Molecular Cloning and Sequencing of D-mandelate Dehydrogenase
Gene from _Rhodotorula graminis_

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Abstract: The yeast _Rhodotorula graminis_ can use D-mandelate as a source of carbon and energy. We have isolated the gene encoding D-mandelate dehydrogenase, one of the two enzymes that stereospecifically catalyze the first step in mandelate degradation. The sequences of the genomic DNA and a cDNA prepared by RT-PCR revealed the presence of three short introns within the coding region. The predicted amino acid sequence of D-mandelate dehydrogenase is 27–33% identical to other members of a large family of NAD-dependent 2-hydroxyacid dehydrogenase from a broad spectrum of bacteria and eukaryotes and it has a wide range of substrate specificities.

Key words: Mandelate dehydrogenase, _Rhodotorula_, gene, cloning, sequencing, yeast, bacteria

Introduction

The ability to utilize mandelate as a source of carbon and energy for growth has been found in a small but diverse range of bacteria and fungi (Fewson, 1988). The pathways for mandelate catabolism vary between different groups of organisms but the first step is generally an oxidation of mandelate (2-hydroxy-2-phenylacetate) to phenylglyoxylate. Several different types of enzymes have evolved to catalyze this reaction (Fewson, 1988). The yeast, _Rhodotorula graminis_, oxidizes both enantiomers of mandelate through the action of stereospecific mandelate dehydrogenase (Durham, 1984). L-mandelate dehydrogenase is a mitochondrial flavocytochrome _b_ that transfers electrons to cytochrome _c_ (Yasim and Fewson, 1993, Smekal et al., 1993). In contrast, D-mandelate dehydrogenase is a cytoplasmic NAD-dependent enzyme (Baker and Fewson, 1989). The purified protein is a homodimer of 38 KDa subunits and crystals diffracting to 0.25 nm have previously been obtained (Bassak et al., 1993). Amino acid sequence determination of about 30% of the polypeptide chain failed to reveal relationships with other known proteins. We have determined the complete sequence from the cloned cDNA to allow a better understanding of the evolution of D-mandelate dehydrogenase from an ancestral 2-hydroxy acid dehydrogenase. D-mandelate dehydrogenase has previously been used for the production of D-mandelate, which is a useful chiral synthon in the production of a range of pharmaceuticals, including semisynthetic β-lactam antibiotics (Yamazaki and Maeda, 1986; Vasil-Racki et al., 1989, Hosono et al., 1990). The yield of enzyme from _R. graminis_ is limited but enzyme production could be greatly enhanced using a heterologous expression system. Production of recombinant enzyme would also allow the preparation of altered forms of D-mandelate dehydrogenase, for example with altered substrate specificity. The objective of this research is to clone, sequence and express D-mandelate dehydrogenase gene from _Rhodotorula graminis_.

Materials and Methods

Strains and plasmids: _Rhodotorula graminis_ GX6000 (ATCC20804) was used as a source of RNA and DNA and was maintained, grown, harvested and stored as described previously (Baker and Fewson, 1989). _E. coli_ strains TC1 and N1 were used as a host for recombinant plasmids. The plasmids pTZ19r and pTZ18r were used for cloning (Rokaech et al., 1998). This project was carried out at University of Edinburgh, Scotland from 1995-1997.

DNA isolation: Chromosomal DNA was isolated from a 10 ml culture of stationary phase culture grown in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose). The cells were harvested by centrifugation and re-suspended in 1 ml of breakage buffer (0.9 M sorbitol/14 mM 2-mercaptoethanol /50 mM sodium phosphate buffer, pH 7.5). The cells were then disrupted by vortexing with acid-washed glass beads. To this suspension 50 μl of 0.5 M EDTA, pH 8.9 was added, vortexed briefly and then 50 μl of 10% SDS and 100 μl of proteinase K solution (5 mg/ml) were added to help lysis. The mixture was mixed well and incubated at 65°C for 30 min then extracted with 1:1 phenol: chloroform and the DNA was precipitated by the addition of 0.5 ml of absolute ethanol. Plasmid DNA and single-stranded plasmid DNA were isolated from _E. coli_ transformants as described previously (Sambrook et al., 1989; Viera and Messing, 1987). M13K07 was used as helper phage for single stranded DNA production.

