

Purification, Characterization and Molecular Studies on Oxalate Decarboxylase from *B. cepacia* inhabits Egyptian soil

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ABSTRACT

Oxalate decarboxylase (OXDC) is an intracellular enzyme that decomposes toxic oxalate into formate and CO₂. So, it is used for medical diagnosis of oxalate and decreasing the oxalate level. OXDC enzyme was purified from *B. cepacia*, characterized and subjected to amino acid analysis. Genomic and plasmid DNA from *B. cepacia* were purified. PCR products of OXDC gene was subjected to DNA sequence analysis. OXDC was purified 18.05 folds to near homogeneity with a specific activity of 4.496 U/ μg protein and showed pure single band with molecular weight 30 kDa. OXDC showed an optimal activity at pH 6, 35C° using Zn⁺² and Mn⁺² as a catalyst. The most abundant amino acid is alanine and the less frequent amino acid is cysteine. Comparing the genomic and plasmid DNA showed that both have the same size. Similarity search results showed that the obtained sequence can detect OXDC gene in many *B. cepacia* genomes.

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Keywords: Oxalate decarboxylase, plasmid, genomic DNA, *B. cepacia*

INTRODUCTION

Oxalate decarboxylase (EC 4.1.1.2) (OXDC) is a primarily intracellular, manganese containing, multimeric enzyme of the cupin protein super family (Webb., 1992; Tanner *et al.*, 2001 & Chang *et al.*, 2004) and its crystal structure was determined (Anand *et al.*, 2002 & Just *et al.*, 2004). It was identified to decompose oxalate (toxic compound causing plant cell death, hyperoxaluria and calcium oxalate urolithiasis) into formate and CO₂. This primarily intracellular enzyme is found in fungi and bacteria, and currently the best characterized enzyme is the *Bacillus subtilis* OXDC (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>). OXDC can be induced by addition of substrate or other carboxylic acids (glycolic, malonic or citric) to the culture medium and / or by lowering

culture medium pH (Micales, 1997; Tanner and Bornemann, 2000; McDowell *et al.*, 2001 & Azam *et al.*, 2002). The commercial uses of oxalate decarboxylase are in the brewing industry and for agronomy to reduce susceptibility of a plant to oxalic acid and to reduce the endogenous oxalic acid concentration in the plant. Subsequently, it is used to reduce plant mortality or destruction from diseases or other phenomenon in which oxalic acid plays a critical invasive role. This enzyme holds special promise because of major scourge in the commercial cultivation of agronomically important plants, for example crops such as sunflowers, were infected by fungal species such as *Sclerotinia* that secrete oxalic acid. These enzymes also have commercial value in estimation of oxalate in clinical samples from patients prone to calcium oxalate

kidney stones. The benefits of this enzyme can be exploited either by plant transformation or by the application of oxalate decarboxylase as a traditional pesticide because it is ecologically safe, non-polluting and does not harm the plants. If an external application of the enzyme is to be used to protect a plant or its part against pathogens, the enzyme can be diluted to form a liquid solution or suspension or mixed with diluent solid to be applied as a dust depending on the particular pathogen (s) and plant (s) targeted. So far, the sources of OXDC enzyme have been limited including only a few fungal and bacterial species. Thus, there is a potential for identification and cloning of new OXDC variants with diverse biochemical properties allowing e.g. more enzyme fitness to process applications

(<http://www.chem.qmul.ac.uk/iubmb/enzyme/>). Oxalate decarboxylase from crude extract and from lysates pellets were combined and purified in a four-step purification procedure including two anion exchange and one hydrophobic interaction chromatography steps and ammonium sulfate protein precipitation (Svedruzic *et al.*, 2005). Oxalate decarboxylase was covalently immobilized to Eupergit (Lin *et al.*, 2011). It was proposed, based on sequence information and alignments with fungal OXDC, that genes from microorganisms such as *Bacillus subtilis*, *Streptococcus mutans* and *Synechocystis* might encode an enzyme with oxalate decarboxylase activity (Dunwell and Gane, 1998, Dunwell *et al.*, 2000). Subsequently, it was confirmed that *B. subtilis* expresses cytosolic OXDC (Tanner and Bornemann, 2000). Next, *B. subtilis* enzymes encoded by genes *yvrK* and *yoaN* (referred to in the newer literature by their more functional designations *oxdC* and *oxdD*, respectively) were over-expressed in *E. coli* and the activity of the recombinant enzymes confirmed (Tanner *et al.*, 2001).

