. 1) to be a potential substrate for CMCase production (13.5±1.5 U g^{-1}) as it was next to pure cellulose (24.4±3.1 U g^{-1}). A similar trend of cellulosic substrate utilization was earlier reported in cassava [18] and apple [6] over other substrates.

Table 1: Optimization of mineral medium for maximum enzyme production in mahua

	CMCase	β-Glucosidase	FPase
Medium	Cellulase enzyme production (U/g)		
CON	44.67±0	56.85±3.4	1.016±1.4
B	30.45±2.8	10.15±2.8	1.0153±0.7
Mo	77.16±17.2	170.56±22.9	68.02±2.8
Cu	52.79±5.74	32.48±5.7	18.78±7.8
Ca	28.42±2.8	140.11±8.6	78.17±12.9
Mn	62.94±8.6	101.53±17.2	34.01±7.8
Mg	24.36±7.3	42.64±20.1	3.14±2.7
Fe	6.09±8.6	115.74±43.07	77.16±5.8
Zn	56.85±22	75.13±25.8	7.11±2.8
MM	54.83±2.8	87.31±6.0	3.04±2.2
BM	67.01±5.8	26.39±14.3	5.58±1.6
BM+MM	89.34±0	276.15±11.48	65.99±7

*MM-Mineral medium, **BM-Basal medium

Optimization of Fermentation Conditions: Substrate concentration of 10% (w/v) was found suitable for CMCase (17.5±1.4 U g^{-1}) and FPase (5.6±0.2 U g^{-1}) production whereas 5% (w/v) for β-glucosidase (15.8±2 U g^{-1}) production (Fig. 2a). The increase in glucose production until the optimum that obtained was due to the availability of cellulose in the medium; while a decrease in production beyond optimum concentration is explained to be as a result of an inhibitory effect of accumulated glucose of low degree of polymerization to the growth medium. It might also be due to the specific binding of the enzymes with the substrates [19]. Optimum CMCase production was observed at pH 4 (2.0±4.3 U g^{-1}), while at pH 5, β-glucosidase and FPase were found 16.03±0.5 U g^{-1} and 10.24±1.3 U g^{-1} (Fig 2b) respectively. The optimum temperature for production of CMCase (30±4.2 U g^{-1}) and FPase (11.2±1.2 U g^{-1}) was at 30°C while the β-glucosidase (25.88±4.8 U g^{-1}) was at 40ºC (Fig. 2c). Earlier, optimum pH and temperature was reported for the release of maximum sugar was pH 6.0 at 45°C [20]. Addition of mineral nutrient Mo^{3+}, Mn^{2+}, Zn^{2+} individually into the growth medium showed positive effect in CMCase production while Mg^{2+}, Fe^{2+}, B^{3+} or Ca^{2+} showed negative effect. For β-Glycosidase addition of Mo^{3+}, Ca^{2+}, Mn^{2+}, Fe^{2+} or Zn^{2+} showed positive effect, but B^{3+}, Ca^{2+}, Mg^{2+} showed negative effect. Whereas, for FPase production, Mg^{2+}, Ca^{2+}, Fe^{2+} showed positive effect, while Mn^{2+}, Mg^{2+}, Zn^{2+} showed negative effect (Table 1). Addition of basal and mineral medium together, enhanced CMCase, β-glucosidase production up to 1.75 and 4 times respectively. However, FPase did not give significant result. Maximum production of CMCase and