Isolation of RNA: RNA was isolated from a 100 ml culture of _R. graminis_ grown until mid-exponential phase in medium containing D, L-mandelate. The cells were harvested and re-suspended in 1 ml of TNE (50 mM Tris.HCl pH 7.5, 100 mM NaCl, 5 mM EDTA). Acid-washed glass beads were used to disrupt the cells with vigorous vortexing for 2 minutes. Then 4 ml of TNE, 0.2 ml 20% SDS and 4 ml phenol were rapidly added and the suspension was vortexed for another 2 min. The mixture was spun for 15 min to separate the phases. The aqueous phase containing RNA was removed and extracted with 1:1 phenol:chloroform until a clear interface was achieved. The upper phase containing the RNA was removed and to this 0.1 volume of 3 M sodium acetate, pH 5.5 and 2 volumes of 100% ethanol were added to precipitate RNA. Before use in RT-PCR, contaminating DNA was removed from RNA by treatment with DNase I. A 100 μl mixture containing 100 μg of total RNA and 10 units DNase I in 20 mM Tris.HCl, pH 8.4, 12 mM MgCl2 and 50 mM KCl was incubated at 37°C for 1 h. The reaction was stopped by heating to 65°C for 10 min and extracted with phenol:chloroform. The RNA was precipitated with ethanol, pellet dried and dissolved in 50 μl H2O.

Polymerase chain reaction: A polymerase chain reaction was performed to synthesize a probe for D-mandelate dehydrogenase gene, containing: 1.7 μg _R. graminis_ DNA; 1 μg each of the forward primer P33 (GGAATTCGATTTCGATCAGAATTAG; corresponding to the amino acid sequence AFQQKFE which is

871
found at positions 21-27 from the N-terminus) and reverse P34 (CCGATCCGACCCACCGCGC; corresponding to AGFDW which is found at position 7-12 of a tryptic peptide); 2.5 units Taq polymerase (Promega) in 1 x reaction buffer (Promega) in a final volume of 100 μl. The DNA was melted at 94°C for 2 min and extension at 72°C for 3 min was performed. A final extension at 72°C for 7 min was carried-out to complete the reaction.

Construction of *R. graminis* DNA libraries: Approximately 10 μg aliquots of *R. graminis* genomic DNA were digested with PstI, BamHI, EcoRI, SmaI, XbaI, SphI and SacI, separated on a 0.8% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham, UK). The membrane was pre-hybridized for 1 h at 65°C, then hybridization was carried-out overnight at 65°C. The probe was prepared by isolating the 320 bp PCR product and labeling by random priming (Feinberg and Vogelstein, 1983). After washing with increasing stringency the membrane was allowed to dry and autoradiographed at ~70°C. The genomic DNA library was constructed by digestion of genomic DNA with SacI. The digested genomic DNA was ligated to plasmid pTZ19r cut with SacI. About 10000 recombinants were screened by hybridization under the same conditions as for the Southern blotting.

First strand cDNA synthesis: A mixture of 1 μl of oligo (dT) 12-18 (500 μg/ml) and 15-20 μg of total *R. graminis* RNA (treated with DNase) in 10 μl of sterile distilled water was heated to 70°C for 10 min and then quickly chilled on ice. The contents of the tube were collected by brief centrifugation, mixed with 4 μl 5x first strand buffer (Gibco BRL, USA), 2 μl of 0.1 M DTT, 2 μl of 5 mm dNTPs and 1μl (200 units) SuperScript™ II RNase H reverse transcriptase (Gibco BRL, USA) and incubated at 37°C for 1 h. The products were used immediately for PCR or stored at 20°C.

