

Identification and Isolation of an Azoreductase from *Enterococcus faecium*

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Abstract

Azo dyes are commonly used in many commercial industries. Some of the azo dyes can produce carcinogenic compounds after being metabolized by azoreductase. Several human intestinal microbiota possess azoreductase activity which plays an important role in the toxicity and mutagenicity of these azo dye compounds. The *acpD* gene product (AzoEf1) responsible for the azoreductase activity of *Enterococcus faecium*, an intestinal bacterium, was heterologously expressed, purified and characterized. The protein sequence shares 67% identity with the azoreductase from *Enterococcus faecalis*, AzoA. Although AzoEf1 possesses many commonalities with AzoA, there are differences in coenzyme preference, residues associated with FMN binding, substrate specificity, and specific activity. AzoEf1 utilized both NADH and NADPH for the reduction of azo dyes, and it contains a leucyl residue at position 104 and threonyl residue at position 19 which differ from AzoA at the active site. Its specific activity was 5095 $\mu\text{M}/\text{min}/\text{mg}$ and its catalytic efficiency for Methyl red reduction was lower than AzoA.

Introduction

An important function associated with intestinal bacteria is their ability to metabolize xenobiotics, such as azo dyes (Chung, K.T. *et al.*, 1978; Brown, J.P. 1981; Cerniglia, C.E. *et al.*, 1982; Manning, B.W., Cerniglia, C.E. & Federle, T.W., 1985). Azo dyes are characterized by the presence of one or more azo (R1-N=N-R2) bonds, and are widely used in the paper, textile, plastic, pharmaceutical, food, cosmetic, enamels, and drug industries (Collier *et al.*, 1993; Dillon *et al.*, 1994; Levine, 1991). The introduction of some of these dyes into the human body results in the reduction of their azo bonds and subsequent biotransformation to carcinogenic metabolites (Cerniglia *et al.*, 1986; Bragger *et al.*, 1997; Brown *et al.*, 1983, Cerniglia, C.E., 1982). Numerous *in vivo* and *in vitro* experiments support the toxicity of these metabolites (Morgan *et al.*, 1984; Chung K.T. 1983). Several intestinal bacteria are known to have azoreductase activity from culture studies (Chung K-T. *et al.*, 1992b), thereby the isolation and characterization of azoreductase genes from these intestinal bacteria will provide information to their biochemical function within the cells and perhaps even allude to their evolutionary origin.

The azoreductase gene has been cloned, expressed and characterized from various intestinal organisms which include *Enterococcus faecalis* (Chen H. *et al.*, 2004),

Escherichia coli (Nakanishi M. *et al.*, 2001), *Shigella* (Ghosh, D.K. *et al.*), and *Staphylococcus aureus* (Chen, H. *et al.*). Interestingly, the properties of these azoreductases vary. For example, the azoreductase from *E. coli* is a 46 kDa homodimer, requires FMN as a cofactor, and uses NADH as an electron donor (Nakanishi M. *et al.*, 2001); compared to *Staphylococcus aureus* which is a tetramer and utilizes NADPH for azo dye reduction (ref). In addition, the deduced protein sequences do not share significant sequence homology.

E. faecalis is a prominent organism found in the intestine, and the function of its azoreductase (AzoA) has been well characterized (Chen, H. *et al.*, 2004, Punj, S. & John G.H., 2008b) including its structure, based on crystallization information (Liu, Z. & Chen, H. *et al.*, 2007 add to reference). On the other hand, the azoreductase activity of *E. faecium* is not as well characterized. Although *E. faecium* is closely related to *E. faecalis*, there are some variations in the whole cell reduction of azo dyes (Punj, S. & John G.H. 2008a, Chung, K.T. *et al.* b), suggesting potential azoreductase differences in terms of catalytic and functional requirements. In addition, it has been shown that the orthologous azoreductases from several species of *Bacillus* possess different substrate specificities (Sugiura W, *et al.*, 2006). Therefore, the present study focuses on characterizing the function of azoreductase from *E. faecium*, revealing catalytic differences between the closely related species, *E. faecalis*.

