**2,4-Dichlorophenol Degradation by Indigenous *Pseudomonas* sp. *P*KZNSA and *Klebsiella pneumoniae Kp*KZNSA: Kinetics, Enzyme Activity, and Catabolic Gene Detection**

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**Abstract**—In this study, two newly isolated 2,4-dichlorophenol(2,4-DCP)-degrading strains, *Pseudomonas* sp.KZNSA (*P*KZNSA) and *Klebsiella pneumoniae* KZNSA (*Kp*KZNSA), were enriched from an activated sludge sample with a known history of contamination with chlorinated organic compounds collected from a wastewater treatment plant located in Durban, South Africa. The strains could use 2,4-DCP as sole carbon and energy source. *P*KZNSA and *Kp*KZNSA degraded 64 and 49% of 2,4-DCP, with the degradation rate constant of 0.14 and 0.03 mg L–1 day–1, respectively. Both *P*KZNSA and *Kp*KZNSA were found to harbor the catabolic genes that encode the enzymes involved in 2,4-DCP degradation via the *ortho*-pathway which is further confirmed by the specific enzyme activity assays. The strains did not possess genes that encode the enzyme maleylacetate reductase, which is involved in funneling the last intermediate (maleylacetate) in the pathway into the Krebs cycle. Findings from this study will be helpful in the exploitation of these microorganisms and/or their enzymes in developing bioremediation strategies for the chlorophenol-polluted environment.

**Keywords:** 2,4-dichlorophenol, *Pseudomonas* sp., *Klebsiella pneumoniae*, phenol hydroxylase, catechol 1,2-dioxygenase

INTRODUCTION

2,4-Dichlorophenol (2,4-DCP), a derivative of phenol, is used as a precursor in the production of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and its derivatives [1] and in the synthesis of higher chlorophenols such as pentachlorophenol [2]. 2,4-DCP is also used as a wood preservative, insecticide, and plant growth regulator [1]. This compound is released during photodegradation of triclosan (antibacterial and antifungal agent) along with the dioxin 2,8-dichlorodibenzo-*p*-dioxin [3]. 2,4-DCP is also released into the environment as a metabolite of the degradation of chemicals applied in agricultural and food sectors [4]. <…> Due to its potential mutagenicity, carcinogenicity, immunogenicity, fertility, and cytotoxic properties, 2,4-DCP has been classified as a priority pollutant by the Agency for Toxic Substances and Disease Registry, WHO (https://www.iarc.who.int/wp-content/uploads/2018/07/pr236\_E.pdf, accessed on February 19, 2021). <…>

Bacterial degradation of 2,4-DCP proceeds via *ortho*-cleavage pathway [10, 18] or *meta*-cleavage pathway [10]. In the *ortho-*pathway, 2,4-DCP is hydroxylated to 3,5-dichlorocatechol by 2,4-DCP hydroxylase [19] and subsequently cleaved to 2,4-dichloro-*cis,cis*-muconate by catechol 1,2-dioxygenase type II [18, 19]. 2,4-dichloro-*cis,cis*-muconate is isomerized to 4-carboxymethylbut-2-en-4-olide (dienelactone)by dichloromuconate cycloisomerase. Dienelactone is further hydrolyzed to 2-chloromaleylacetate by dienelactone hydrolases [19]. In the last stage of the degradation, 2-chloromaleylacetate is dechlorinated to maleylacetate and then to 3-oxoadipic acid by maleylacetate reductase [20]. In the *meta*-cleavage pathway, 2,4-DCP is first oxidized to 3,5-dichlorocatechol and is subsequently degraded to 2-hydroxy-3,5-dichloro-6-oxohexa-2,4-dienoic acid, as illustrated in *Cupriavidus necator* strain JMP222 [10]. <…>