RT-PCR: The forward primer N1179 (CAAGAAGTCTGCTGCTGCCTGCCGT) and reverse primer R13 (CCACTGAGCTCAGTTAGGGCGGAGAAGC) were designed for amplification of the complete D-mandelate dehydrogenase coding sequence. These incorporated cleavage sites for EcoRI and PstI, respectively, to facilitate cloning of the PCR product. PCR was performed in a 50 μl reaction with 15 mM MgCl₂, 5 pmol of each primer, 200 μM dNTPs and 1 μl of the reverse transcriptase reaction products. After denaturing at 95°C for 5 min, 3 cycles were carried-out with: 95°C for 40 sec, 50°C for 30 sec and 72°C for 90 sec. A further 40 cycles were completed under the same conditions except that the annealing temperature was raised to 64°C. The reaction was completed by further 7 min incubation at 72°C.

DNA sequence determination and analysis: The DNA sequence was determined on both strands using dyeoxy chain termination methods (Sanger et al., 1977) with the Sequenase (US Biochemical Corp.) T7 polymerase. DNA sequence analysis was carried out using Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison and Wisc.

Western blotting: Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a nylon membrane (Hybond-N as described previously (Haed and Süssa, 1984). D-mandelate dehydrogenase was detected using antiserum raised in rabbit followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bic-Rad) as secondary antibody. After washing, enzyme activity was visualized using o-dianisidine as a substrate.

Enzyme assay: D-mandelate dehydrogenase activity was determined following the reverse reaction in 1.4 ml assay mixtures containing 200 mM phosphate buffer (pH 5.85), 200 μM-NADH, 1 mM phenylglyoxylate and enzyme (crude cell extract). The oxidation of NADH was monitored at 340 nm.

Results and Discussion

PCR amplification of a gene fragment: N-terminal amino acid sequence was obtained from purified D-mandelate dehydrogenase and from three tryptic peptides, enabling us to design fully degenerate oligonucleotide primers for the polymerase chain reaction. A PCR with genomic DNA from *R. graminis* amplified a DNA fragment of 320 bp that was then cloned into M13mp19. The sequence of this fragment was shown to encode the N-terminal region of D-mandelate dehydrogenase. A small intron was also detected within this region by comparing the DNA sequence with amino acid sequence (Fig. 1).

Isolation and sequencing of the D-mandelate dehydrogenase gene: *Rhodotorula graminis* chromosomal DNA was digested with seven different restriction enzymes. None of the restriction enzymes were used to digest the genomic DNA cut within 320 bp fragments. Southern blot hybridization was carried-out on the digested chromosomal DNA. The blot was probed with 32P-labeled by random priming. The radioactivity of the Southern blot (Fig. 2) showed that at least part of the D-mandelate dehydrogenase gene was contained within 4.4 kb SacI fragment (Fig. 2, lane 9).

A genomic library was constructed from *R. graminis* DNA digested to completion with SacI. The digested DNA was ligated to pTZ19r that had also been cut with SacI. Transformants containing plasmids with inserts were identified as white colonies on plates containing X-gal and IPTG. Colony blotting using the same probe as for Southern blot screened approximately 10000 recombinants. A single positive clone (pR11) was identified. Plasmid from this positive clone was purified, cut with SacI and shown to contain an insert of the expected size, 4.4 kb.

The entire D-mandelate dehydrogenase coding region was sequenced on both DNA strands. Subclones of the 4.4 kb SacI fragment in pTZ18r and pTZ19r were sequenced with a universal primer. The remaining sequence was obtained using primers that were designed according to the experimentally determined sequence. The complete coding sequence is contained within the cloned SacI fragment. The 1630 bp of assembled sequence includes the PCR fragment previously sequenced. Translation in all three reading frames identified peptide sequences corresponding to those that had been determined experimentally but in different reading frames, indicating the presence of further introns within the coding region, in addition to the one already identified in 320 bp PCR fragment. Isolation of cDNA was therefore necessary for the unambiguous determination of the protein coding sequence and for expressions of recombinant D-mandelate dehydrogenase.

