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Production and characterization of carboxymethyl cellulase from *Paenibacillus polymyxa* using mango peel as substrate

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Abstract

Mango peel, a solid mango processing waste, comprises 15-20% of total fruit weight. This, being a rich source of lignocelluloses, was used as substrate for carboxymethyl cellulase (CMCase) production using *Paenibacillus polymyxa*. Maximum CMCase production (7.814 U mg^{-1}) was observed in a medium containing 7% mango peel (w/v) with 1.5% ammonium sulphate (w/v) at 37°C and pH 5.5. Purification to an extent of 28.24 fold was achieved by affinity column chromatography. Bands corresponding to 26.5 and 34.0 kDa molecular sizes were observed on 12% denaturing Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) while of 72 kDa on 10% non- denaturing Native-PAGE, proving its heteromeric multienzyme nature. The enzyme was stable over a range of 20-60°C and pH of 4.0-7.5. Michaelis-Menten equation constant (K_m and V_{max}) values of purified CMCase were 8.73 mg ml^{-1} and 17.805 mM ml^{-1} min^{-1}, respectively.

Key words

Carboxymethyl cellulase, Fruit waste, Mango peel, *Paenibacillus polymyxa*

Introduction

Carboxymethyl cellulase (CMCase), a major constituent of cellulase complex is widely used in chemicals, fuel, food, animal feed, brewery and wine, textile and laundry, pulp and paper and agro-based industries (Maurer *et al*., 1997; Lynd *et al.,* 2002; Sukumaran *et al.,* 2005). In India, most of the enzymes are imported at huge costs which warrant an absolute need for its commercial indigenous production to reduce the market price (Sukumaran *et al.,* 2005). For decades, bacteria such as *Bacillus subtilis, Pseudomonas flourescens* and fungi; *Aspergillus niger*, *Trichoderma viride* etc. have been commercially used for production of extra- cellular enzymes (Szengyel *et al*., 2000; Ariffin *et al*., 2006). The biggest obstacle in commercial success of enzyme production is the high cost of raw material used as substrate which could be over come by resorting to microbial fermentation technology using low valued biological substrates including agro- waste, *viz*., rice straw, wheat straw, rice bran, wheat bran and baggasse etc. (Nigam and Singh, 1996; Ikram-ul-Haq *et al*., 2006) and fruit processing waste such as apple pomace and grape pomace, pineapple waste (Hang and Woodams, 1994; Krishna, 1999; Omojasola *et al.,* 2008; Sun *et al.,* 2010).

Mango peel, a solid waste of mango fruit processing industry is not efficiently utilized for any specific purpose. It is mainly used as a cattle feed or deposited as landfills, eventually causing environmental pollution (Garg and Ashfaque, 2010). Since, it is a rich source of lingocellulosic fibre (Tandon *et al.,* 1995), its potential use as substrate for CMCase production under optimized conditions was worked out in the study. The enzyme was fractionally precipitated, partially purified and biochemically characterized for determining the kinetic properties.

Materials and Methods

Mango peel was dried (until 5-7% moisture content) and powdered to 50 mesh size particles. Thirty two cellulolytic microbial isolates from different soil samples, compost and other degrading cellulose rich substrates were screened for cellulase activity as per

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the protocol of Au and Chan (1986). A bacterial isolate exhibiting maximum enzyme activity identified as *Paenibacillus polymyxa* from Microbial Type Culture Collection (MTCC), Chandigarh, India, and numbered as MTCC10056 was taken up for further studies. The medium used in the study unless otherwise stated was a modified mineral salt medium (Rowe *et al.*, 1975) having mango peel or other carbohydrate as substrate. After the completion of fermentation (7th day), the culture filtrate was centrifuged at 14,000 rpm, 4°C for 20 min and the supernatant was assayed for CMCase activity. Different carbohydrate sources and agro-waste were compared for potential use as substrate for cellulase production.

Enzyme assays were performed at 30°C for 1 hr. The CMCase activity was determined as per method of Miller (1959) using carboxy methyl cellulose as substrate. The amount of released reducing sugar was quantified using glucose as standard for determining enzyme activity and expressed in terms of U gm^{-1} of substrate utilized. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µm equivalent of glucose $minute^{-1}$ g^{-1} of substrate utilized. Protein concentration was estimated by method of Lowry *et al.* (1951). The Michaelis-Menten equation constant (K_m and V_{max}) were determined by Line Weaver-Burke plot using carboxy methyl cellulose as substrate. Reaction was carried out at 30°C in 50 mM acetate buffer pH 5 at various concentrations of substrate from 0-3.0%.

