Cellulases (CMCases) and polyphenol oxidases from thermophilic *Bacillus* spp. isolated from compost

Lungisa Mayende, Brendan S. Wilhelmi and Brett I. Pletschke

Abstract

In composting, organic matter is degraded by cellulases and ligninolytic enzymes at temperatures typically above 50 °C. This study isolated thermophilic microorganisms from a compost system that were then screened for cellulase and polyphenol oxidase activity. Temperature optima for the cellulases and polyphenol oxidases were determined as 70 and 40 °C, respectively. Maximal cellulase activity was determined as 1.333 mg glucose released ml^{-1} min^{-1}. Maximal polyphenol oxidase activity attained was 5.111 nmol phenol ml^{-1} min^{-1}. Cellulases were found to be stable over a period of 1 h. The isolated compost microorganisms were identified as strains of *Bacillus* using 16S ribosomal DNA sequence analysis.

Compost is organic matter that has been biologically decomposed by microorganisms. Along with humus-like end products, the microorganisms convert organic material into carbon dioxide, biomass and thermal energy (Tuomela et al., 2000). The compost process typically undergoes a series of stages; these include a rapid increase in temperature, a period of sustained high temperature, followed by the slow cooling of the compost (Dees and Ghiorse, 2001).

Compost microorganisms that have previously been identified include *Bacillus* spp., *Actinomycetales*, *Thermus* spp., *Streptomycetes* spp., *Aspergillus* spp. among others (Strom, 1985; Beefa et al., 1996; McCaig et al., 2001; Song et al., 2001). Interest in these microorganisms has increased due to potential commercial applications, which include biodegradation and the production of bioactive compounds such as antibiotics and enzymes (Malherbe and Cloete, 2002). Thermophilic enzymes are important in bioremediation because higher temperatures in the treatment of wastes have the advantage of increased solubility of the substrates (Harayama, 1998).

The aims of this study were to isolate thermophilic microorganisms from compost, to screen the isolates for cellulase and polyphenol oxidase activity, to perform temperature optimum and temperature stability studies on the enzymes and to identify the thermophilic microorganisms with enzyme activity using 16S rDNA sequence analysis.

Compost samples were taken from a simple lucerne and garden refuse compost heap (50 °C) supplemented with layers of Albany soil at Rhodes University, Grahamstown, South Africa. The microorganisms present in the compost were isolated on a 10% (w/v) malt extract, 4% (w/v) yeast extract and 20% (w/v) agar medium, and grown in 24% (w/v) malt extract and 5% (w/v) yeast extract broth for subsequent enzyme assays and DNA extraction. Bacterial growth (ranging from 700 to 900 cfu ml^{-1}) was observed within a 24 h incubation period at 50 °C. No fungal or actinomycete growth was observed during this period. Approximately 20% of all the isolates exhibited high cellulase activity, while 10% exhibited polyphenol oxidase activity.

The colorimetric assay used for the determination of cellulase activity was the dinitrosalicyclic acid (DNS) method (Miller, 1959). The DNS was prepared as described by Bhat and Wood (1988). Carboxymethylcellulose (CMC) was used as substrate (Damaso et al., 2003). Samples (isolated microorganisms of 700–900 cfu ml^{-1} that were re-suspended in 0.05 M citrate buffer, pH 4.8) were screened for cellulase activity and those with the highest activity selected for further studies (denoted as LM01, LM02, LM03 and LM04). Temperature optimum studies were performed over a temperature range of between 20 and 80 °C (except for LM03). Samples were incubated at the respective temperatures for 30 min with CMC substrate, after which DNS was added and the samples were boiled for 5 min and place on ice for 10 min. The absorbance was determined at 540 nm. Temperature stability studies were performed at the temperature optima for up to 1 h. For polyphenol oxidase activity determination, a volume of 200 µl of each sample was added to 50 µl L-3,4-dihydroxyphenylalanine (L-DOPA) (2.5 mg ml^{-1}) to all but the negative controls. The absorbance was read at 30 s intervals at 500 nm for 5 min at 25 °C. Mushroom tyrosinase was used as a positive control and for assay optimization. Temperature optimum studies were performed over a temperature range of between 20 and 80 °C.

The genomic DNA of the samples exhibiting cellulase and polyphenol oxidase activity was isolated using the method described by Pospiech and Neumann (1995). The 16S rDNA was amplified by PCR (primers: 9f 5′-GATTTGATCCTGGCTCAG-3′ and 1451r 5′-AAGGAGGTGATCCAGCC-3′). The PCR amplication products were cloned into pGEM®-T Easy Vector Systems (Promega). Plasmids were purified using a QAIprep® Spin Miniprep kit (Qiagen). Purified plasmids were prepared for cycle sequencing using ABI PRISM® BigDye V3.1 (Applied Biosystems). Protocols were followed as per the suppliers' instructions. Samples were sequenced using a 3100 ABI Genetic analyser. The 16S rDNA sequences were compared to sequences in nucleotide databases using a nucleotide-nucleotide BLAST program.