Isolation and sequencing of cDNA: Two PCR primers were designed based on the known N-terminal sequence of the protein and the C-terminal sequence predicted from the genomic DNA. An EcoR restriction site was incorporated in the forward primer and a PstI restriction site in the reverse primer to facilitate the cloning of product. Total RNA from *Rhodotorula graminis* was used as a template for synthesis of single-stranded cDNA by reverse transcription (see Materials and Methods) and this was then used in PCR. The polymerase chain reaction was carried-out at 95°C for 5 min initial denaturing then 35 cycles of: 95°C for 40 sec, denaturing, 64°C annealing for 30 sec and 72°C extension for 1.5 min. Finally another 5 min extension at 72°C was carried-out to complete the reaction. The resulting fragment of approximately 1 kb was treated with Klontow fragment then cut with EcoRI and ligated with pTZ19r that had been cut with EcoRI and SmaI to generate the recombinant plasmid pR13. The cDNA in pR13 was recloned into pTZ18r to obtain the alternative orientation (pR14) for sequencing the second strand. The same primers that were used to sequence the genomic DNA encoding D-mandelate dehydrogenase were also used to sequence the cDNA with single
Illias et al.: Sequence and expression of D-mandelate dehydrogenase

P33
EcoRI
GAATTCGACTTTGCAGCAGAAAAATTGAATAGTCATCCCTGCAACCTGACACCACGACGG
AsPheGluGlnLysPheGluValIleProAlaAsnLeuThrThrHisAspGly

TTTAATACAGGGCTTTCGAGAAGCGGTGCGGTTGTGTTGGCCTACCGGCTGCTTTTC
PheLysGlnAlaLeuArgGluLysAr

CGCTGACGCCATTTCGCGCAGCATCATTTCTGCTCTCTCCCCTGGTACGAGCTATGGCC
INTRON
gTyrGlyA

ACTTCGAAGCCATCACTCAAGCTTCGCGGAGAACGCGCCGAGGCTATCCCTGGGAGCC
spPheGluAlaIleIleLysLeuAlaValGluAsnGlyThrGluSerTyrProTrpAsnA

CCGACCTCATCTCGACCTCCCTCCCTCGAGCTTTCTTTGGCGCGCGCCGCGCGAGGT
laAspLeuIleSerHisLeuProSerSerLeuLysValPheAlaAlaAlaGlyAlaGlyP

TTGATTTGGCGGATCC
heAspTrp BamHI
P34

Fig. 1: The sequence of a 320 bp D-mandelate dehydrogenase gene fragment amplified from R. graminis genomic DNA. The regions corresponding to the PCR primer (P33 and P34) sequences are underlined – in the case of P34 the sequence shown is the complement of the primer sequence. The amino acid sequence predicted from the DNA sequence matches perfectly the sequenced determined directly from the protein assuming the presence of an intron as indicated in bold.

Fig. 2: Southern blot hybridization of R. graminis genomic DNA. Aliquots of R. graminis DNA were subjected to 0.8% agarose gel electrophoresis without treatment (lane 1) or after digestion with PstI (lane 2), BamHI (lane 3), EcoRI (lane 4), Smal (lane 5), XbaI (lane 6), SphI (lane 7) or SacI (lane 8). The gel was blotted onto Hybond-N nylon membrane and hybridized with the cloned 320 bp PCR fragment that had been labeled with [32P]-dCTP by random priming.

873
Fig. 3: The complete sequence of the D-mandelate dehydrogenase-coding region. The sequence of the genomic DNA (top line) is aligned with cDNA sequence (second line), clearly showing the positions of the three introns. The predicted amino acid sequence is shown below the cDNA sequence. These sequences have been submitted to the EMBL database with the reference number of AJ001428.
Introns in DMDH gene from *R. graminis*

<table>
<thead>
<tr>
<th>Introns</th>
<th>Position</th>
<th>5'</th>
<th>Branchpoint</th>
<th>3'</th>
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<tr>
<td>1</td>
<td>282-367</td>
<td>GT</td>
<td>CTGAC</td>
<td>CAG</td>
</tr>
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</tr>
<tr>
<td>3</td>
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Introns in *S. Cerevisiae*

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</thead>
<tbody>
<tr>
<td>TACTAAC</td>
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Introns in *Neurospora crassa*

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>ACTAACA</td>
<td>CAG</td>
</tr>
<tr>
<td>GCTGACT</td>
<td>CAG</td>
</tr>
</tbody>
</table>

Fig. 4: Comparison of *R. graminis* introns with other introns. The sequences of the 5' and 3' ends and the putative branch points of the introns in the D-MDH gene are indicated along with their position in the sequence numbering as shown in Fig. 3. The corresponding sequences for other genes from *S. cerevisiae* (Ruby and Abelson, 1991) and *Neurospora crassa* (Orbach et al., 1986) are shown for comparison.

