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Cloning, expression and characterization of catechol 1,2-dioxygenase from *Burkholderia cepacia*

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Abstract

The present study reports on the cloning, expression and characterization of catechol 1,2-dioxygenase (CAT) of bacterial strains isolated from dioxin-contaminated soils in Vietnam. Two isolated bacterial strains DF2 and DF4 were identified as *Burkholderia cepacia* based on their 16S rRNA sequences. Their genes coding CAT was amplified with a specific pair of primers. Recombinant CAT (rCAT) was expressed in *E. coli* M15 cells and its activity was confirmed by the detection of *cis,cis*-muconic acid, a product from catechol, by high-performance liquid chromatography (HPLC) analysis. The rCAT of DF4 had an optimal pH and temperature of 7 and 30ºC, respectively. Metal ions, such as Zn^{2+} and Mn^{2+}, and surfactants, such as SDS, Tween 20 and Triton X100, strongly inhibited enzyme activity, while K^{+} slightly increased the activity.

Keywords

Burkholderia cepacia, catechol 1,2-dioxygenase, *cat* gene, dibenzofuran

Introduction

Dioxins are a large group of chloroaromatic compounds, mainly consisting of polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyl. It is well known that dioxins are harmful to human health, causing birth defects, mutagenesis and carcinogenesis (Hiraishi, 2003; Wang et al., 2004; Kishida et al., 2010). In particular, dioxins can be degraded by physicochemical techniques including thermal remediation, photodegradation, hydrolysis and dichlorination using metal catalysts. However, the application of these treatments is still challenging (Hiraishi, 2003).

Recently, a biological approach to dioxin degradation, including phytoremediation, and microbial and fungal degradation, has attracted many studies, due to their capability to transform, and further detoxify dioxins and dioxin-like compounds (Hiraishi, 2003; Chang, 2008; Rodenburg et al., 2015; Lopez-Echartea et al., 2016). Several soil bacteria have exhibited dioxin degradation, such as *Burkholderia* sp. (Arfmann et al., 1997; L'Abbee et al., 2005), *Sphingomonas* sp. (Arfmann et al., 1997; Chai et al., 2016), *Pseudomonas* sp. (Hong et al., 2004; Jaiswal et al., 2011), *Ralstonia* sp. (Wesche et al., 2005), *Janibacter terrae* (Jin et al., 2006), *Comamonas* sp. (Ji et al., 2017). One of the intermediate compounds in the degradation pathway of dioxin-like compounds is catechol, which is toxic by dermal and oral routes of exposure, harmful if inhaled, causes skin irritation and serious eye damage, may cause an allergic skin reaction, and is suspected of causing genetic defects.

Catechol 1,2-dioxygenase [EC 1.13.11.1] catalyzes the intradiol cleavage of the aromatic ring at 1,2-(ortho) position of catechol, yielding *cis,cis*-muconic acid. It has been reported that catechol 1,2-dioxygenase has a great bioremediation potential to remove wastewater contaminated with phenol, benzoate, fluorocatechol, bromocatechol, chrlorocatechol, methylcatechol, herbicides (diuron), polychlorinated biphenyls, chloroethanes and others (Durána and Esposito, 2000; Silva et al., 2013). This enzyme can be found in bacteria such as *Mycobacterium fortuitum* (Silva et al., 2013), *Candida tropicalis* (Long et al., 2016), *Rhodococcus ruber* (Wang et al., 2017), *Pseudomonas putida* (Li et al., 2018) and others. Because Vietnamese soil has been contaminated with dioxins for a long time, it is possible that there are better CAT degraders as well as dioxin degraders (Thanh et al., 2019).

The present study concerns the cloning and expression of the catechol 1,2-dioxygenase (*cat*) gene involved in catechol degeneration, from *Burkholderia cepacia* strains isolated from dioxin-contaminated soils in Vietnam.

Materials and methods

Isolation of dibenzofuran-degrading bacteria

A total of 21 soil samples were collected in sterile polythene bags from the topsoil of 5 different places in the Aso area (16^o13'38.3"N and 107^o16'03.5"E, 16^o13'38.7"N and 107^o16'03.6"E, 16^o13'39.0"N and 107^o16'04.3"E, 16^o13'39.2"N and 107^o16'03.7"E, and 16^o13'38.7"N and 107^o16'04.3"E), A Luoi district (Thua Thien Hue province, Vietnam). Potential dibenzofuran degrading bacteria were isolated as described by Hong et al (2004) with slight modifications. 200 mg of each soil sample was put in 5 mL of minimal salt medium (MSM, per liter: 3.5 g $Na_2HPO_4.2H_2O$, 1 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2.6H_2O$, 0.05 g $Ca(NO_3)_2.4H_2O$, and trace elements such as EDTA, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, H_3BO_3, $C°Cl_2.6H_2O$, $CuCl_2.2H_2O$, $NiCl_2.6H_2O$ and $Na_2MoO_4.2H_2O$) supplemented with 0.5 mM dibenzofuran (Sigma-Aldrich, Singapore) as the carbon source for microbial growth, and incubated at $30^{°C}$ with a shaking speed of 185 rpm for 7 days. 100 µL of supernatant was then transferred into fresh medium and the previous step was repeated. After repeating this three times, 100 µL of from the final step was spread onto agar plates having the same composition as the liquid medium, and incubated at $30^{°C}$ for 7 days. Colonies were isolated from the agar plates subcultured in 5 mL of LB medium and incubated under the same conditions of temperature and shaking speed as the culture in the liquid MSM medium. Bacterial genomic DNA was extracted by the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) and 16s rRNA sequences of isolates were used for molecular identification.