Recently, a structural genomics study of proteins encoded by putative open reading frames in *Thermatoga maritima* has identified an oxalate-binding monocupin as a putative bacterial oxalate decarboxylase (Schwarzenbacher *et al.*, 2004). Till date, OXDC from *C. velutipes*, *A. niger* and *B. subtilis* are the best characterized.

MATERIAL AND METHODS

Organism and growth conditions:

Burkholderia cepacia was isolated, purified and grown under aerobic conditions in Schlegel's basal mineral medium containing 0.4% potassium oxalate at 30°C, pH 7 with shaking at 105 rpm for 72 hrs. The yield from 15 liters of Schlegel's basal mineral medium was typically about 7.824 g (wet weight) of cells harvesting by centrifugation, the cells were washed once in buffer A (0.1M KH₂PO₄ to 1 mM dithiothreitol adjusting pH at 6.8). The cell pellets were suspended in 4 ml of buffer B (buffer A plus 1 mM phenylmethylsulfonyl fluoride, 10 mM MgCl₂, and 1 mM thiamine pyrophosphate) per gram of cells (Abu Shady *et al.*, 2011).

Purification of oxalate decarboxylase:

Preparation of the enzyme:

Purification was performed according to Dashek (1997). The cells were broken by sonication three times for 30 seconds. Cell debris was removed by centrifugation at 20,000 x g for 10 min. The cell free extract was treated with 50 mg of protamine sulfate per gram of cells to precipitate nucleic acids that were removed by centrifugation (20,000 x g for 10 min) (Dashek and William, 1997). The precipitate obtained was dialyzed overnight and lyophilized until used.

Ammonium Sulphate Precipitation:

The cell free extract was then subjected to ammonium sulfate precipitation between 10 and 80% saturation through stirring slowly in an ice bath at 4°C for at least 1 hour, the

precipitate was removed by centrifugation at 12,000 rpm for 30 minutes and dissolved in a minimal amount of buffer pH 6.7 (0.1 M NaH₂PO₄, 1mM dithiothreitol, 10 mM MgCl₂, and 1 mM TPP). The protein content and the oxalate degrading activity were determined (Blackmore and Quayle, 1970 & Bradford, 1976).

Sephadex G- 75 Gel filtration Chromatography:

The dialyzed oxalate decarboxylase was applied to the Sephadex G-75 (1.5 × 24 cm) column equilibrated with buffer C, pH 7.8 (pH 6.7). The enzyme was eluted using the same buffer, pH 6.7 at a flow rate of 2 ml/min. The eluted active fractions were pooled and dialyzed. The protein content and the enzyme activity were determined.

The Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS -PAGE) was performed according to Sambrook *et al.* (1989) using 10% polyacrylamide gel and protein was visualized by staining the gel with Coomassie blue staining to determine the purity of the protein and relative molecular weight using standard molecular weight markers (Sambrook *et al.*, 1989).

Assay of oxalate decarboxylase:

Oxalate decarboxylase can be quantified by measuring the rate of oxalate disappearance (Micales, 1995). One unit of oxalate decarboxylase is the amount of enzyme needed to degrade 1 mg of oxalate per minute at 28°C.

Determination of protein content:

The protein content of dialyzed samples was measured using Bio-Rad protein assay reagent (Bradford, 1976).