Methods

Bacterial strain, plasmids and culture conditions

Enterococcus faecium ATCC 6569 was grown in Brain Heart Infusion (BHI) broth supplemented with 0.05% (w/v) Vitamin K solution and 2% (w/v) yeast extract under anaerobic conditions at 37°C. The pCR2.1-TOPO (Invitrogen) plasmid was used for cloning the PCR fragment and pET-15b (Novagen) containing a hexa-histidine tag at the N-terminal was used for overexpression of the protein. For transformation, *E. coli* TOP10 (Invitrogen) competent cells and NovaBlue (DE3) (Novagen) cells were used. The *E. coli* strains were grown in Luria-Bertani (LB) medium containing 50 μg ampicillin (Amp) ml^{-1} at 37°C.

Genome analysis and PCR amplification of the *acpD* gene

Genomic DNA was extracted from *E. faecium* using phenol-chloroform (Wilson 2001). The protein-protein BLAST tool at the National Center for Biotechnology Information (NCBI) was used to perform homology searches. The AzoA protein sequence (GenBank - AAR38851.1) of *E. faecalis* was used as the query sequence to identify a similar protein from the data base of *E. faecium*. A putative *acpD* gene product was identified and the *acpD* gene, renamed *azoEf1* was amplified using PCR with the *E. faecium* genomic DNA as template. The forward primer (AzoEf1f) 5'GGGGAGAAGGTAAGCATATGGCATCTG

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3'contained the *NdeI* restriction site upstream of the start codon, and the reverse primer (AzoEf1r) 5'CCCCAAAAGAGGGATCCGGGAAAATATCC 3'contained the *BamHI* site downstream of the stop codon. The PCR was performed in a Perkin Elmer DNA Thermal Cycler 480, and the conditions were 30 cycles: each cycle consisting of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 mins. The PCR reaction mix contained sterile distilled water, 1X PCR buffer (Invitrogen), 1.5 mM Magnesium chloride (Invitrogen), 0.2 mM dNTP mix (Fischer Scientific), 10 ng genomic DNA, 50 pmol of forward and reverse primers, 2.5 Units Taq DNA polymerase (Invitrogen) in a final reaction volume of 100 μ L. The amplified PCR product was run on a 0.8 % (w/v) agarose gel and the DNA eluted from the gel using the QIAquick® Gel Extraction kit (Qiagen).

Cloning and expression of *AzoEf1*

The amplified PCR product was directly cloned into the pCR2.1-TOPO vector which was subsequently transformed into *E. coli* TOP10 cells. The recombinant plasmid was extracted using the QIAprep® Spin Miniprep kit (Qiagen) and digested with the restriction enzymes *NdeI* and *BamHI* (Invitrogen) to release the insert. The released insert was eluted from a 0.8 % (w/v) agarose gel and the purified DNA was then ligated into the pET-15b vector, using a 1:6 vector to insert ratio. Ligation was carried out by T4 DNA ligase (Invitrogen) at 4°C for 16 h. The recombinant plasmid, pAzoEf1 was transformed into NovaBlue (DE3) *E. coli* expression cells. The *azoEf1* gene was sequenced from the pET-15b using the T7 promoter and terminator primers (Novagen).

Purification of the enzyme

NovaBlue (DE3) cells containing pAzoEf1 were inoculated in a LB/Amp (100 μ g ml⁻¹) medium and incubated overnight

at 37°C at 300 rpm. The cells were harvested, resuspended in 1 ml LB/Amp (100 μ g ml⁻¹) and transferred to 1 L of LB/Amp (100 μ g ml⁻¹). The 1 L culture was incubated at 37°C 300 rpm. Upon reaching an OD₆₀₀ = 0.6, the cells were induced for 3 h with Isopropyl-1-thio-P-galactopyranoside (IPTG), final concentration of 1 mM. The induced culture was centrifuged at 6000 x g for 10 mins at 4°C to harvest the cells. The supernatant was discarded, and the pellet was frozen at -20°C.

The pellet was thawed, and resuspended in 10 ml lysis/wash buffer (20% glycerol, 50 mM sodium monobasic phosphate, 750mM sodium chloride, 20mM Imidazole in double-distilled water pH, 8.0) Lysozyme (final concentration of 0.1 mg ml⁻¹) was then added and the sample was incubated on ice for 30 mins. The cells were disrupted by sonication using a Sonic 300 dismembrator with an intermediate tip and relative output of 60%. Sonication was performed 10 times for 5 sec on ice. The sample was centrifuged at 10,000 x g for 30 min at 4°C to remove cell debris. The supernatant was then subjected to batch purification using 50% Ni-NTA slurry (50% suspension in 30% ethanol, precharged with Ni²⁺).