MATERIALS AND METHODS

**Sample collection, enrichment, and isolation of bacterial isolates.** An activated sludge sample with a known history of contamination with chlorinated organic compounds was collected from a wastewater treatment plant located in Durban, South Africa. Samples collected in 500 mL bottle and immediately were stored at 4°C until used for enrichment setup. The mineral salt medium (MSM) used for the culture enrichment was consist of (mg/L): KH2PO4, 800; Na2HPO4, 800; MgSO4·7H2O, 200; (NH4)2SO4, 500. pH was adjusted to 7.5. One mL of trace elements which comprising (mg/L): FeSO4·7H2O, 5.0; ZnSO4·7H2O, 4.0; MnSO4·4H2O, 0.2; NiCl·6H2O, 0.1; H3BO3, 0.15; CoCl2·6H2O, 0.5; ZnCl2, 0.25; EDTA, 2.5 was added using syringe filter (0.2 µm pore) into MSM. Ten percent of the sludge sample was inoculated into MSM supplemented with 40 mg/L of 2,4-DCP in a 250 mL Erlenmeyer flask and incubated at 30°C with shaking at 150 rpm for a week [11]. <…> Pure cultures were obtained by streaking individual morphologically different colonies on nutrient agar plates. The pure colonies were stored at –70°C as 20 vol % glycerol stocks.

**Growth and degradation studies.** Growth and degradation assays for the 2,4-DCP-degrading isolates were set up in an Erlenmeyer flask. The isolates were grown to their late log phase in nutrient broth (NB) composed of (g/L): beef extract, 1.0; yeast extract, 2.0; peptone, 5.0 and NaCl, 5.0 at 30°C with shaking at 150 rpm, harvested by centrifugation at 8000 × g for 10 min, washed twice with MSM, resuspended in the same medium and standardized to OD600 of 1.0 based on McFarland turbidity standards. About 10 vol % of the standardized inoculum was added separately into 90 mL of MSM supplemented with 40 mg/L of 2,4-DCP and the flasks were incubated for 22 days at 30°C at 150 rpm. *Cupriavidus necartor* JMP134 was used as a positive control while MSM plus 2,4-DCP without the inoculum was used as a negative control to test for abiotic loss. Unless otherwise stated, all growth and degradation assays in this study were conducted in triplicates. The growth of the cultures in the flasks was monitored spectrophotometrically at 600 nm. To measure the 2,4-DCP concentration in the flask, 1.5 mL aliquots were withdrawn from each flask at 48 h interval and centrifuged at 20000 × g for 10 min. The supernatants were *trans*ferred into a quartz cuvette (Hellma Analytics, Germany) and the OD200-350 was measured using a UV­–Vis spectrophotometer at scanning mode. 2,4-DCP having absorption maximum at 284nm used to perform calibration curve.2,4-DCP concentrations in sample at each sampling period were extrapolated from the linear regression equation. <…>

**Determination of chloride released during degradation.** Chloride release during 2,4-DCP degradation was quantified spectrophotometrically as described previously [11] with some modifications. Two hundred µL 250 mM Fe(NH4)(SO4)2·12H2O in 6 M nitric acid was added to the supernatant, followed by the addition of 400 µL of saturated mercuric thiocyanate in ethanol. The development of a red color is an indication of the presence of free halide which was measured at OD460. NaCl standard curve was plotted, and the concentration of the chloride released during degradation studies was calculated by extrapolation from the linear regression equation.

**Identification and phylogenetic analysis of the bacterial isolates.** Two isolates showing highest degradation for 2,4-DCP were identified by PCR amplification, sequencing, and analysis of 16S rRNA gene sequences. Genomic DNA was isolated and purified (Genomic DNA Purification Kit, Thermo FisherScientific, USA), and used as a template. The primers used were: 63F-5'-CAGGCCTAACACATGCAAGTC-3' and 1387R-5'-GGGCGGTGTGTACAAGGC-3' [21]. <…>