**Stranded pR13 and pR14 templates as appropriate.** The complete sequences of the genomic DNA and cDNA are aligned in Fig. 3 along with the predicted amino acid sequence of D-mandelate dehydrogenase.

**DNA sequence features:** The G + C content of the cloned genomic DNA is high at 63%. Although this gave rise to some problems in reading the sequence with standard Sequenase reaction conditions, the use of dTTP in place of dGTP in the sequencing reactions allowed the DNA sequence to be read unambiguously. Comparison of the genomic DNA and cDNA sequences identifies the positions of three introns within the D-mandelate dehydrogenase-coding region, varying in length from 66 to 86 nucleotides (Fig. 3). Each of these begins with the dinucleotide GT and ends with the trinucleotide CAG. These features are conserved in many other protein-coding genes that have been sequenced from *Rhodotorula* and *Rhodosporidium* species (Filipula et al., 1988, Rasmussen and Orum, 1991). The introns within these genes also contain a conserved sequence, CTGAC that presumably defines the RNA splicing branch point and bears some similarity to the conserved TACTAAC branch point sequence found in *Saccharomyces* introns (Ruby and Abelson, 1991). The first and third introns in the D-mandelate dehydrogenase gene also contain this exact sequence, whereas no perfect match is found in the second introns (Fig. 4). However, in later case, the sequence CTCAC is found, which has a single mismatch compared with the consensus. We have identified similar divergence from the consensus sequence in several introns within the L-mandelate dehydrogenase coding sequence from *R. graminis* (Illias et al., 1998).

**Amino acid sequence:** The cDNA encoding D-mandelate dehydrogenase predicts a protein of 351 amino acids with a calculated molecular weight of 38591 Daltons. This compares with the molecular weight of 38 KD found by SDS-PAGE (Fawson and Baker, 1989). A computer search of protein sequence data banks with the program FASTA, using the D-mandelate dehydrogenase as the query sequence, indicated extensive amino acid sequence similarity with a range of D-2-hydroxy acid dehydrogenases. Alignment with other proteins in the Swissprot database demonstrated that *Rhodotorula graminis* D-mandelate dehydrogenase exhibits 27-33% identity to each of: the D-3-phosphoglycerate dehydrogenase from *Haemophilus influenzae* (SER5_HAELN), D-glycerate dehydrogenase from *Hyphomicrobium methyllovorum* (DHGY_HYFM), D-lactate dehydrogenase from *Lactobacillus delbrueckii* (LDHD_LACDE), formate dehydrogenase from *Hansenula polymorpha* (FDH_HANPO), D-3-phosphoglycerate dehydrogenase from *E. coli* (SER8_ECOLI), formate dehydrogenase from *Emericella nidulans* (FDH_NEUCI), formate dehydrogenase from *Neurospora crassa* (FDH_NEUCF), D-lactate dehydrogenase from *Lactobacillus casei* (DLD2_LACCA) and D-3-phosphoglycerate dehydrogenase from *S. cerevisiae* (SER5_YEAST). All of these enzymes utilize 2-D-hydroxy acids as substrate with the exception of formate, which has a single carbon atom. D-mandelate dehydrogenase from *Rhodotorula graminis* thus clearly belongs to the D-isomer specific 2-hydroxy acid dehydrogenase family. The two closest known relatives to D-mandelate dehydrogenase appear to be from other yeast but are sequences identified from genome sequencing projects. These are the putative products of a gene on the *Saccharomyces cerevisiae* chromosome XIV (EMBL accession number: Z71559; *Saccharomyces Genome Database reference YNL274C*) and a gene from *Schizosaccharomyces pombe* (accession number: D99185). The predicted amino acid sequences show 33% identity with D-mandelate dehydrogenase over 340 amino acids but are more closely related to each other with 46% identity. The substrates for these two genes are currently unknown. Multiple alignments of the sequences of D-mandelate dehydrogenase with other members of the family clearly show their relations with several amino acid residues being strictly conserved in each protein (Fig. 5). The crystal structures of three members of D-2-hydroxy acid dehydrogenase family have been determined. D-lactate dehydrogenase from *Lactobacillus pentosus*, formate dehydrogenase from *Pseudomonas* sp. 101 (Lamzin et al., 1992, Lamzin et al., 1994) and D-glycerate dehydrogenase from *Hyphomicrobium methyllovorum* (Golberg et al., 1994) each have a two-domain structure that is typical of NAD+-dependent enzymes. Sequence alignment indicates that the
Fig. 5: Multiple alignments of the sequenced D-mandelate dehydrogenase and other D-2-hydroxy acid dehydrogenases. The sequence of D-mandelate dehydrogenase from R. graminis (dmadh), D-glycerate dehydrogenase from Hyphomicrobium methyllovorum (gldh; Swissprot: DHGY_HYPM), formaldehyde dehydrogenase from Pseudomonas sp.101 (fdh; Swissprot: FDH_PESR) and the predicted products (271556 and D89185) of reading frames identified in the genomes of Saccharomyces cerevisiae (Scer) and Schizosaccharomyces pombe (Spom.) were aligned using the MILEUP algorithm within the Wisconsin package. The asterisks above the aligned sequences denote residues that are identical in all five sequences. The numbering to the right indicates the dmadh residue number at the end of each line.