Cloning catechol 1,2-dioxygenase

A gene encoding catechol 1,2-dioxygenase (*cat*) involved in dibenzofuran degradation was searched for available genomic databases in the GenBank. A set of specific primers with forward sequence is 5'-GATCCCCACACGACAACCGACA-3' and reverse sequence is 5'-TCCTTGCGTTGTCGATTCGTCG-3' was designed based on the full-length *cat* gene of *B. cenocepacia* for PCR amplification. PCR was performed with 40-ng genomic DNA, 10-pmol each primer and 1× PCR Master Mix (Promega, Madison, WI, USA) in a total volume of 25 µL. The PCR condition was: an initial denaturation at $95^{°C}$ for 10 min; 30 cycles of $95^{°C}$ for 30 s, $55^{°C}$ for 60 s, and $72^{°C}$ for 90 s; and a final extension of $72^{°C}$ for 10 min. The amplicon was purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and cloned in pGEM-T Easy vector (Promega). The recombinant vector was then

introduced into *Escherichia coli* TOP10 by a heat-shock method (Sambrook and Russell, 2011). The nucleotide sequence of the amplicon was analyzed by the dideoxy chain termination method on the Applied Biosystem 3130 (Thermo Fisher Scientific, Waltham, MA, USA).

Expression of the catechol 1,2-dioxygenase gene

A set of specific primers was designed for PCR amplification of the coding DNA sequence region of the *cat* gene with overhang recognition sites of *Bam*HI and *Hin*dIII at the 5' end (underlined), in which the forward primer is 5'-GGATCCATGGACAAGCAAGCCA-3' and the reverse primer is 5'-AAGCTTCGCCTGCGCGCGGCCT-3'. pQE-30 vector harboring His-tag for purification (Qiagen, Hilden, Germany) after linearization by *Bam*HI and *Hin*dIII was fused with the amplicon as mentioned above and then introduced into *E. coli* strain M15 (Qiagen, Hilden, Germany) by a heat-shock method (Sambrook and Russell, 2011). Transformed *E. coli* cells were proliferated in 5 mL of LB (Luria-Bertani) medium containing 50 μg/mL ampicillin and 50 μg/mL kanamycin at 37°C with a shaking speed of 190 rpm overnight. 50 μL of overnight culture was then subcultured in the same medium to continue growth until the OD_{600} reaches a value of 0.5 to 0.8. Expression of the *cat* gene was induced by the addition of 0.5 mM of IPTG during 4 h. The cell biomass was harvested by centrifugation at 4°C (6,000 rpm for 10 min), then washed twice with 100 mM sodium phosphate buffer (pH 7). The cells were ground in liquid nitrogen until a fine powder resulted, and then 1 mL of 100 mM sodium phosphate buffer (pH 7) was added and well mixed. Following this, the total soluble protein was recovered by centrifugation at 4°C (13,000 rpm for 25 min). Recombinant enzyme with His-tag was purified by mixing the extracted total soluble protein with Ni-NTA agarose. Ni^{2+} ion was used as a ligand in agarose chromatography column to link with an affinity tag (His-tag) of enzyme. The procedure of purification was performed following manufacture's instruction (Qiagen, Hilden, Germany). The purified enzyme was qualified by SDS-12% PAGE (polyacrylamide gel electrophoresis) and investigated the biochemical properties as describing below.

Enzymatic assay

Catechol 1,2-dioxygenase activity was determined spectrophotometrically by measuring the amount of *cis*,*cis*-muconic acid released during the reaction, as described by Long et al. (2016). 10 μL of enzyme was mixed with 980 μL of 100-mM sodium phosphate buffer (pH

7) and 10-mM β-mercaptoethanol, and the mixture was then pre-incubated at 30°C for 10 min. The reaction was initiated by adding 10 µL of 20-mM pyrocatechol (Merck, Darmstadt, Germany) as a substrate (Haroune et al., 2002) and incubated at 30°C for 10 min. The formation of *cis*,*cis*-muconic acid was determined at a wavelength of 260 nm by a UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). *cis*,*cis*-muconic acid purchased from Sigma-Aldrich (Singapore) was used as the standard. One unit of catechol 1,2-dioxygenase was defined as the amount of enzyme required to release 1 nmol of *cis*,*cis*-muconic acid per min at 30°C . The total soluble protein concentration was estimated by Bradford's method (Bradford, 1976) with serum albumin as the standard. The specific activity of catechol 1,2-dioxygenase was obtained by dividing the units of enzyme by the total soluble protein in the sample.

Characterization of catechol 1,2-dioxygenase

The effect of pH (5-11) on the activity of the enzyme was tested with a sodium phosphate buffer (pH 5-7), a Tris-HCl buffer (pH 8-9), and Tris-NaOH (pH 10-11). The preparation of the sodium phosphate buffer was as follows: 0.69 g of $NaH_2PO_4.H_2O$ was dissolved in 100 mL of distilled water (acid component of buffer). 1.06 g of Na_2PO_4 was dissolved in 150 mL of distilled water (base component of buffer). The pH was adjusted to be 5-7 by slowly adding acid to base. To evaluate the effect of temperature, the enzyme was pre-incubated in the optimal pH buffer at 15-50°C for 10 min. Metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+} and Zn^{2+}) and surfactants (SDS, Tween 20 and Triton X100) were used for the investigation of their effect on the enzymatic activity. The enzyme was pre-incubated with 5 mM of metal ion or surfactant for 10 min at the optimum temperature and pH (Nadaf and Ghosh, 2011), and the remaining activity of enzyme was measured as described above.