**Preparation of crude extract to determine enzyme activities.** The crude extract was prepared as described previously [11] with some modifications. The modified MSM (pH 7.0) contained (g/L): K2HPO4, 2.75; KH2PO4, 0.1; NH4Cl, 0.2; MgSO4·7H2O, 0.01; CaCl2·2H2O, 1.0; NH4Cl, 0.5, yeast extract, 1.0, and 1 mL of filter-sterilized micronutrients. The micronutrients contained (mg/L): FeSO4·7H2O, 5.0; ZnSO4·7H2O, 4.0; MnSO4·4H2O, 0.2; NiCl·6H2O, 0.1; H3BO3, 0.15; CoCl2·6H2O, 0.5; ZnCl2, 0.25, and EDTA, 2.0. The inoculum was prepared as described above and 10 vol % inoculated in 1 L MSM supplemented either with 600 mg/L phenol or 40 mg/L 2,4-DCP in separate flasks. The flask was left for 36 h at 30oC at 200 rpm. The cells were harvested at the late exponential phase of growth by centrifugation at 12096 × *g* for 15 min at 4°C. The cells were washed twice with 50 mM Na-phosphate buffer, pH 7.5 containing 1 mM EDTA and 1 mM β-mercaptoethanol to halt the protease activity. The cell pellet was collected and re-suspended in 100 mL of the same buffer. Cell-free extracts were prepared by lysing the pellet by sonication with 400 ultrasonicator (OMNI International, USA) using 8 cycles each with a pulse of the 30 s on/off for 4 min. The cell extract was centrifuged at 20442 × *g* for 30 min at 4°C. The clear supernatant was kept on ice to prevent inactivation of the enzymes and used as a crude extract for enzyme assays or stored at –20°C.

**Enzyme activity assays.** Enzyme activities were measured by determination of the amounts of substrates utilized or metabolites formed and/or decrease/increase in OD using Shimadzu UV-1800 UV–Vis spectrophotometer (Japan) fitted with temperature controller CPS-240A unit [10, 11, 17, 18, 20]. The specific activity was calculated using the equation:

Specific activity = ((Δ*A*/min) × *v*) × (ε × *c* × *l*)–1,

where Δ*A*/min is the change in OD; *v* is volume of reaction mixture; ε is the molar extinction coefficient; *c* is the amount protein; and *l* is the path length (cm). Extinction coefficients were: 6300 M−1 cm−1 at 340 nm for NADH, 16800 M−1 cm−1 for *cis*,*cis*-muconate at 260 nm, 33000 M−1 cm−1 for 2-hydroxymuconic semialdehyde at 375 nm, 17000 M−1 cm−1 for *cis*-dienelactone at 280 nm, 15625 M−1 cm−1 for *trans*-dienelactone at 280 nm.<…>

***cis*-Dienelactone hydrolase (cDLH) and *trans*-dienelactone hydrolase (tDLH) activity assay.**cDLH and tDLH activities were measured using 5 µM of *cis*- and *trans*-dienelactones as the substrates. About 100 µL of crude enzyme was added to 900 µL 50 mM Tris-HCl buffer (pH 7.0) containing 0.5 µM of the substrate and incubated for 30 min at 30oC. The decrease in the OD280 of the reaction mixture was monitored indicating the decrease in substrate concentration because of enzyme activity. One U of enzyme activity was defined as the amount of the enzyme that hydrolysed 1 µM of *cis*- and *trans*-dienelactone per min under standard assay conditions.

***Maleylacetate reductase activity assay.*** Maleylacetate reductase activity was measured in 1 mL reaction mixture as described previously [25]. The reaction mixture contained 100 µL of cell lysate and 0.2 mM NADH in 50 mM Tris-HCl (pH 7.5) in a quartz cuvette. Nonspecific oxidation of NADH was measured at 340 nm, after incubation for 2 min followed by commencing of the reaction by adding 0.1 mM maleylacetate as a substrate. NADH oxidation after 30 min incubation was measured continuously. The maleylacetate reductase activity was then calculated as the difference in the oxidation rates of the NADH with and without the presence of maleylacetate. One U of enzyme activity was defined as the amount of the enzyme that reduced 1 µM of NADH per min under standard assay conditions.