All protein purification steps were carried out at 4°C. In a polypropylene test tube, 4 ml of supernatant was mixed with 1 ml of the Ni-NTA slurry. The 50% Ni-NTA slurry was washed with lysis/wash buffer to remove ethanol. To allow the protein to bind to the resin, the tubes containing the samples were fixed horizontally to Abidance (New Brunswick Scientific) and incubated for 1 h with constant rotation. The sample was centrifuged for 2 min at 3800 rpm using a Sorvall SS-34 rotor. The pellet was then washed three times with lysis/wash buffer, and centrifuged as above. The protein was eluted by resuspending the pellet in 1.5 ml of elution buffer (20% glycerol, 50 mM sodium monobasic phosphate, 750 mM sodium chloride, 250 mM Imidazole in double-distilled water, pH 8.0) and centrifuged at 3900 rpm using a Sorvall SS-34 rotor. This step was repeated

Table 1. Specific activity^a of AzoM using different substrates

Substrate	NADH (μ M min ⁻¹ mg ⁻¹)	NADPH (μ M min ⁻¹ mg ⁻¹)
Methyl Red	5095 (\pm 151.5)	24.29 (\pm 1.507)
Congo Red	682.2 (\pm 33.78)	17.02 (\pm 3.496)
Acid Red 88	76.63 (\pm 3.992)	23.23 (\pm 1.819)
Ponceau BS	20.70 (\pm 0.916)	ND ^b
Orange II	18.18 (\pm 1.350)	ND
Direct Blue 15	3.699 (\pm 0.591)	3.575 (\pm 2.335)
Ponceau S	ND	ND
Tartrazine	ND	ND
Amaranth	ND	ND

^aAll tests were performed at room temperature, pH 7.0, with 20 μ M of each dye, 0.1 mM NADH or NADPH without externally added FMN. Results are reported with four significant figures. 1 μ M of azo dye degraded per min per mg protein.

^bND – not detected (<1 μ M min⁻¹ mg⁻¹)

four times and the protein was collected after each elution. All samples were stored at -20°C. The concentration of the protein was determined using a calibration curve using bovine serum albumin (BSA) as a standard curve.

SDS-PAGE

SDS-PAGE analysis was performed as previously described (Laemmli, 1970). A 1 ml culture (induced and noninduced) was collected and the cells were prepared for analysis by adding a final 1X concentration of SDS-PAGE loading buffer (4X=0.06 M Tris-HCL, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) 2 β -mercaptoethanol, 0.0025% (w/v) bromophenol blue), boiling the sample for 15 min, and loading 20 μ l onto a 12.5% SDS-PAGE gel to confirm the expression of the protein.

Enzyme assay

The assay was carried out in a polystyrene semi-micro cuvette (Sigma-Aldrich, St. Louis, MO) in a total reaction volume of 1 ml. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 20 μ M of azo dye and enzyme (0.5-1 μ g). The reaction was initiated by the addition of 0.1 mM NAD(P)H. The extinction coefficient for Methyl Red at 430 nm was 23,360 M⁻¹cm⁻¹. The concentrations for the remaining dyes in Table 1 were calculated from a calibration curve for each dye. The enzyme activity was measured by a decrease in absorbance over a 2 min period using a UV-visible spectrophotometer (UV-1601PC, Shimadzu). The enzyme activity was defined as the decrease in azo dye concentration (μ M) per min (For the control, the enzyme was denatured by boiling for 15 mins, followed by the addition of a few drops of hydrochloric acid (HCl).

A

<i>E. faecium</i>	1	MA SVLVVVKGHPLTAEE SRTVKALTSFLTSYKENHPDDEVTVLELYR--DD
<i>E. faecalis</i>	1	MSKLLVVKAHPLTKEE SRSVRALETFLASYRETNE SDEIEILDVYAPETN
<i>E. faecium</i>	49	IPEIDEELLSGWEALRAGAEFTSLSESQQQKIARFNE LTDQFLAADKIVI
<i>E. faecalis</i>	51	MPEIDEELLSAWGALRAGAEFTLSENQQQKVAREFNE LTDQFLSADKIVI
<i>E. faecium</i>	99	ANALWNLNIPTKLKAWFDTVNVAGKTFRYTENGPEGLVTGKKALHIQSN
<i>E. faecalis</i>	101	ANPMWNLNVPTRLKAWFDTVNVAGKTFQYTAEGPKELTSGKKALHIQSN
<i>E. faecium</i>	149	GVYNGQDFASQYVKGILNFGIDQVDQLFIEGIDYDPDRADELMQNALDK
<i>E. faecalis</i>	151	GFYEGKDFASQYIKAILNFGVDQVDGLFIEGIDHFPDRAEELLNTAMTK
<i>E. faecium</i>	199	AAALGKSF
<i>E. faecalis</i>	201	ATEYGKTF