Characterization of the purified enzyme:

The effects of pH, temperature and various metal ions on the purified oxalate decarboxylase activities were performed (Micales, 1995). Optimum temperature

was determined by preincubating the purified oxalate decarboxylase at different temperatures between 20 and 40°C for 1 h, followed by determination of the residual activity and protein content. Optimum pH was determined by preincubating the purified oxalate decarboxylase at various pH levels (3–8), followed by determination of the residual activity and protein content. The effects of metal ions including Ca²⁺, Mg²⁺, Al³⁺, Co²⁺, Cd²⁺, Fe³⁺, Na⁺, Zn²⁺, Hg²⁺, and Cu²⁺ 125 [5mM]) were investigated by incorporating them in to assay mixture and incubated at pH 6.0 for 1hour followed by determination of the residual activity and protein content.

Amino acid analysis of oxalate decarboxylase:

Amino acid analysis of oxalate decarboxylase was done in agricultural research center using HPLC technique (Lindroth and Mopper, 1979).

Checking of Plasmid DNA presence in *B. cepacia*:

Presence of plasmid DNA in *B. cepacia* was checked according to Sambrook *et al.* (2001) using the QIAprep® Spin Miniprep Kit (Qiagen, USA) (Sambrook and Russell, 2001).

Genomic DNA purification from *B. Cepacia*:

Isolation of genomic DNA from *B. cepacia* was done according to McDowell *et al.* (2001) with brief modification using DNAeasy Blood & Tissue Kit from QIAGEN. Kit buffers preparations and genomic DNA extraction was done according to the manufacturer's instructions with including RNase A (200µg/ml) in extraction buffer (Mehta and Datta, 1991). Extracted genomic DNA was kept on-70°C till use.

Amplification of gene coding for the oxalate decarboxylase Enzyme (PCR):

The DNA sequence of oxalate decarboxylase gene was obtained from GenBank Accession No: NC_008391 (Bamb_4014). The primers were

designed using Gene Runner program Version 3.05 (Hasting software, USA). The primers were designed to flank the complete sequence encoding the enzyme. The primers were ordered from Gibco BRL (Gibco BRL custom primers, Grand Island, NY). The sequence of sense primer was 5'-AATCTTTCTCGACGCAGG-3', while the reverse primer was 5'-TTACAGCGGCATGACATC-3'.

Restriction sites that do not found to cut inside target sequence were chosen to be added to the primers. *Sac I* (complementary strand 5'-GAGCTC-3') site was added to the 5' end of the forward primer to be 5' GAGCTC - AATCTTTCTCGACGCAGG -3' and *Sal I* (complementary strand 5'-GTCGAC-3') site was added to the 5' end of the reverse primer to be 5'GTCGAC - TTACAGCGGCATGACATC-3'.

Polymerase chain reaction (PCR) was performed according to Sambrook *et al.* (1989) using QIAGEN® Fast Cycling PCR Kit (Qiagen) (Sambrook *et al.*, 1989).

Gel analysis:

The PCR product was run in 1.3% gel. The gel was photographed and

analyzed using gel documentation system (ProXima AQ-4).

DNA sequencing of PCR products and sequence similarity search:

DNA sequence analysis of PCR products was performed using the automated sequencer ABI PRISM (model 310) in Agricultural Research Institute, Ministry of Agriculture, and Giza, 167 Egypt. Results obtained from sequence analysis were subjected to a similarity search using standard nucleotide BLAST option of Internet (<http://www.ncbi.nlm.gov/BLAST>) (Altschul *et al.*, 1997).

RESULTS

Purification of oxalate decarboxylase:

The purification procedure of oxalate decarboxylase is summarized in Table (1). The enzyme was purified 18.05 -fold to near homogeneity with a specific activity of 4.496 U/ μ g protein using ammonium sulphate (40% saturation). The purified enzyme was homogenous showing a single band on SDS-PAGE with a molecular weight of 30 kDa as shown in fig.(1).