***Muconate cycloisomerase activity assay.*** The *cis*,*cis*-muconate cycloisomerase activity was measured in 1 mL reaction mixture containing 30 mM Tris-HCl (pH 8.0), 2 mM MnSO4, 100 µL enzyme extract, and 100 µM *cis*,*cis*-muconate as a substrate. The enzyme is reported to be totally inactivated by at 1.3 mM EDTA concentration. One mM EDTA added in the buffer in crude extract was further diluted 10-fold in reaction mixture, as well as 2 mM MnSO4 added to neutralize its effect on enzyme activity. Further Mn2+ was reported to be necessary for *cis*,*cis*-muconate cycloisomerase activity [18, 26]. The activity of muconate cycloisomerase was measured at OD260 and 30°C. One U of the enzyme activity is defined as the amount of enzyme that caused decrement of 1 µmol of *cis*,*cis*-muconate per min under standard assay conditions [18, 27].

RESULTS

**Isolation, screening, and selection of 2,4-DCP degrading bacteria.** The initial enrichment experiments revealed the disappearance of 2,4-DCP in the flasks as well as increase in cell density. After 5 rounds of the enrichment steps and dilutions, 6 dominant bacterial strains were isolated and screened for their 2,4-DCP degradation potential. Two isolates, A and B, showing highest degradation potential were identified and used for further studies.

**Identification and phylogenetic analysis of the isolates based on 16S rRNA gene sequencing.** The 16S rRNA gene sequences of the isolates were blasted using the NCBI nucleotide BLAST program. The programme BLASTN 2.8.1 displayed a comprehensive lineage and taxonomy report of the organisms. The taxonomy report of 16S rRNA gene sequences from isolate A showed the 110 (45) number of hits matched with domain bacteria, out of which 100 (43) number of hits were phylum Gammaproteobacteria. Ninety eight (42) number of hits belonged to genus *Psedomonas*. In *Psedomonas*, most of the bacteria were found to be *Psedomonas fluorescence* (36 hits, total number 7). The rest were found to be *Psedomonas* sp. and uncultured bacterium. Paranthesis above mean the total number of organisms.<…>

**Growth rate and 2,4-DCP degradation kinetics.** Two strains, *P*KZNSA and *Kp*KZNSA were further used for growth and degradation kinetic studies by monitoring cell growth, 2,4-DCP degradation rate and Cl– released. *P*KZNSA demonstrated an increase in OD600 during growth in MSM supplemented with 2,4-DCP attaining a peak OD600 of 0.23 after 13 days after which a decline in growth was observed. *P*KZNSA was able to degrade 64% 2,4-DCP after 18 days with 440 µM of Cl– released in the medium (Fig. 2a). *Kp*KZNSA showed almost the same pattern as *P*KZNSA to degrade 2,4-DCP (Fig. 2b). *Kp*KZNSA demonstrated an increase in OD600 during growth in MSM supplemented with 2,4-DCP, attaining a peak OD600 of 0.21 after 13 days, later a decline in growth was observed. The organism was able to degrade 49% 2,4-DCP after 16 days, showing 455 µM of Cl– released in medium. *P*KZNSA and *Kp*KZNSA exhibited the ability to degrade the 2,4-DCP gradually.

*P*KZNSA showed a specific growth rate of 0.05 per dayand doubling time of 0.007 days, with the highest degradation rate constant at 0.14 mg L–1 day–1. *Kp*KZNSA showed a specific growth rate of 0.03 per day and doubling time of 0.004 days. The strain degraded 2,4-DCP with degradation rate constant of 0.03 mg L–1 day–1.<…>

**Detection of genes involved in the 2,4-DCP degradation pathway.** The retrieving of gene sequences from the whole genome data available at NCBI for closely related strains of *P*KZNSA and *Kp*KZNSA led to the designing of specific primers for genes involved in 2,4-DCP degradation pathways (Table 1). The whole genome data of sister strains for both, *P*KZNSA and *Kp*KZNSA showed the presence of gene sequences encoding phenol hydroxylase, catechol 1,2-dioxygenase, muconate cycloisomerase, dienelactone hydrolase but no strains showed the presence of gene sequences encoding for maleylacetate reductase. The primers successfully amplified the fragments for phenol hydroxylase, catechol 1,2-dioxygenase, muconate cycloisomerase and dienelactone hydrolase genes from the genomic DNA isolated from *P*KZNSA (Fig. 3a) and *Kp*KZNSA (Fig. 3b). Figure 3c shows the amplification of muconate cycloisomerase gene in *P*KZNSA and *Kp*KZNSA. PCR experiments also showed no amplification of genes designed for catechol 2,3-dioxygenase (the enzyme that facilitates *meta*-pathway) nor maleylacetate reductase (the enzyme that is involved in funneling the compounds to the Krebs cycle).