High performance liquid chromatography

Catechol was added to 5 mL of the purified catechol 1,2-dioxygenase to a final concentration of 1 mM. The reaction mixture was incubated at 30°C for 2 h and then acidified to pH 2 by adding HCl and extracted by ethyl acetate with a ratio 1:1. Anhydrous Na_2SO_4 was added to the mixture, and then the reaction solution was dried at 40°C and dissolved in 2.5 mL of ethanol. The amount of *cis*,*cis*-muconic acid was determined by reversed phase HPLC analysis, using a C18 column (5 µm, 4.6 × 250 mm) with a photodiode detector, a flow-rate of 0.8 mL/min and a runtime of 60 min. Mobile phase compositions were 1% acetic acid in water and 1% acetic acid in methanol (90:10, v/v). The stationary phase was silica gel. The

cis,*cis*-muconic acid was detected at a wavelength of 260 nm (Long et al., 2016). The HPLC analysis was carried out at an ambient temperature on an LC-20 Prominence system (Shimadzu, Kyoto, Japan) with an SPD-20A UV-VIS detector using an LC-Solution software. All solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany). *cis*,*cis*-muconic acid (Sigma-Aldrich) was used as the standard for the determination of the *cis,cis*-muconic acid content released in the reaction.

Results and discussion

Bacterial isolation and screening

A total of more than 200 dibenzofuran-positive colonies was obtained from different sampling places in the Aso area, including fungi and bacteria. Among the isolates, six bacterial colonies that showed the strongest growth were chosen for further identification. Data from the sequencing of the 16S rRNA gene of these colonies, and phylogenetic tree analysis (Fig. 1) indicated that they included two strains of *Burkholderia cepacia* complex, named DF2 (Accession number in NCBI: MG768914) and DF4 (MG768915), and several other strains such as *Enterobacter cloacae* DF3 (MG774409), *Staphylococcus* sp. DF5 (MG774408), *Achromobacter* sp. DF6 (MG774410) and *Pseudomonas* sp. DF1 (MG774407). *Burkholderia cepacia* DF2 and DF4 were used to isolate the *cat* gene which is involved in the pathway of dibenzofuran metabolism (Wang et al., 2004).

Cloning catechol 1,2-dioxygenase gene

The 16S rRNA gene sequences of *B. cepacia* DF2 and DF4 showed high identities with that of *B. cenocepacia* MSMB384WGS from GenBank database, 100% and 99%, respectively. The reference strain MSMB384WGS has a *cat* gene encoded catechol 1,2-dioxygenase. The specific primers for the *cat* gene in *B. cepacia* were designed for PCR amplification, and putative *cat* genes of *B. cepacia* DF2 and DF4 were amplified. The nucleotide sequences of them (both being 903 bp) indicated that the two putative *cat* genes of DF2 and DF4 shared 99% identity at the nucleotide sequence level (MF953296 and MF953301, respectively), while they showed a 93% identity with that of MSMB384WGS. The phylogenetic tree of *cat* genes from DF2, DF4, and some other microorganisms, are shown in Fig. 2.

The deduced amino acid alignment from the two *cat* genes of DF2 and DF4 indicated that they had two different amino acids at the positions of 175 and 180 (Fig. 3). The protein models of CAT from DF2 and DF4 were analyzed by the Protein Homology/analogY

Recognition Engine program (ver. 2.0) (Kelley et al., 2015). The results showed that these two CAT enzymes had high relevant levels with deduced CAT enzymes from *B. cenocepacia* (AOK37598), *Pseudomonas arvilla* (PDB: 2AZQ), *Acinetobacter radioresistens* (PDB: 2XSU), *Burkholderia vietnamiensis* (PDB: 5TD3), and *R. opacus* (PDB: 3HJ8) (Fig. 3). Comparison of a putative three-dimensional structure of CAT enzymes of the two strains, DF2 and DF4, with that of *P. arvilla*, shows that four amino acid residues for Fe ligand were conserved at the positions of 156 (tyrosine), 190 (tyrosine), 214 (histidine), and 216 (histidine) (Matera et al., 2010). Moreover, active amino acid residues such as Leu66, Ala69, Thr97, Ile98, Gly100, Pro101, Leu102, Trp148, Arg211, Gln230, and Ala244 are also highly conserved in the CAT enzymes of two *B. cepacia* strains and *P. arvilla*.

Moreover, we also isolated some other genes involved in dibenzofuran degeneration from *B. cepacia* DF2 and DF4, such as biphenyl 2,3-dioxygenase, anthranilate 1,2-dioxygenase, hydroxyquinol 1,2-dioxygenase small subunit, and hydroxyquinol 1,2-dioxygenase large subunit. These genes have been deposited in the database of GenBank (unpublished data).

Expression of catechol 1,2-dioxygenase

Four transformants of *E. coli* were randomly chosen and induced the expression of CAT by IPTG. The total soluble proteins were analyzed by SDS-PAGE, showing that each of the recombinant 33-kDa CAT proteins were overexpressed (Fig. 4A and 4B).