Table 1: Summary of oxalate decarboxylase purification procedures:

Purification Steps	oxalate decarboxylase activity (U/ ml)	Total protein (μ g/ ml)	Specific activity (U/ μ protein)	Purification fold
Crude extract	140	703	0.249	1
Ammonium sulphate fractionation (40%)	310	349	0.888	3.56
Dialysis against phosphate buffer	417	153	2.725	10.94
Gel-Filtration (Sephadex)	652	145	4.496	18.05

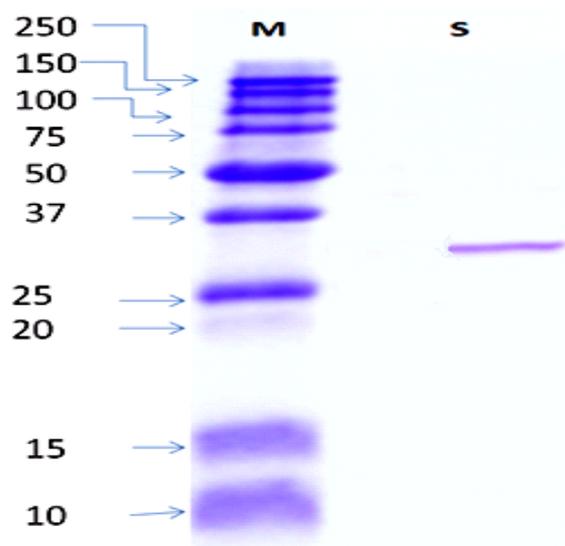


Fig. 1: Electrophoresis pattern of oxalate decarboxylase. Lane M: Precision protein standards in kilo Dalton and Lane S: Purified oxalate decarboxylase after Gel filtration chromatography.

Characterization of the oxalate decarboxylase:

The optimum pH value and optimum temperature for the oxalate decarboxylase is 6 and 35°C, respectively. Zn²⁺ and Mn²⁺ are the most efficient activators for oxalate decarboxylase activity whereas Hg²⁺ is the most inhibitor for the enzyme.

Amino acid analysis:

Amino acid analysis revealed that the most abundant amino acid is alanine

and the less frequent amino acid is cysteine. In addition, the number of basic amino acids was 47 and number of acidic amino acids was 54. The polar and the non-polar amino acids are 186 and 232, respectively.

Checking of Plasmid DNA presence in *B. cepacia*:

Checking of any plasmid in *Burkholderia cepacia* showed that it contains only mega plasmid (genomic) DNA with a size of 12,437.6 bp (Fig. 2).

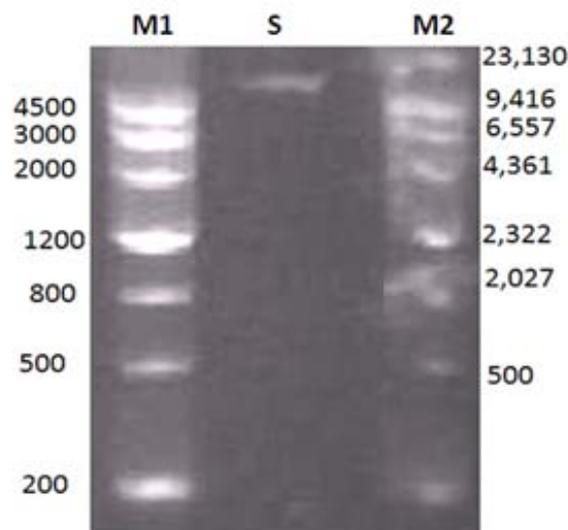


Fig. 2: Agarose gel electrophoresis of purified plasmids from *Burkholderia cepacia*. Lane M1: Gel Pilot Wide Range Ladder; Lane S: Purified plasmid DNA and Lane M2: *Lambda Hind III* DNA marker.

Detection of genomic DNA from *B. cepacia*:

The genomic DNA of *B. cepacia* consists of one replicon whose a size

12,437 bp. Comparison of genomic and plasmid DNA showed that both have the same size as shown in Fig. (3).