The selected microorganisms were thermophilic (60 °C) or extremely thermophilic (70 °C), as defined by Sonnleitner and Fiechter (1983). The temperature optimum for samples LM01 and LM04 was 60 °C; indicating thermophilic cellulase activity (Fig. 1). The maximum activity obtained was 0.535–1.085 mg glucose released ml^{-1} min^{-1}. The temperature optimum for both samples LM02 and LM03 was 70 °C (Fig. 1). The maximum activity obtained was 1.215–1.333 mg glucose released ml^{-1} min^{-1}. Sample LM03 produced an extremely thermophilic cellulase, with high cellulase activity still evident at 80 °C. Fig. 2 shows the Arrhenius plots constructed for samples LM01, LM02, LM03 and LM04; the activation energies were calculated as 1.773, 1.181, 5.404 and 1.216 J mol^{-1}, respectively. The cellulase-containing fractions were stable for a period of up to 1 h. Table 1 shows a typical temperature stability profile.

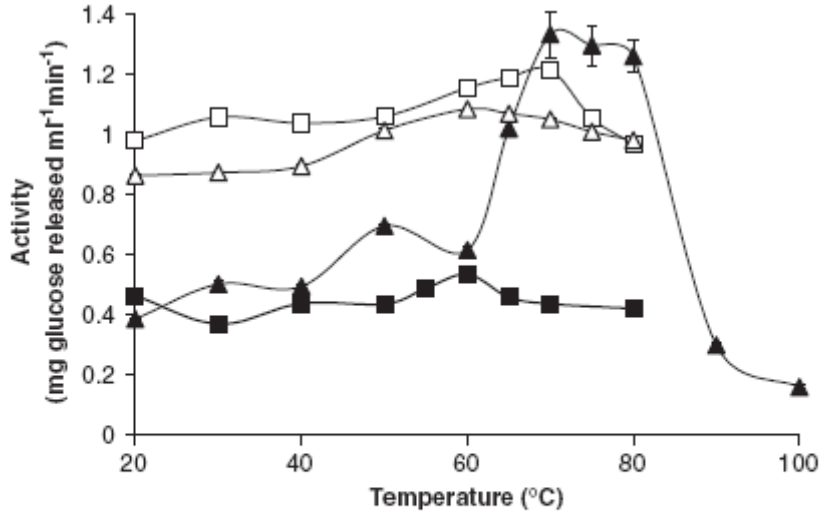

Fig. 1. Temperature optimum profiles for cellulase activity in samples LM01 (■), LM02 (□), LM03 (▲) and LM04 (△). Values are expressed as means ± SD ($n = 3$).

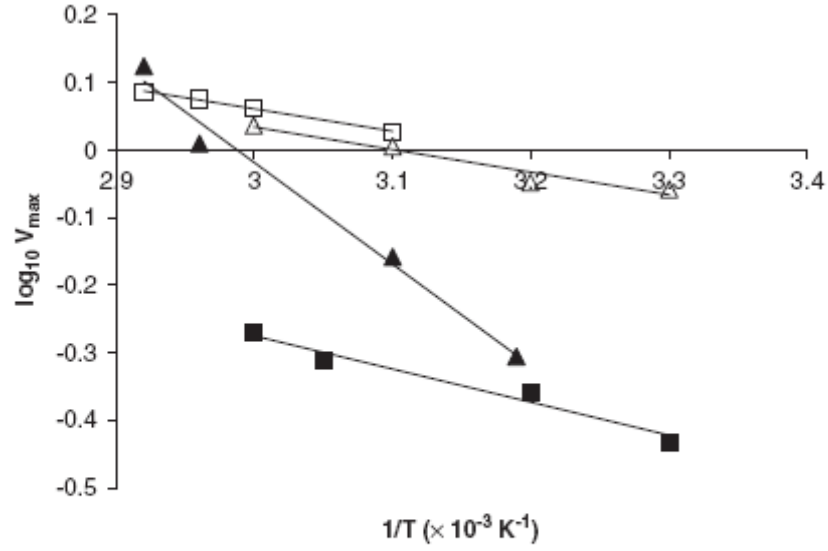

Fig. 2. Arrhenius plots obtained for cellulase activity in samples LM01 (■) ($r^2 = 0.9673$), LM02 (□) ($r^2 = 0.993$), LM03 (▲) ($r^2 = 0.9836$) and LM04 (△) ($r^2 = 0.9464$).