The purity of the CAT enzyme, which was recovered from the affinity chromatography with the Ni^{2+} column, was checked by SDS-PAGE. The results showed that only one band of protein appeared with a molecular weight of about 33 kDa (Fig. 4C). *cis,cis*-muconic acid was detected in the reaction mixture using recombinant and wild-type CAT, while it was not in the negative control (Fig. 5).

Characterization of CAT enzyme

Some fundamental properties of the CAT enzyme of DF4 were characterized, including the effect of pH, temperature, and metal ions on the activity of the CAT enzyme. CAT had the highest relative activity at pH 7 (100%) but the acidic environment significantly reduced its activity (only about 20% at pH 5). On the other hand, the relative activity of CAT can be maintained at 80% in an alkaline environment (pH 9) (Fig. 6A). The optimal temperature for CAT activity was 30°C (relative activity: 100%) and this significantly decreased at temperatures above or below 30°C (Fig. 6B), showing 40-50% of relative activity at 15°C or 40°C.

The optimal pH of the CAT activity of DF4 was similar to that of *R. ruber* OA1 (pH 7), but the optimal temperature was different, with that of *R. ruber* OA1 being 25°C (Wang et al., 2017). The optimal pH and temperature for the CAT activities were slightly higher (pH 7.5 and 30-35°C) in *P. putida* N6 and *C. tropicalis* JH8, respectively (Guzik et al., 2011; Long et al., 2016). As shown in Fig. 6C, most of the tested metal ions and surfactants decreased the enzymatic activity compared with the control. The activity of CAT was completely lost in the case of Zn^{2+} and SDS, while the relative activity remained about 12, 40, and 49% under the effect of Triton X100, Mn^{2+} and Tween 20, respectively. CAT of *Sphingomonas xenophaga* QYY was also inhibited by Zn^{2+}, and partially lost its activity in the presence of Mn^{2+} [Gou et al., 2009]. On the contrary, Wang et al. (2017) showed that Mn^{2+} could increase the CAT activity of *R. ruber* OA1 up to 5 fold, while Zn^{2+} did not affect its activity. Our results also show K^{+} caused a slight increase of CAT activity (approx. 110%), while 81% of CAT activity remained in the presence of Mg^{2+}. However, according to Wang et al. (2017), these two ions strongly inhibited the activity of catechol 1,2-dioxygenase of *R. ruber* OA1. A study on the CAT characterization of *Rhodococcus* sp. NCIM 2891 showed that an increasing metal ion, including Fe^{3+}, resulted in enzyme inhibition (Nadaf and Ghosh, 2011). This inhibition may include changes of enzyme conformation, or that metal ions bind to thiol groups of enzyme structure, causing enzyme deactivation. Thus, further investigations to understand the mechanism of how metal ions affect CAT activity may help to develop the resistance of the enzyme to inhibitors such as metal ions, thereby enabling the enzyme to be applied to degrade aromatic xenobiotics in the environment (Guzik et al., 2013).

Conclusion

The present study has reported on the cloning, expression and characterization of CAT from *B. cepacian*, which was isolated in dioxin-contaminated soils. HPLC analysis confirmed that recombinant CAT catalyzed the oxidation of pyrocatechol to *cis,cis*-muconic acid. The recombinant CAT exhibited an optimal pH and temperature of 7 and 30°C, respectively. Metal ions such as Zn^{2+} and Mn^{2+}, and surfactants including SDS, Tween 20 and Triton X100, strongly inhibited enzyme activity, while K^{+} caused a slight increase in the activity.

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Figure 1. Phylogenetic tree of 16S rRNA genes from DF2, DF4 and various *Burkholderia* strains. The tree was generated using Mega 7.0 software with a Maximum Likelihood analysis method.

Figure 2. Phylogenetic tree of catechol 1,2-dioxygenases genes from *B. cepacia* DF2 and DF4, *B. cenocepacia* MSMB384WGS (AOK37598), *P. arvilla* C-1 (PDB: 2AZQ), *A. radioresistens* LMG S13 (PDB: 2XSU), *B. vietnamiensis* LMG 22486 (PDB: 5TD3), *R. opacus* 1CP (PDB: 3HJ8), *C. tropicalis* JH8 (AJD79085), and *P. putida* (AAK33066). The tree was generated using Mega 7.0 software with a Maximum Likelihood analysis method.

Figure 3. The deduced amino acid sequence alignment of *cat* genes of *B. cepacia* DF2 and DF4 and other *cat* genes from *B. cenocepacia* MSMB384WGS (AOK37598), *P. arvilla* (PDB: 2AZQ), *A. radioresistens* (PDB: 2XSU), *B. vietnamiensis* (PDB: 5TD3), and *R. opacus* (PDB: 3HJ8). Identical amino acids are shown in black letters. The amino acid residues for conservation of Fe ligand as Tyr156, Tyr190, His214, and His216 in catechol 1,2-dioxygenases are shown as closed inverted triangles. The active amino acid residues of catechol 1,2-dioxygenases as Leu66, Ala69, Thr97, Ile98, Gly100, Pro101, Leu102, Trp148, Arg211, Gln230, and Ala244 are shown as closed circles.