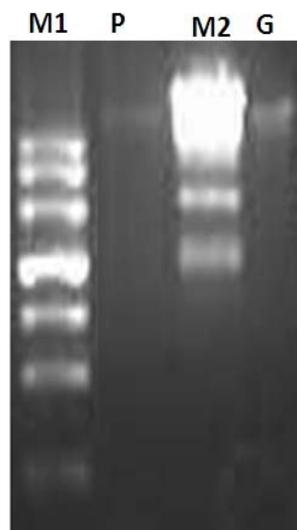


Fig. 3: Electrophoresis pattern of plasmid and genomic DNA of *Burkholderia cepacia*. Lane M1: Gel Pilot Wide Range Ladder; Lane P: Plasmid; Lane M2: Lambda Hind III DNA and Lane G: Genomic DNA.

Polymerase chain reaction:

The expected size of oxalate decarboxylase gene which amplified

from genomic DNA was 1305 bp as presented in Fig. (4).

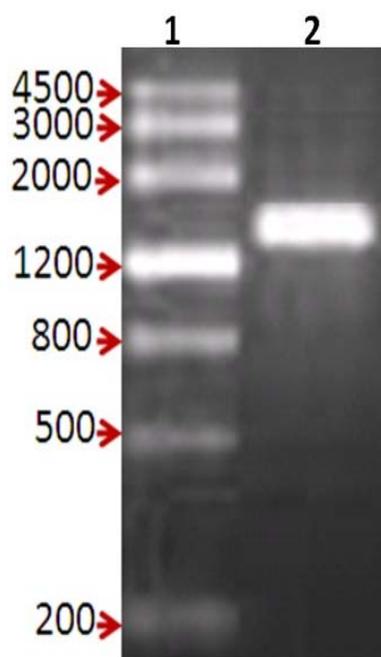


Fig. 4: Agarose gel electrophoresis of PCR products after amplification of Oxalate decarboxylase gene from genomic DNA. Lane 1: Gel Pilot Wide Range Ladder, Lane 2: PCR products of amplified Oxalate decarboxylase gene.

DNA Sequence analysis and similarity search:

Using the blast option of the Internet, a search for similarity of the obtained DNA sequence of oxalate decarboxylase gene from *B. cepacia* in the Genbank and other associated sites showed that the obtained sequence can detect oxalate decarboxylase gene from many *B. cepacia* genomes. Deduced amino acid sequence of the obtained DNA sequence analysis of oxalate decarboxylase is presented in fig. (5).

The obtained sequences (forward and reverse) and amplification primers (forward and reverse) were submitted to EMBL Nucleotide Sequence Database (EBI), Hinxton, UK and obtained the accession numbers as follow:

Burkholderia cepacia Seq1 gene for oxalate decarboxylase Accession#:

HE653274

Protein id="CCF72305.1"

Burkholderia cepacia Seq2 gene for oxalate decarboxylase Accession#:

HE653275

Protein id="CCF72306.1"

PCR primers= forward name: *MomaKF*,

PCR primers= reverse name: *MomaKR*.

DISCUSSION

Through the purification process the ammonium sulphate precipitation and gel permeation chromatography fractionation steps cause some loss of total OXDC, but both steps are necessary to obtain highly purified protein with high specific activity of OXDC. Tanner *et al.* (2001) recorded the pH optimum for the oxalate decarboxylase enzyme is 5 which is close to our results (Tanner *et al.*, 2001). In the time, Kathiara *et al.* (2000) recorded the pH optimum for the oxalate decarboxylase enzyme 3.6 (Kathiara *et al.*, 2000). Whereas pH optimum for oxalate decarboxylase is 3.0 (McDowell *et al.*, 2001).

The optimal temperature of oxalate decarboxylase is 35°C. This result

is concordant with Kathiara *et al.* (2000) who investigated that the optimal temperature of oxalate decarboxylase is 35°C (Kathiara *et al.*, 2001). Whereas Svedruzic *et al.* (2007) investigated that optimum temperature of the oxalate is 23°C (Svedruzic *et al.*, 2007). As a bicupin OXDC has a high thermal stability and is resistant to proteases, features characteristic of a member of the cupin superfamily. This stability has been attributed to a structure with a large number of subunit contacts, hydrophobic interactions, hydrogen bonding, efficient packing, short loops and fewer cavities (Dunwell *et al.*, 2001).