B

AzoR <i>E. coli</i>	1	MSKVLVVKSSILAG-YSQSNQLSDYFVEQWREKHSAD EITVRDLAANEIP
acpD <i>E. faecium</i>	1	MA SVLVVVKGHPLTAEE SRTVKALTSFLTSYKENHPDDEVTVLELYRDDIP
AzoR <i>E. coli</i>	50	VLDGELVG---ALRP--SDA BLTPRQCEALALSDELIAELKAHDVIVIAA
acpD <i>E. faecium</i>	51	EIDEELLSGWEALRAGAEFTSLSESQQQKIARFNE LTDQFLAADKIVIAN
AzoR <i>E. coli</i>	95	PMYNFNISTQLKNYFDLVARAGVTFRYTENGPEGLVTGKKALVITSRGGI
acpD <i>E. faecium</i>	101	ALWNLNIPTKLKAWFDTVNVAGKTFRYTENGPEGLVTGKKALHIQSN
AzoR <i>E. coli</i>	145	HKDGP TDLVTPYLLSTFLGF IGITDVK FVFAEG IAYGPBMAAKAQSDAKAA
acpD <i>E. faecium</i>	151	YNG--QDFASQYVKGILNFGIDQVDQLFIEGIDYDPDRADELMQNALDK
AzoR <i>E. coli</i>	195	IDSIVSA-
acpD <i>E. faecium</i>	199	AAALGKSF

Fig. 1. Amino acid sequence homology. a) 67% sequence identity between *E. faecium* AcpD and *E. faecalis* AzoA, b) 42% sequence identity between *E. coli* AzoR and *E. faecium* AcpD.

Table 2. Apparent Michaelis constants (K_m) and maximal velocities (V_{max}) of recombinant AzoEf1*

Substrate	K_m (μM)	V_{max} ($\mu\text{M}/\text{min}/\text{mg}$ protein)	k_{cat} (s^{-1})	k_{cat}/K_m $\mu\text{M}^{-1}\text{s}^{-1}$
Methyl red	16.4 (\pm 2.2)	22410 (\pm 1557)	17	1
NADH	171.4 (\pm 27.9)	13555 (\pm 1605)	10	0.06

*Measurements with respect to methyl red; measured at 430nm.

Effect of pH and temperature on enzyme activity

To determine the effect of pH on the activity of the enzyme, a pH range from 4.0 to 9.0 was used. The three different buffer systems used for the experiments were: pH 4 and 5 sodium acetate (pKa 4.76), pH 6 and 7 potassium phosphate (pKa 6.86) and pH 8 and 9 Tris- HCl (pKa 8.06). For the temperature study, four different temperatures (12°C, 28°C, 38°C and 53°C) were tested. All reaction mixtures were preincubated at the selected pH and temperature for five minutes. The reaction was initiated by the addition of 0.1 mM NADH. Specific activities for each pH and temperature were determined as above.

Results

Identification of the *E. faecium* *acpD* gene

The AzoA protein sequence from *E. faecalis* and the AzoR from *Escherichia coli* were initially identified as an acyl carrier protein phosphodiesterase (AcpD). Thus, the AzoA was used as the query in a BLAST search against the *E. faecium* (strain DO) database. The search identified a single AcpD protein that was 206 amino acids in length and showed 67% primary structure identity with AzoA (Fig. 1a). The amino acid residues involved in FMN binding, leu at position 104 and thr residue at position 19 differ from Ser-Met-O and Ser in AzoA respectively (Liu ZJ *et al*, 2007. In

comparison with the AzoR from *Escherichia coli*, a 42% identity was observed (Fig. 1b).