Figure 4. SDS-PAGE of the total soluble protein from transformed *E. coli* M15 cells containing pQE-30 vector containing *cat* gene from *B. cepacia*. M: PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA), lane 9: control (proteins of non-transformed *E. coli* M15 cells). (A): proteins of DF2 strain; lanes 1, 3, 5 and 7: proteins of cells with IPTG; lanes 2, 4, 6 and 8: proteins of cells without IPTG. (B): proteins of DF4; lanes 1, 3, 5 and 7: proteins of cells without IPTG; lanes 2, 4, 6 and 8: proteins of cells with IPTG. (C): purified CAT by His-tag affinity chromatography; lane 1: crude extract of CAT enzyme (control); lane 2: purified CAT.

Figure 5. HPLC analysis profile of catechol conversion by wild type CAT and recombinant CAT. A: Standard *cis,cis*-muconic acid (30 μm/mL), B: *cis,cis*-muconic acid produced by recombinant CAT, C: *cis,cis*-muconic acid produced by wild type CAT. 10 μL of dissolved reaction mixture was used to evaluate the concentration of *cis,cis*-muconic acid.

Figure 6. Effect of pH (A), temperature (B), and metal ions and surfactants (C) on the activity of CAT from DF4. Data represent the means of three experiments, and error bars represent means ± standard errors.

Fig. 1

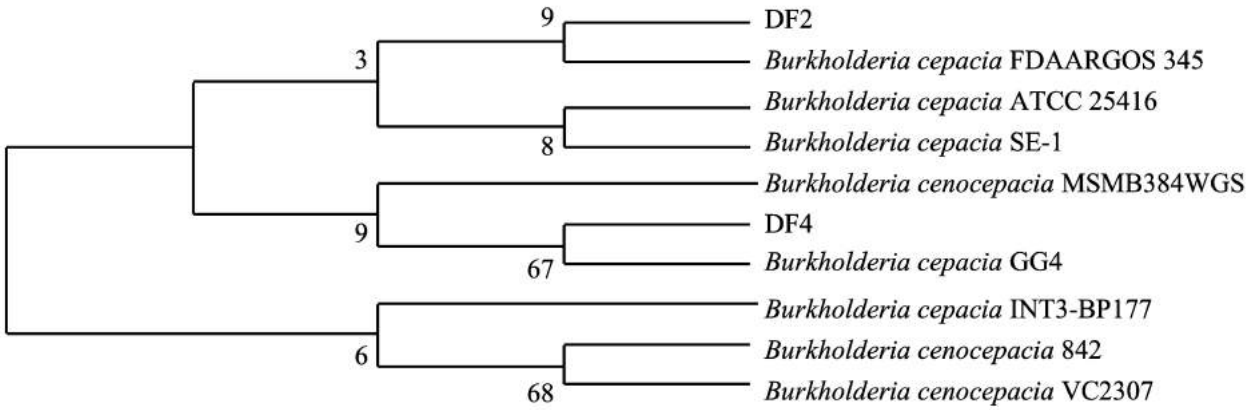
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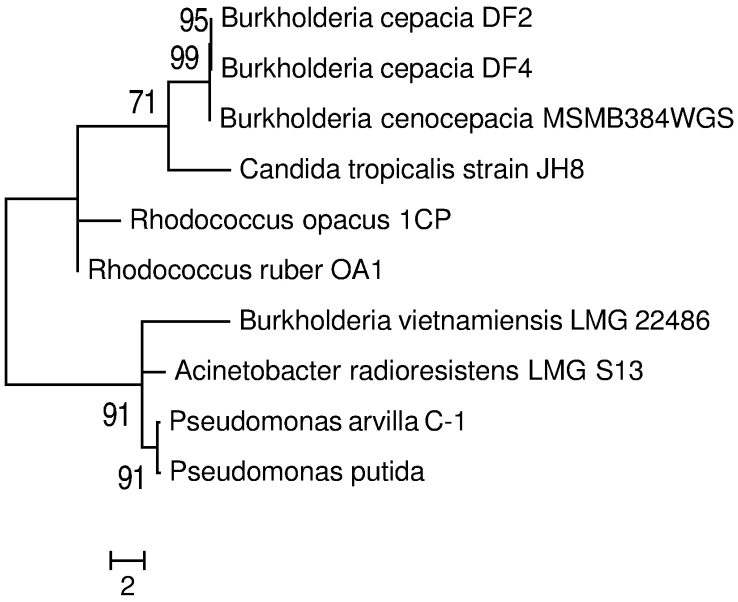
Fig. 2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Rhodococcus ruber OA1
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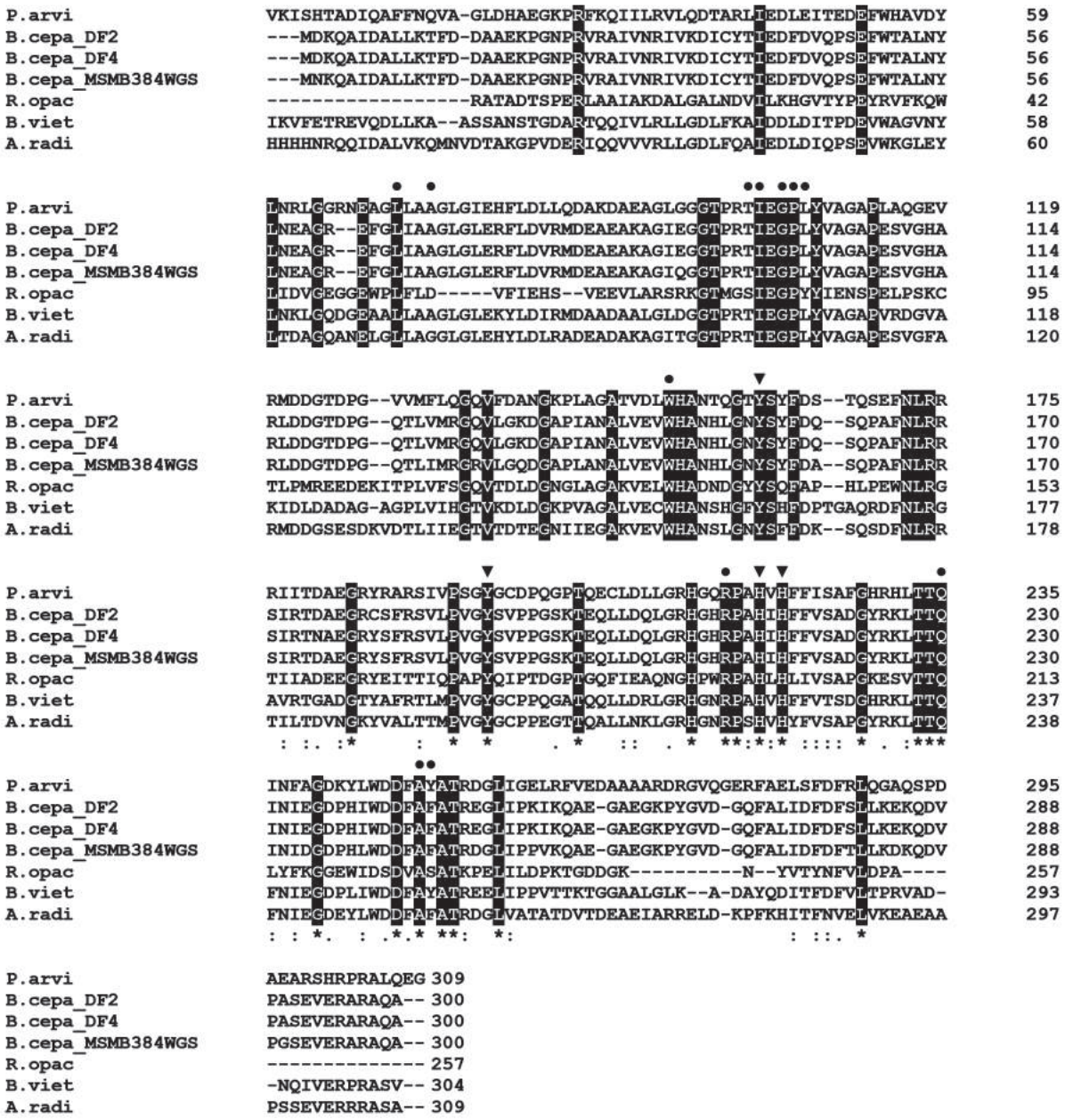
Pseudomonas arvilla C-1
Pseudomonas putida
2

