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Cloning, sequence analysis and heterologous expression in *Pichia pastoris* of a gene encoding a thermostable cellobiose dehydrogenase from *Myriococcum thermophilum*

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Abstract

We cloned and expressed a gene encoding a thermostable cellobiose dehydrogenase (CDH) from the thermophilic ascomycete *Myriococcum thermophilum.* The 2904 bp long open reading frame contained six introns located either close to the 5′- or 3′-end of the ORF. The corresponding cDNA of 2487 bp was cloned into the expression vector pPICZαB to achieve inducible heterologous expression and secretion of the recombinant flavocytochrome in the methylotrophic yeast *Pichia pastoris.* Transformants were selected on media with normal and 10-fold increased zeocin concentration, and selected clones were tested for inducible extracellular production of the recombinant oxidoreductase. The maximally obtained volumetric activity was 0.25 U/ml in YPM (rich) medium and 2.15 U/ml in production stage (minimal) medium in a fed-batch fermentation. Recombinant CDH was purified in two consecutive chromatographic steps leading to a final specific activity of up to 7.4 U/mg protein at 40 °C. Kinetic properties of the recombinant CDH were characterized and the temperature optimum for the recombinant CDH was determined at 63 °C. Certain properties of the sequence of MtCDH are discussed in context with thermal and proteolytic stability.

Keywords: Cellobiose dehydrogenase; GMC-flavoenzyme superfamily; Heterologous expression; *Pichia pastoris*

Cellobiose dehydrogenases (CDH; E.C. 1.1.99.18; cellobiose:[acceptor] 1-oxidoreductase)[2] are extracellular fungal flavocytochromes originally described in basidiomycetes in connection with early events in lignocellulose degradation [1,2]. The majority of CDH producers investigated to date are white-rot fungi capable of CDH secretion mainly during the exponential growth phase, when cellulose is the main carbon source used by the fungi [3]. High-level expression of this oxidoreductase is a common feature of various lignocellulose-degrading fungi [4]. In the case of *Phanerochaete chrysosporium* CDH represents about 0.5% of the total extracellular protein produced under cellulolytic conditions ranking it among the most intensely expressed proteins of the lignocellulose degrading enzyme system [5]. Both phytopathogenic and saprotrophic fungi, many of which are ascomycetes, are also know to be CDH producers. Much less is known about both production and biochemical properties of ascomycete CDHs, and only few enzymes were characterized so far (and still fewer genes isolated). All currently known CDHs consist of an N-terminal heme-containing

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[2] *Abbreviations used:* CDH, Cellobiose dehydrogenases; DCIP, 2,6-dichloroindophenol.

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domain, a linker of approximately 20–30 amino acids and a C-terminal flavin-containing domain. The latter, initially taken for a separate enzyme, used to be referred to as CBQ, cellobiose-quinone-oxidoreductase. This term is no longer recognized by the Enzyme Commission.

Thermostable CDHs, often produced by thermophilic ascomycetes, offer a clear advantage over their mesophilic counterparts for biotechnological applications, mainly due to their increased operational stability. For CDH the main fields of application are biosensors [6] and biofuel cells [7], where thermostable variants may be particularly advantageous. A thermostable CDH from *Sporotrichum thermophile* (*Thielavia heterothallica*) has previously been purified and the gene encoding the enzyme cloned and sequenced [8]. The thermophilic fungus under study here, namely *Myriococcum thermophilum* (synonym *Papulospora thermophila*, Fergus) grows mainly in mushroom composts [9] together with other thermophilic fungi predominantly from the family Chaetomiaceae. Due to its extraordinary degradative abilities it promotes the growth of white button mushroom *Agaricus bisporus* in composts [10]. None of the secreted enzymes of *M. thermophilum* have been investigated to date.