Cloning, expression and purification of the *acpD* gene from *E. faecium*

Specific primers designed to amplify the *acpD* gene, renamed as *azoEf1*, successfully amplified the predicted 730 bp fragment from the genomic DNA of *E. faecium*. The amplicon was directly cloned into the TOPO-TA vector and the sequencing results showed that the insert contained the complete open reading frame (ORF) for the gene. However, the ATCC 6569 strain contained 10 base pair differences resulting in two amino acid changes in the insert when compared to the sequence from *E. faecium* strain DO. The amino acid changes were at residues 88 and 174 (both Glutamic acid to Aspartic acid). Reamplification and sequencing results revealed the same differences. The sequence was deposited into GenBank (accession number GQ479040). The pET-15b vector was used for overexpression and purification of the recombinant protein in DE3 *E. coli*.

The crude induced DE3 cell extract exhibited azoreductase activity and yielded a yellow coloration (data not shown). The protein was purified using Ni-NTA resin, and the concentration of the protein was determined to be 12.5 mg ml⁻¹. The SDS-PAGE gel showed the protein to be approximately 23 kDa in size (Fig. 2). The purified protein also possessed a yellow coloration and the absorbance spectrum yielded a typical flavoprotein peak at 457 nm which is slightly shifted from the free flavins which have a peak at 445 nm (data not shown).

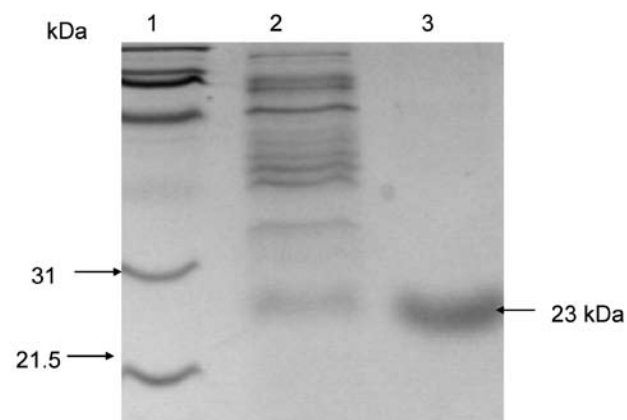


Fig. 2. SDS-PAGE showing the induced, purified and dialyzed 23kDa protein. Lane 1: SDS-PAGE Broad range marker, Lane 2: induced cell extract, Lane 3: Purified AzoEf1.

Substrate specificity

Table 1 shows that AzoEf1 utilizes either NADH or NADPH to reduce Methyl Red, Congo Red, Acid Red 88, and Direct Blue 15. However, Ponceau BS and Orange II were reduced in the presence of NADH only. The specific activity was highest for Methyl Red (5095 $\mu\text{M min}^{-1} \text{mg}^{-1}$) when NADH was used as the electron donor. The specific activity in the presence of NADPH for Methyl Red, Congo Red, Acid Red 88, were significantly lower. Interestingly, the specific activity for Direct Blue was the same for both NADH and NADPH (approximately 3.6 $\mu\text{M min}^{-1} \text{mg}^{-1}$). No activity was observed for Ponceau S, Tartrazine, and Amaranth in the presence of either electron donors. The optimal pH was determined to be between 7.0 and 8.0 at 25°C for the reduction of Methyl Red using NADH as the electron donor,

and the optimum temperature was approximately 38°C (data not shown).

Apparent kinetic constants

The enzyme reactions were conducted by varying the concentration of one substrate, Methyl red or NADH, and maintaining a fix concentration of the other substrate. The K_m and V_{max} values were obtained from Lineweaver-Burk plots. As shown in Table 2, the enzyme possesses greater activity and affinity for Methyl Red as indicated by its higher turnover number ($k_{cat} = 17$) and catalytic efficiency ($k_{cat}/K_m = 1$) compared to NADH ($k_{cat} = 10$, $k_{cat}/K_m = 0.06$) as a substrate. No external addition of FMN was required for the assays which suggested that the FMN moieties already bound to the enzyme was sufficient for its activity.

Discussion

Knowledge of bacterial azoreductase is not fully understood. Azoreductase reductively cleaves a wide range of azo dyes and their activity is associated with numerous intestinal microorganisms, including the genera *Enterococci* (Chung K.T. *et al.*, 1992 b, Walker R. *et al.*, 1971). The isolation and characterization of azoreductase genes from these prominent Gram positive cocci is lacking, thereby their catalytic function and influence on human health is not fully understood. To date, only *E. faecalis* has been shown to possess an *azoA* gene, which encodes for an azoreductase that has broad substrate specificity.