The effect of substrates concentration 1-10% (w/v), nitrogen addition (0.5-1.5% w/v), incubation temperature (20-70 C) and pH (3-8) on cellulase production using mango peel as substrate was determined. All the experiments were conducted in triplicate and the mean values are expressed. Temperature and pH stabilities of CMCase were determined as per method of Au and Chan (1986). The culture filtrate was precipitated using ammonium sulphate at 20-70% saturation and was dialyzed twice against the 50 mM of acetate buffer (pH 5.0). The concentrated protein was fractionated using affinity column (1.5 x 26 cm) having agarose matrix pre-equilibrated with 50 mM acetate buffer (pH 5) and protein fractions of 1.5 ml were collected upto 40 ml. The flow rate was maintained at 1 ml min^{-1} and the protein values were monitored spectro-photometrically at 280 nm. The enzyme activity of all the fractions was estimated and the fractions showing the enzyme activities were pooled up. Electrophoresis in 10% native-polyacrylamide gels and 12% sodium dodecyl sulphate-polyacrylamide gels were carried out in the discontinuous buffer systems as described by Laemmli and Favre (1973).

Results and Discussion

Out of thirty two microbial isolates, *Paenibacillus polymyxa* MTCC 10056, a bacterium isolated from degrading citrus peel, exhibited maximum enzyme activity (6.069 U mg^{-1}). The selected isolate was further confirmed for enzyme production ability by growing it on pure substrates and other agro-wastes. Among the synthetic carbon substrate tested, maximum CMCase production was observed with carboxymethyl cellulose (24.5U mg^{-1}) and minimum with glucose (4.722 U mg^{-1}). On the other hand, among agro wastes, mango peel (5.7 U mg^{-1}) was found most promising where as least production (2.6 U mg^{-1}) was observed in banana waste medium (Table 1).

The CMCase production increased with increase in substrate (mango peel) concentration upto 7% (6.3 U mg^{-1}). Decrease in CMCase production was observed at 10% substrate concentration (3.7 U mg^{-1}) which might be due to the catabolite repression and/or inhibition due to phenolics accumulation in the medium. Similar repression of synthesis of cellulolytic enzymes has been demonstrated in many organisms (Narasimha *et al.*, 2006).