The methylotrophic yeast *Pichia pastoris* has previously been used for the heterologous expression of a wide variety of recombinant proteins [11] ranging from intracellular bacterial proteins (e.g. [12]) to mammalian secretorial proteins (e.g. [13]). The expression of basidiomycetous CDH-encoding genes in *P. pastoris* has already been demonstrated [14,15]. Furthermore, *P. pastoris* expression allows regulated inducible expression in cultures grown to an extraordinarily high cell density. In this work we describe the isolation and heterologous expression of a gene encoding a thermostable CDH from *M. thermophilum.*

Material and methods

Organisms and culture conditions

The fungus *M. thermophilum* CBS 208.89 was obtained from Centraalbureau voor Schimmelcultures (Utrecht, Netherlands) and was maintained on PHC medium (0.5% meat peptone, 0.5% yeast extract, 0.1% magnesium sulphate, 2% α-cellulose pH 5.4). *P. pastoris* X33 is a component of the *Pichia* Easy Select Expression System and was obtained from Invitrogen (Carlsbad, CA).

Isolation of CDH encoding genomic DNA and cDNA

Myriococcum thermophilum was grown for 90 h in liquid PHC medium at 37 °C, harvested by filtration and shock-frozen in liquid nitrogen. Genomic DNA from 100 mg aliquots was isolated using the DNeasy Plant Mini System (Qiagen, Valencia, CA) according to the manufacturer's instructions. Restriction enzymes, polymerases and DNA modifying enzymes were obtained from Fermentas (St. Leon-Rot, GER) and used as recommended by the manufacturer. Nucleic acid amplifications were performed using *Pfu* proof-reading thermostable polymerase, dNTP mix, oligonucleotide-primers (VBC Biotech, Vienna, Austria) and a Biometra TRIO thermocycler (Biometra, Göttingen, Germany). Degenerate primers CDH07 (forward) and CDH06b (reverse, see Table 1 for details) were designed according to a sequence analysis of conserved regions in all known *cdh* genes [16] and used to amplify a fragment of the *cdh*-gene. 30 PCR cycles at temperatures of 94 °C (40 s), 55 °C (1 min) and 72 °C (1 min), respectively, were employed with genomic DNA as template. A product of approximately 1100 bp was obtained and ligated into the pCR Blunt II TOPO vector (Invitrogen) and sequenced by a commercial service provider (VBC Biotech). A fragment of genomic DNA containing the entire coding region of *cdh* was obtained using the primers ThecdhNTFOR and ThecdhCTREV (Table 1) designed from the highly similar *cdh* gene of *T. heterothallica* (GenBank Accession No. AF074951).

Total RNA was isolated from a culture grown in cellulose-rich medium using the RNeasy Plant Mini System (Qiagen), and first strand cDNA was obtained with the Revert Aid First Strand cDNA Synthesis kit (Fermentas) using either an oligo-dT-primer or random-primer-mixtures. cDNA fragments were amplified using primers ThecdhNTFOR with MthcdhREV3 (5′-part of the coding region), and MthcdhFOR2 with ThecdhCTREV (3′-part of the coding region), and joined by fusion-PCR using overlapping fragments as templates and terminal primers.

Sequence analysis

The translated amino acid sequence of the *M. thermophilum* CDH-encoding cDNA was analyzed using the programs Compute pI/mW, SignalP, NetNGlyc and NetOGlyc 3.1 at http://www.cbs.dtu.dk/services/ [17–20].

Table 1
List of oligonucleotide primers used in this work; introduced restriction sites are in bold print

Name	Sequence (from 5′to 3′)	Remark
CDH07fwd	TGYGARAAYTGYYTNCARTGG (Y = T or C, R = A or G)	Conserved region in cyt. domain
CDH06b	CCNACNGGNARRTTDATCCA (D = A,G,T)	Conserved region in DH domain
ThecdhNTFOR	CCAAGATGAGGACCTCCTCTC	Amplification of the entire CDH-ORF
ThecdhCTREV	CTCACAAGCACTGCGAGTACCA	Amplification of the entire CDH-ORF
MthcdhREV3	GCAGCATCCGTGTTGAGCTGG	Amplification of partial cDNA
MthcdhFOR2	GCACCTTCGGCAGTGCAAAGATTC	Amplification of partial cDNA
CDH-XhoI	TCT**CTCGAG**AAAAGACAGAACAATGTCCCG	Insertion in expression vector (forward primer)
CDH-XbaI	GAATTCGC**TCTAGA**CCCAAGCACTGCGAG	Insertion in expression vector (reverse primer)