The current study investigated the azoreductase activity from a related intestinal microorganism *E. faecium* which was shown to grow without inhibition in azo dyes (data not shown), as well as to reduce mono and bis-azo sulfonated azo dyes, indicating azoreductase was functionally expressed in the bacterium. Interestingly, the azoreductase genes from *E. coli* and *E. faecalis* were initially described as a putative acyl carrier protein phosphodiesterase (AcpD). Therefore, the protein BLAST search used the azoreductase AzoA from *E. faecalis* to identify an AcpD conserved domain protein from the *E. faecium* genomic database (DO strain). Interestingly, the ATCC 6569 culture strain had 10 base pair differences with the *E. faecium* DO strain, resulting in two amino acid changes at residues 88 (Glx>Asx) and 174 (Glx>Asx). The putative azoreductase identified (AzoEF1) showed 67% amino acid sequence identity with AzoA from *E. faecalis*. The purified AzoEf1 azoreductase is suggested to be associated with FMN as the absorption peaks were similar to a flavodoxin fingerprint motif (Chen.H., *et al.*, 2004 and 2005). AzoEf1 reduced 6 of the 9 azo dyes tested (Table 1) and did not require any exogenous addition of FMN.

In contrast to AzoA (Chen.H *et al.*, 2004), AzoEf1 did not reduce Amaranth and Ponceau S (Table I). This may be due to subtle differences in the active site of AzoEf1 compared to AzoA. AzoEf1 reduced the mono azo dye Methyl red in the presence of both cofactors NADH and NADPH but the specific activity for NADH was much greater. AzoA was also shown to prefer NADH over NADPH as the electron donor for the reduction of Methyl red with a similar K_m but with almost twice the catalytic efficiency for the dye (Punj S. & John GH, 2008b).

In conclusion, our results showed that although AzoEf1 shares many similarities with AzoA, critical alterations in the active site of these enzymes can alter substrate specificity and catalytic rates. In spite of the close evolutionary link

between AzoA and AzoEf1, a further investigation of the differences in the active site of these two enzymes will provide a better understanding of the distinguishing factors involved in substrate specificity of azoreductase. Further, AzoEf1 has the ability to use both NADH and NADPH as an electron donor. The azo dye specificity showed differences as AzoEf1 was not specific for Panceau S and Amaranth, while AzoA has been reported to show activity for those azo dyes. Finally, the presence of an azoreductase in these two intestinal microorganisms is likely to play an important and varying role in azo dye metabolism in the intestine, and possibly impact human health.

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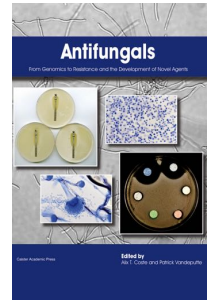
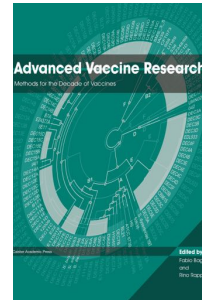
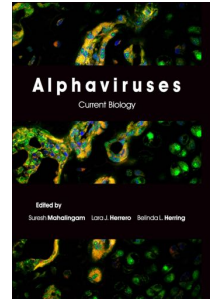
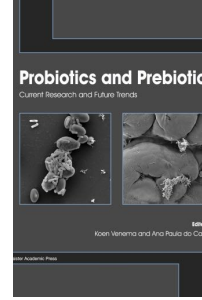
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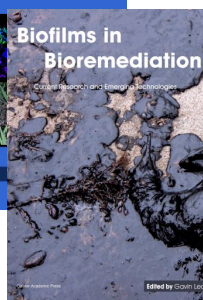
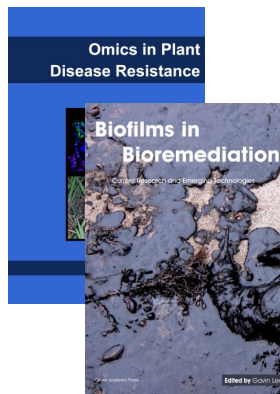
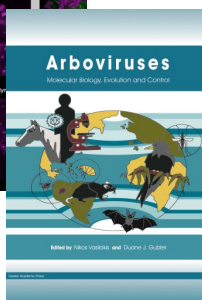
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