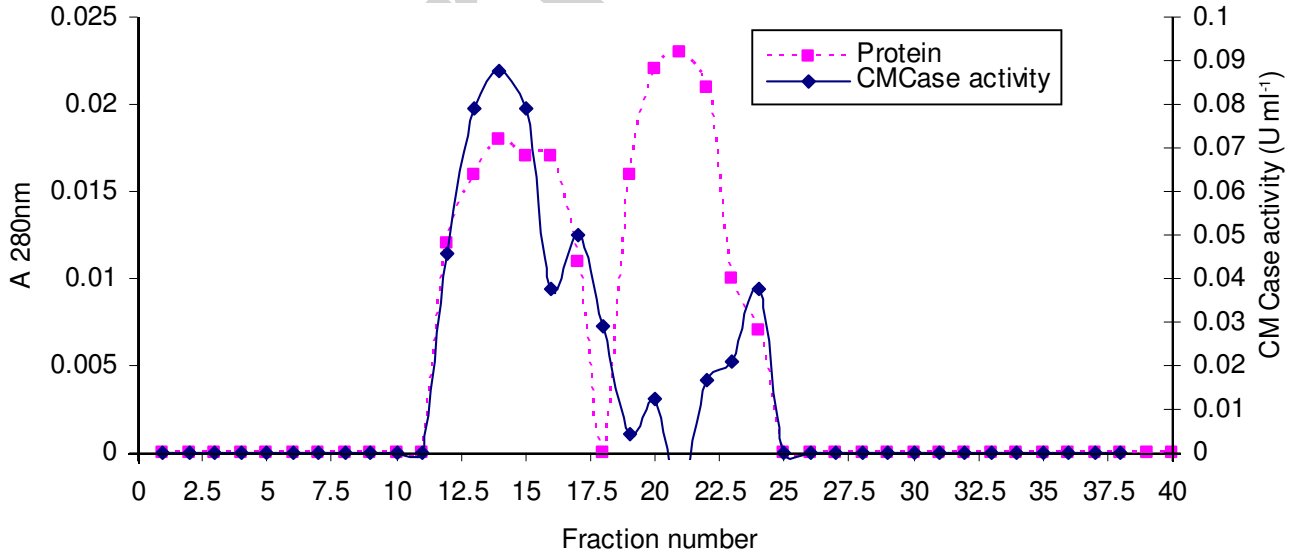

Fig. 1: Elution profile of protein fractions with CMCase activity

Table - 1: CMCase production by *Paenibacillus polymyxa* using different carbohydrate sources as substrate.

Substrate	Specific activity of cellulase (U mg^{-1})
Sucrose	6.94±0.30*
Starch	10.06±1.82*
Carboxymethyl cellulose	24.44±3.24*
Glucose	4.72±0.24*
Cotton	11.33±1.28*
Filter paper	8.89±1.67*
Cellulose	11.67±1.94*
Maltose	9.63±1.53*
Cellulobiose	13.98±1.34*
Banana waste	2.59±0.45*
Bael[1] fibre	3.38±1.18*
Mahua[2] pomace	1.67±0.92*
Mango peel	5.71±1.30*
Aonla[3] pomace	2.87±0.97*
Maize corn	5.05±0.84*

1 = *Aegle marmelos,* 2 = *Madhuca indica,* 3 = *Emblica officinalis,*

* Values are mean of three replicates ± S.D

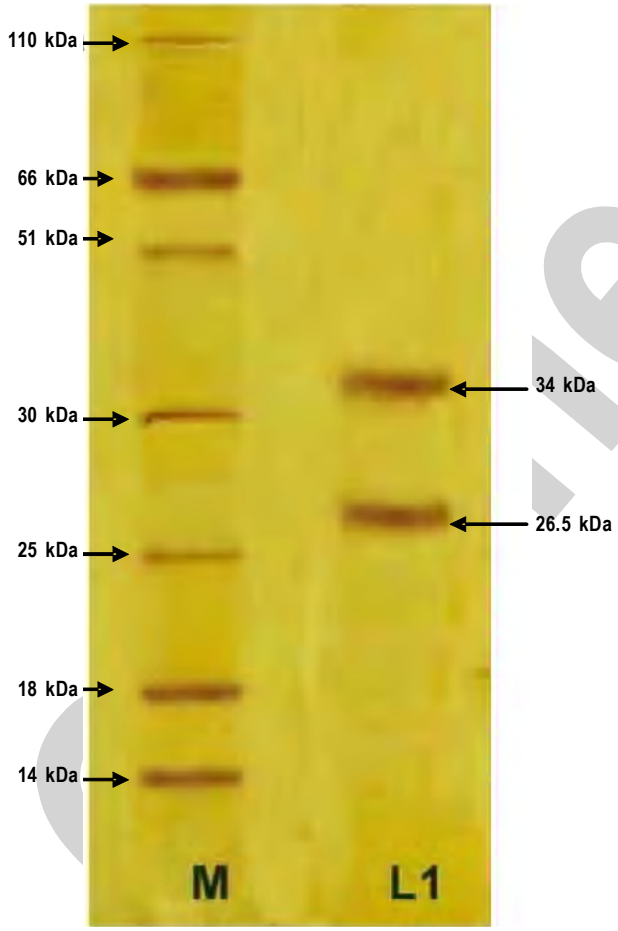

Fig. 2: Electrophoretic banding pattern of protein on 12% SDS-PAGE (M = Marker, L1 = Purified cellulase)

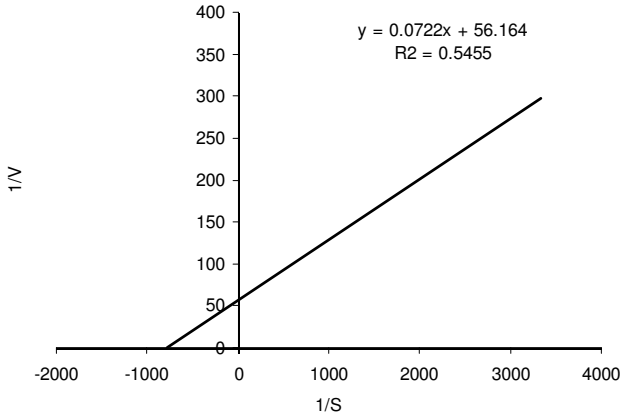

Fig. 3: Lineweaver-Burke plot for determination of Km and Vmax using carboxy methyl cellulose as substrate (1/V=Reciprocal of velocity, 1/S= Reciprocal of substrate concentration), Km= Michaelis constant)