Table 1
Temperature stability profile of cellulase activity in sample LM04 at 60 °C

Time (min)	Total activity (mg glucose released $ml^{-1} min^{-1}$)	Standard deviation (SD)
5	0.671	0.008
10	0.642	0.006
20	0.689	0.020
30	0.881	0.050
40	0.883	0.037
50	0.742	0.020
60	0.976	0.046

Polyphenol oxidase activity was only observed in isolates LM03 and LM04 and the temperature optimum of both was 40 °C, thus these enzymes were only thermotolerant (Fig. 3). The maximum polyphenol oxidase activity obtained was 3.667–5.111 nmol phenol ml^{-1} min^{-1}. Activation energies for polyphenol oxidase activity for samples LM03 and LM04 were 14.368 and 28.702 J mol^{-1}, respectively (Fig. 4). Polyphenol oxidases may be active during the first mesophilic stage of composting or after the thermophilic stage (Hammel, 1997; Dees and Ghiorse, 2001). The low polyphenol oxidase activity suggests that the microorganisms isolated did not utilize lignin as a primary substrate.

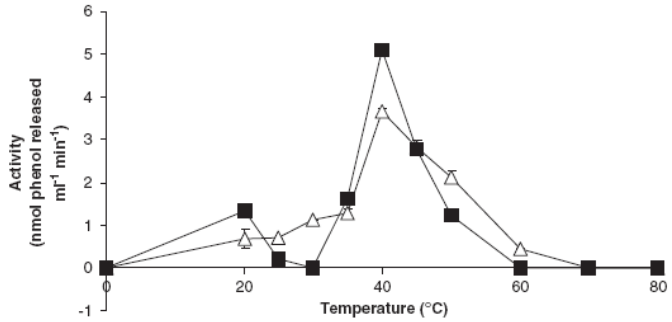

Fig. 3. Temperature optimum profiles for polyphenol oxidase activity in samples LM03 (△) and LM04 (■). Values are expressed as means ± SD ($n = 3$).

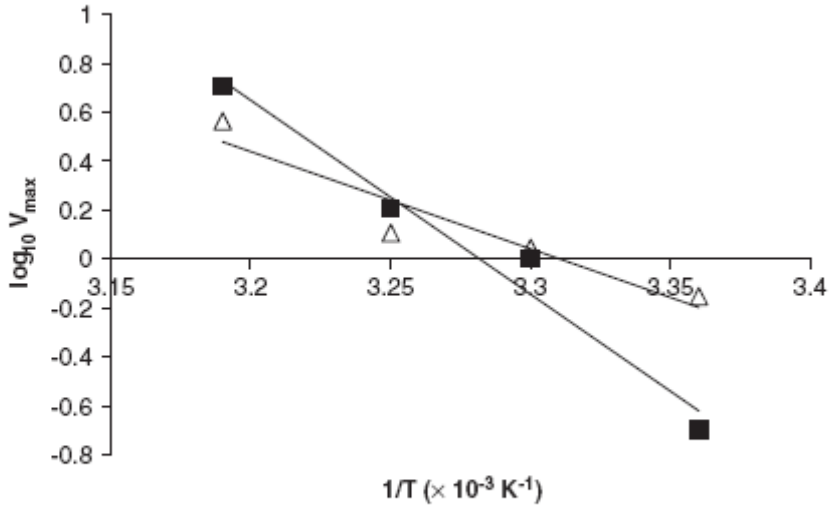

Fig. 4. Arrhenius plots obtained for the polyphenol oxidases in samples LM03 (△) ($r^2 = 0.9015$) and LM04 (■) ($r^2 = 0.9712$).

The isolated microorganisms were identified by 16S rDNA sequence analysis. The sequences were entered into the nucleotide-nucleotide BLAST (NCBI BLAST) and percentage identities established. The highest identity for sample LM01 was 99% with *Bacillus subtilus*. For samples LM02 and LM03 the highest identity was 97% with *Bacillus* spp. Sample LM04 had the highest identity with *B. subtilus* JM4 of 99%.

Blanc et al. (1999), Dees and Ghiorse (2001), and McCaig et al. (2001) have demonstrated the presence of *Bacillus* spp. in compost. The alkaliphilic properties of cellulases produced by *Bacillus* spp. have been studied, due to the possible application of these enzymes in the detergent industry (Horikoshi, 1999; Ozawa et al., 2001). The emphasis of our research was to determine whether the isolated *Bacillus* strains contained cellulases and polyphenol oxidases with thermophilic or thermotolerant properties. Mawadza et al. (2000) isolated thermophilic *Bacillus* strains with cellulolytic activity, but performed their enzyme stability studies at 50 °C and not at the respective temperature optima.

In conclusion, the results show that thermophilic microorganisms isolated from a composting environment were able to produce thermophilic and extremely thermophilic cellulases. These cellulases have potential application in the biotechnology industry due to their thermophilic characteristics and relatively high activities. In contrast, the polyphenol oxidases exhibited low activities and were found to be thermotolerant. The isolate with the highest cellulase activity and polyphenol oxidase activity was a *Bacillus* sp. Although most cellulases currently used in industry are of fungal origin (Uhlig and Linsmaier-Bednar, 1998), this study indicates that bacterial cellulases produced by *Bacillus* strains may have a potential industrial use.

We acknowledge SASOL and the National Research Foundation of South Africa for financial support.

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