Fig. 3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B.cepa_MSMB384WGS ---MNKQAIDALLKTFD-DAAEKPGNPRVRAIVNRIVKDICYTIEDFDVQPSEFWTALNY 56
R.opac ----------RATADTSPERLAAIAKDALGALNDVILKHGVTYPEYRVFKQW 42
B.viet IKVFETREVQDLLKA--ASSANSTGDARTQQIVLRLLGDLFKAIDDLDITPDEVWAGVNY 58
A.radi HHHHNRQQIDALVKQMNVDTAKGPVDERIQQVVVRLLGDLFQAIEDLDIQPSEVWKGLEY 60
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B.cepa_DF4 LNEAGR--EFGLIAAGLGLERFLDVRMDEAEAKAGIEGGTPRTIEGPLYVAGAPESVGHA 114
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R.opac LIDVGEGGEWPLFLD-----VFIEHS--VEEVLARSRKGTMGSIEGPYYIENSPELPSKC 95
B.viet LNKLGQDGEAALLAAGLGLEKYLDIRMDAADAALGLDGGTPRTIEGPLYVAGAPVRDGVA 118
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R.opac TLPMREEDEKITPLVFSGQVTDLDGNGLAGAKVELWHADNDGYYSQEAP--HLPEWNLRG 153
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Fig. 4