Our findings revealed that the Zn²⁺ and Mn²⁺ are the most potent ions catalyzing decarboxylation reaction leading to the highest enzyme activity while Hg²⁺ acting as inhibitor for enzyme activity. This is may be due to Mn²⁺ ion that is the part of the structure of the enzyme where the enzyme is composed of two cupin domains, each of which contains a Mn²⁺ ion (Moomaw *et al.*, 2009). It is known that several decarboxylases utilize a divalent metal ion to catalyze decarboxylation reactions. Other findings establish a novel type of biological decarboxylation reaction that is transition metal-dependent and nonoxidative. Many enzymes utilize an inorganic cofactor, namely, a transition or non-transition metal center. In regard to transition metal-dependent decarboxylation, previous knowledge has been limited to the cases where an oxidant is required to extract electrons from the primary substrate (Liu and Zhang, 2006).

The molecular weight of the oxalate decarboxylase is discordant with Shen *et al.* (2008) and Kathiara *et al.* (2000) who investigated a major protein band at 43 and 64 kDa, respectively (Shen *et al.*, 2008 & Kathiara *et al.*, 2000). The result of amino acid analysis is confirmed by

Mäkelä *et al.* (2009) who stated that the protein was rich with alanine. Checking of any plasmid present in *Burkholderia cepacia* showed that only mega plasmid (genomic) DNA with a size of 12,437.6 bp was detected. Comparison of genomic and plasmid DNA showed that both have the same size indicating that there were no extra plasmids but only mega plasmid (genomic) DNA with a size of 12,437.6 bp. Also, there may be many genomic plasmids at more or less similar sizes which could not be observed because of overlapping. Genomic DNA together with plasmid present in the strain gives an overall estimate for genome size of approximately 24.874bp. While Cheng and Lessie (1994) investigated that the genome of a strain of *B. cepacia* (ATCC17616) consists of three replicons whose respective size are 3.4, 2.5 and 0.9 Mb, which, together with the 170 – Kb cryptic plasmid present in the strain gives an overall estimate for genome size of approximately 7Mb (Cheng and Lessie, 1994). On the other hand the genome of another strain of *B. cepacia* (ATCC25416) was analyzed and found to contain four circular replicons of sizes 3.65, 3.17, 1.07 and 0.2 Mb (Rodly *et al.*, 1995), the values total 8.1 Mb. In many instances, the degradation of toxic compounds and environmental pollutants are controlled by genes located in plasmids. A 50 Kb plasmid is responsible for the degradation of para-nitrophenol by *B. cepacia*. A 70 Kb plasmid in *B. cepacia* strain 2CBS carries a gene cluster with the determinant of an enzyme able to catalyze double hydroxylation of 2- halbenzoates (Haak *et al.*, 1995). Sahin *et al* (2000) detected Strains harboring plasmids size of 7 and 10 to 12 MDa (NS05, NS06, NS07, NS08) and 24 to 29 MDa (NS01, NS03, NS04, NS10) (Sahin *et al.*, 2000). Similar or higher size of plasmids result showed 92% similarity with *Burkholderia ambifaria* AMMD chromosome 2, complete sequence for

oxalate decarboxylase (Accession number CP000441.1) and also *Burkholderia glumae* signaled as pHG1, pTA19 and pTA07 have been reported by Jenni *et al.* (1988) (Jenni *et al.*, 1988).

CONCLUSION

This study aimed to purify and characterize the oxalate decarboxylase enzyme. Amino acid analysis of the enzyme also was conducted. Comparison of genomic and plasmid DNA of *B. cepacia* was done. Sequence similarity search results showed that the obtained sequence can detect oxalate decarboxylase gene from many *B. cepacia* genomes.

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