876
coenzyme-binding domain of D-mandela dehydrogenase comprises residues 111-307. The 'catalytic' domain is formed by both N- and C-terminal portions of the polyepptide chain (residues 1-110 and 308-351). Within the family of D-2-hydroxy acid dehydrogenases, the NAD+ binding domain is highly conserved than the catalytic domain. D-glycerate dehydrogenase was crystallized as the apo-protein whereas the structure of formate dehydrogenase was solved with both NAD+ and azide present. Azide is an inhibitor that presumably binds in place of formate (Lamzin et al., 1994). We can thus predict, by comparison with known structures and sequences that several residues are involved in NAD+ binding (Gly170, Gly172, Gin175, Asp194, Pro229) which is achieved by a remarkably similar topological arrangements not only in D-2-hydroxy acid dehydrogenase but also in other NAD+-dependent enzymes, despite a very low level of sequence similarity (Popov and Lamzin, 1994). In contrast, the catalytic subunits of D-2-hydroxy acid dehydrogenase are quite different in both sequence and structure from other dehydrogenase. Several residues have been identified as important for catalysis in the D-2-hydroxy acid dehydrogenase by examination of crystal structures (Lamzin et al., 1992; Goldberg et al., 1994), chemical modification (Kochhar et al., 1992b) and analysis of mutant enzymes (Kochhar et al., 1992a). The catalytically important residues (His304, Glu286 and Arg257) are conserved in D-mandelate dehydrogenase.

Expression of D-mandelate dehydrogenase in E. coli: The D-mandelate dehydrogenase cDNA in pR13 was transferred into the expression vector pRC23 (Crowl et al., 1985) as an EcoRI BamHI fragment to generate pR16. The plasmid directs expression under control of the Pt promoter from bacteriophage λ but expression is repressed at 30°C in E. coli N17, which synthesizes the thermosensitive class protein (Stanley and Luzio, 1984). After growth to mid-exponential phase (OD600 about 0.6), expression was induced by shifting to 42°C and continuing growth overnight. Expression of D-mandelate dehydrogenase was detected by Western blotting (data not shown). The protein was active with readily measurable activity for the reverse reaction; phenylglyoxylate-dependent oxidation of NADH. Specific activity of the recombinant D-mandelate dehydrogenase in crude extract is 0.0528 U/mg of total protein present. This activity was produced from a yield of 1370 mg total protein obtained from 17 gram wet cells. The development of this expression system opens the way to large-scale production of recombinant enzyme for more detailed biochemical and biophysical studies in addition to its potential use as a biocatalyst.

References