The effectiveness of nitrogen source in supporting CMCase production along with growth and secretion of extracellular protein has also been reported in many microorganisms (Narasimha *et al.,* 2006). Since, mango peel has a poor protein status (2.9%) the effect of addition of nitrogen on CMCase production was worked out. The addition of ammonium sulphate (1.5%) as nitrogen supplement enhanced CMCase production by 28.2%.

For CMCase production, pH of 5.5 and incubation at 37°C were found to be optimum (7.81 U mg^{-1}). Increase or decrease in pH or temperature significantly affected the enzyme production. Baig *et al.* (2004) have reported pH 6.0 as optimum for maximum cellulase production from *Trichoderma lignorum* using banana waste. Optimum temperature for CMCase production was reported at 30°C by *Bacillus sp.* and *Pseudomonas fluorescens* (Khyami-Horami 1996; Bakare *et al.,* 2005).

After ammonium sulphate precipitation of culture filtrate, the crude protein obtained was purified to the extent of 28.24 fold with 1.99% recovery (Table 2) by affinity chromatography on agarose column. The elution profile of protein fractions revealed that fractions numbering 10 to 25 showed peak CMCase activity (Fig. 1). Purification to extent of 24-26 folds had been reported earlier in cellulase and pectinase (Bakare *et al.*, 2005; Ariffin *et al.,* 2006; Arotupin, 2008).

The fraction exhibiting enzyme activities were pooled and analyzed on 12% SDS-PAGE and 10% Native PAGE. Two bands corresponding to 34 and 26.5 kDa were observed in 12% SDS-PAGE (Fig. 2) and it was observed as single band in 10% Native gel. Earlier, 29 kDa alkaline cellulase and 30-65 kDa cellulase was reported in *Bacillus* sp and *Bacillus pumilus*, respectively (Khyami-Horami, 1996; Ariffin *et al.,* 2006) in denaturing PAGE; where as 60-70 kDa cellulase has been obtained in 10% Native PAGE (Giorgini, 1992). Similarly, Chen *et al.* (2004) has reported CMCase of 94 kDa in *Sinorhizobium fredii* while Coral *et al.* (2002) identified 83 and 50 kDa CMCase in *Aspergillus niger* wild type strain Z10.

Table - 2: Summary of CMCase purification by *Paenibacillus polymyxa*

Purification step for purification	CMCase activity (U mg^{-1})	Total protein (mg)	Specific activity (U mg^{-1})	Yield %	Fold purification
Culture filtrate (Crude)	24.6	140	0.18	100	1
Ammonium sulphate	10.46	14.07	0.74	42.27	4
Affinity chromatography	0.5	0.1	4.82	1.99	28.24

Since cellulase is a heteromeric multienzyme complex, these two bands might correspond to different components of cellulase enzyme complex in *P. polymyxa.* CMCase of *P. polymyxa* exhibited maximum cellulase activity at 30°C (8.4 U mg^{-1}) and at pH-5 (9.4 U mg^{-1}). The purified enzyme was found to be stable over range of 20-60°C, at pH 4.0-7.5, though, only 25% activity was retained at pH 7.5. Enzyme showed a temperature stability range between 20-60°C. The stability declined at temperature higher than 60°C while negligible enzymatic activity was observed at 70°C. The K_m and V_{max} for cellulase from *P. polymyxa* was 8.73 mg ml^{-1} and 17.805 mM min^{-1}, respectively (Fig. 3). Earlier, K_m values for CMCase have reported from *Alternaria alternata* as 16.64 mg ml^{-1} (Macris, 1984) and *Candida peltala* as 66 mg ml^{-1} (Saha, 1996). Lower K_m value has the advantage that the enzyme maintains sufficient degradation rate which leads to better substrate transformation. The study revealed that mango peel may serve as substrate for CMCase production using *P. polymyxa* with optimized conditions.

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