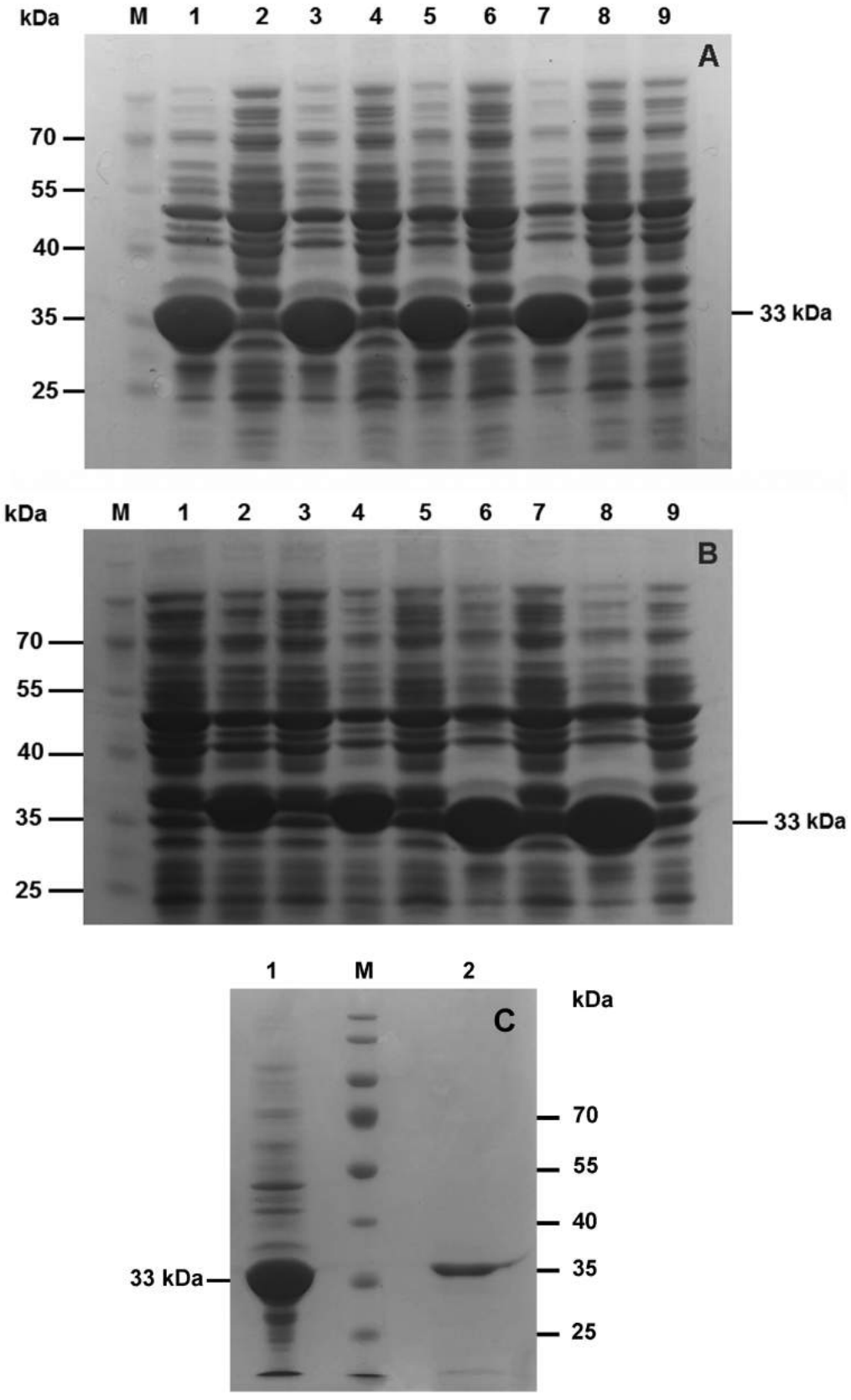
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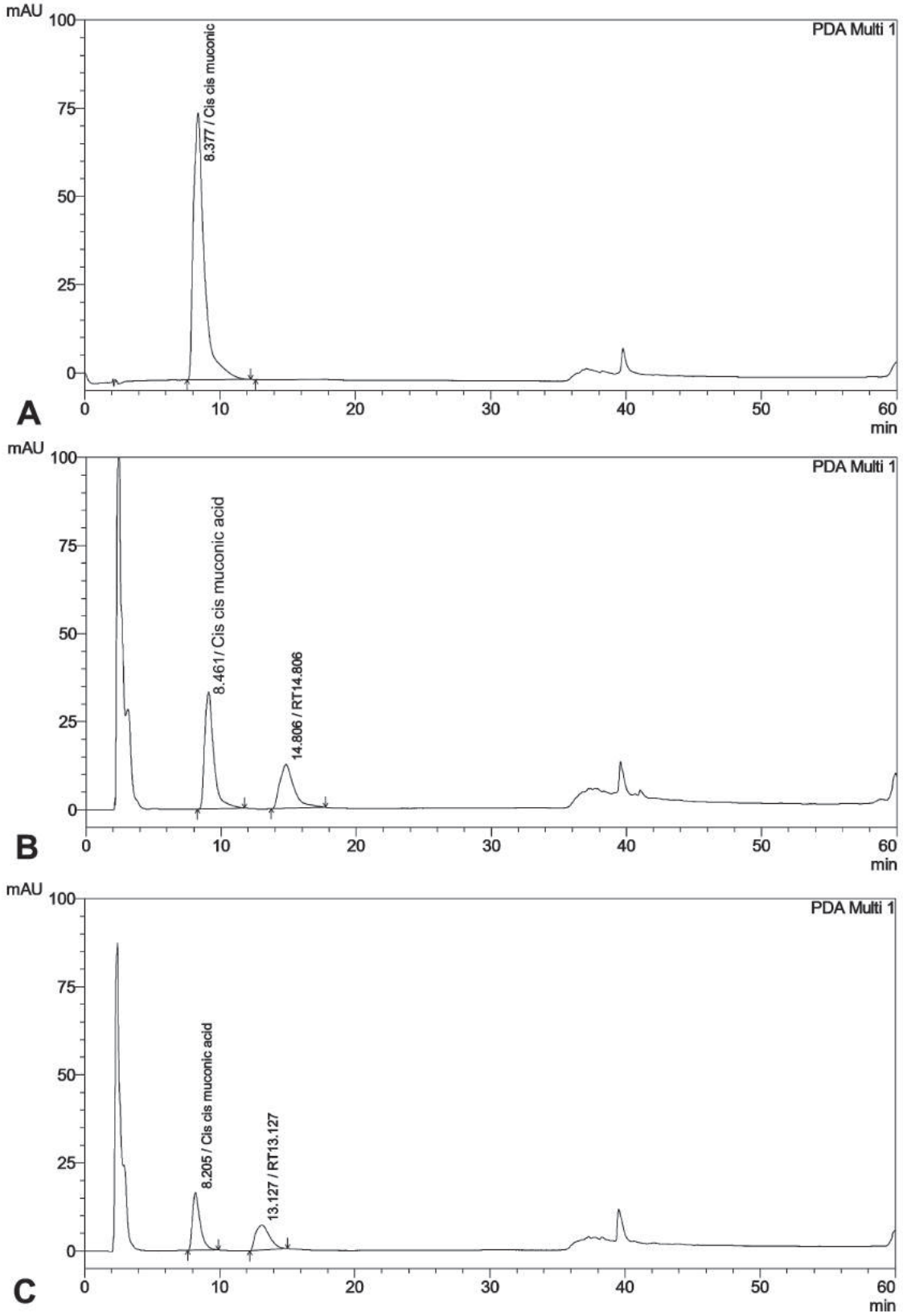
Fig. 5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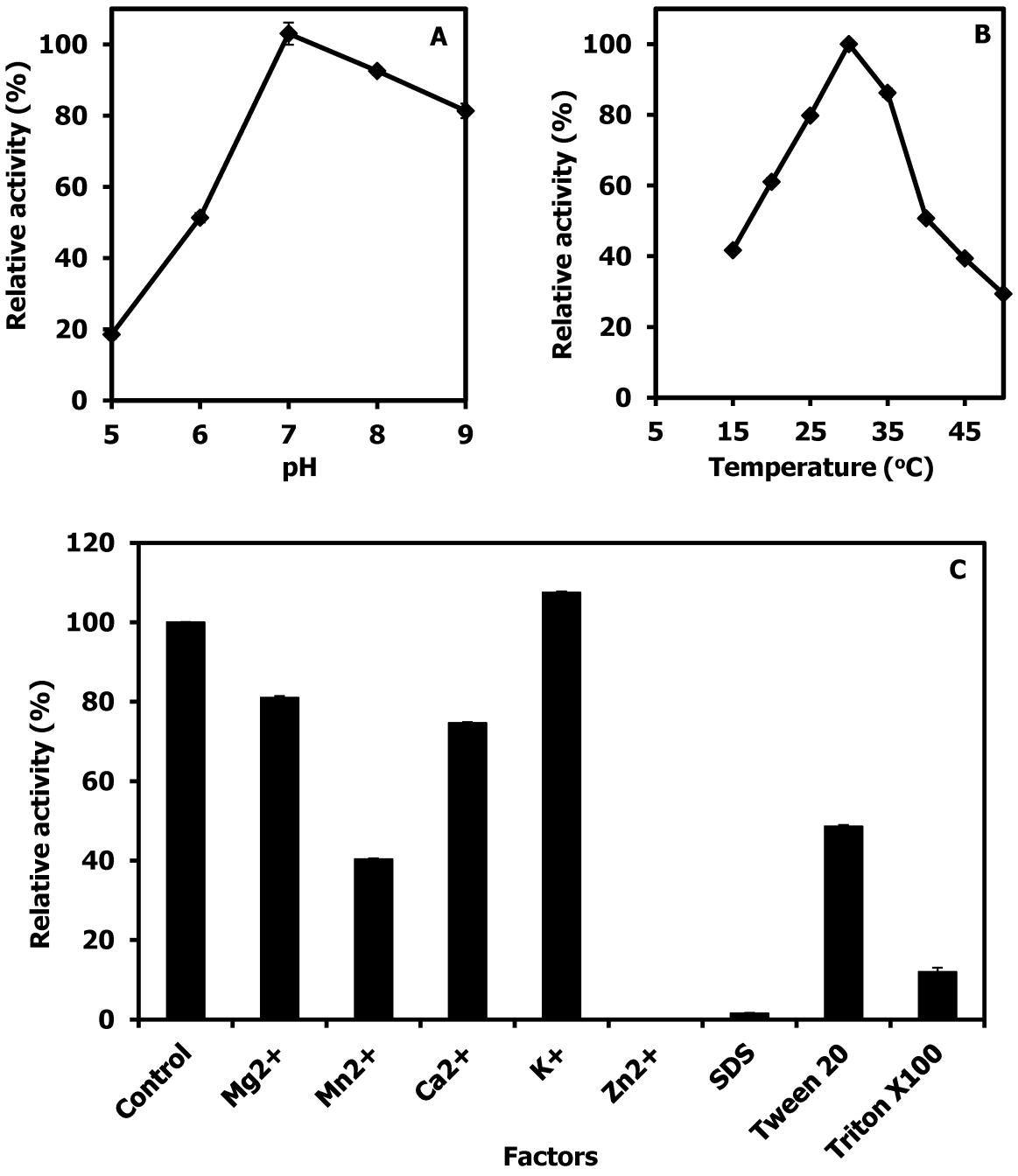
C
min

Fig. 6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8
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pH
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40
20
0
5
15
25
35
45
Temperature (°C)
C
Relative activity (%)
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100
80
60
40
20
0
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Mn2+
Ca2+
K+
Zn2+
SDS
Tween 20
Triton X100
Factors