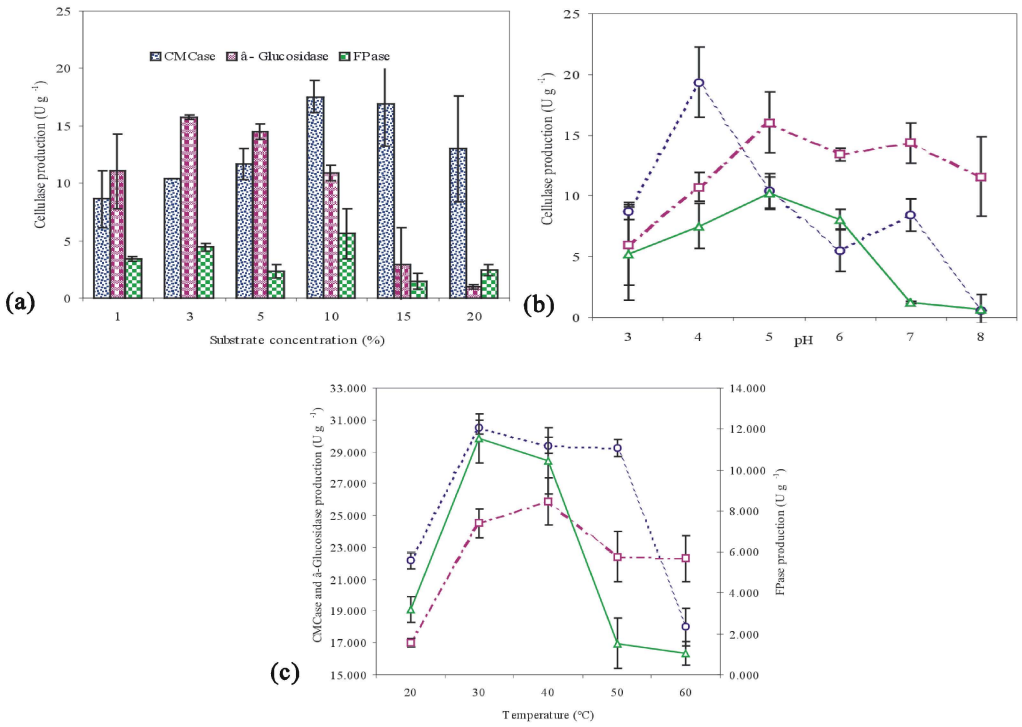

Fig. 2: Optimization of (a)Substrate, (b) pH and (c) temperature for maximum enzyme production from mahua peel using *Trichoderma* harzianum NAIMCC-F-02957 under submerged condition (°) CMCase (U/ml); (□) β-glucosidase (U/ml); (Δ) FPase (FPU ml^{-1})

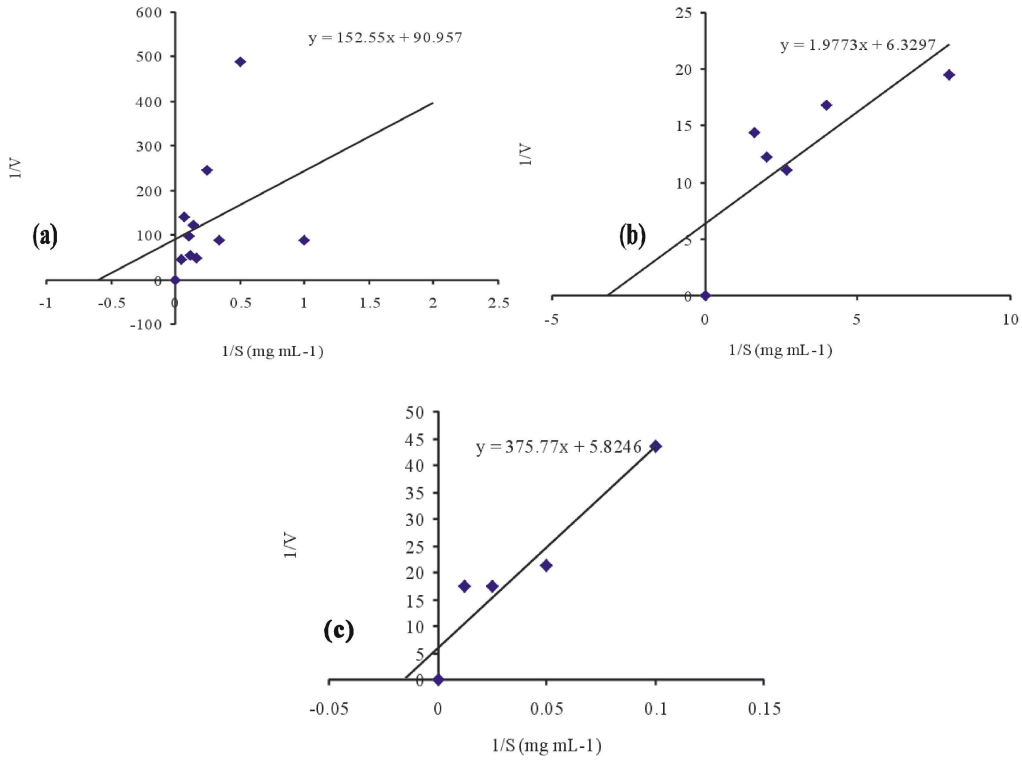

Fig. 3: Enzyme Kinetic of cellulase production in submerge fermentation in mahua pomace using *Trichoderma herzianum* NAIMCC-F-02957 under submerged condition (a) CMCase; (b) β-glucosidase; (c) FPase.

β-Glycosidase was achieved on 7th day while that of FPase on 5th day of incubation. Decline in production after optimum incubation period might be due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation. Omojasola, *et al.* [21] also gave similar time course reports of maximum glucose yield on 7th day of fermentation using *Tricoderma logibrachiatum*.

Partial Purification of Crude Enzyme: Cellulase was purified to apparent homogeneity by 70% ammonium sulphate precipitation which resulted in purification of 5.056 fold and 43.6% yield. This was followed by dialyses which exhibited maximum activity at 40°C in CMCase (0.103±0.033 U ml^{-1}) but 30°C was found optimum for β-glucosidase (0.218±0.001 U ml^{-1}) and FPase (0.02±.033 U ml^{-1}) activities. CMCase was stable at pH 5.5 (0.233±0.009 U ml^{-1}) but β-glucosidase and FPase were at 4.5 (0.371±0.022 U ml^{-1}) and 5.5 pH (0.133±0.026 U ml^{-1}), respectively. CMCase was stable at 60°C for 60 min while β-glucosidase and FPase was only for 40 min each. The instability of these enzymes at very low or very high pH values may be due to the fact that they are proteins which are generally denatured at extreme pH values [22].

Enzyme Kinetics: At optimum pH, the K_m and V_{max} value of CMCase were found 1.67 mg g^{-1} of 0.011 μmole g^{-1} min^{-1} of protein, respectively from β-glucosidase with cellobiose as the substrate these values were found, 0.31 at 0.16 μmole g^{-1} min^{-1} while FPase found 66 mg g^{-1} at Filter paper as substrate were 0.177 μmole g^{-1} min^{-1} protein (Fig 3 a, b, c,). The K_m values for CMCase and β-glucosidase wers lower than that of *Alternaria alternata* [23] and *Candida peltala* [24] 16.64mg ml^{-1}, 66mg ml^{-1} respectively. Lower K_m value has advantage as the enzyme maintains sufficient degradation rate even at a lower substrate concentration, which leads to better substrate transformation.

CONCLUSION

Agricultural cellulosic wastes which are the most abundant renewable biomass in the biosphere, is used for the production of several valuable products by microorganisms. The present study has proved that mahua pomace is also a substrate for pilot scale production of three forms of cellulase, *viz.*, CMCase, FPase and β-glucosidase by *T. harzianum*. These results also highlight the potentials of using mahua pomace as a component of low value raw material in scaling up large scale industrial production of cellulase by *T. harzianum* through fermentation. The additional advantage for the industry would be that the left out biomass after enzyme extraction could be formulated as a low cost biological control preparation since the organism is already well-established and efficient biocontrol agent commercially successful in the market.

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