DISCUSSION

In this study, indigenous bacteria capable of degrading 2,4-DCP were isolated from activated sludge samples in Durban, South Africa. Studies have reported that microorganisms isolated from the groundwater and activated sludge [28] are capable of degrading chlorophenols. A total of 6 isolates showed the capability to grow in MSM supplemented with 2,4-DCP (40 mg/L) but only 2 were capable to degrade 2,4-DCP effectively. However, none of the isolates could completely mineralize 2,4-DCP, possibly due to substrate inhibition [13].<…>

Microorganisms facilitate the degradation of chlorophenolic compounds by the genes encoding the specific enzymes [11]. *P*KZNSA and *Kp*KZNSA showed the expression of phenol hydroxylase, catechol 1,2-dioxygenase, muconate cycloisomerase, cDLH and tDLH when induced with either phenol or 2,4-DCP. The phenol along with 2,4-DCP was chosen as the growth substrate for biomass production because phenol is precursor of many derivatives like 2,4-DCP and a wide variety of microorganisms are known to be capable of metabolizing or mineralizing phenol under aerobic and/or anaerobic conditions. <…> In the present study, a high concentration of substrate *cis*,*cis*-muconates of 10 mM was used as dichloromuconate cycloisomerase is reported to show low activity towards *cis,cis*-muconate due to high *K*m value of about 4 mM. Likewise, catechol 1,2-dioxygenase with relaxed substrate specificity and high activity against chlorocatechols was identified as a key activity in many organisms and named catechol 1,2-dioxygenase type II [18] to be differentiated from classical catechol 1,2-dioxygenases. It is highly possible that *P*KZNSA and *Kp*KZNSA express catechol 1,2-dioxygenase type II when induced with phenol or 2,4-DCP and showed high specific activity towards un-substituted substrates. *P*KZNSA showed high catechol 1,2-dioxygenase specific activity (15.63 U/mg of protein) almost similar (17.14 U/mg of protein) as reported in *P. putida* strain N6 [34] but contrary to reported in other *Pseudomonas* strains where very low specific activity is reported [11]. <…>

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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TABLES

**Table 1.** Primers and annealing temperatures used for the detection of catabolic genes involved in 2,4-DCP degradation in the bacterial isolates in this study

|  |  |  |  |
| --- | --- | --- | --- |
| Enzyme | Nucleotide sequence (5ʹ-3ʹ) | Expected size, bp | *T*m, oC |
| *P*KZNSA | | | |
| Phenol hydroxylase | pPhe-F-ATGAAGCTGCTCGCCGTCCGC  pPhe-R-CAGGTCGAGTTCGTCGTAGATGG | 600 | 60 |
| Catechol 1,2-dioxygenase | pC120-F- AATCTCGAGATGTCTATCCGAATTTCCCAG  pC120-R- AATGCTTAGCTTAGTCTTCGAGGGCGCG | 467 | 62 |
| Muconate cycloisomerase | pMuc-F- ATTCAGCTCAGGCAGTCAAAGCAAA  pMuc-R- CGAGCAATCAACTGTTCGACGCTT | 651 | 60 |
| Dienelactone hydrolase | pDh-F-CTGGCCATCGACATGTACGGCGA  pDh-R-TGTTGTAGCCAATGTCCGGCCC | 491 | 60 |
| *Kp*KZNSA | | | |
| Phenol hydroxylase 1 | kPhe1-F-TGCTGTTCTGGATGCCAAACC  kphe1-R-CGGAACACCTGGGTGAAGAA | 715 | 55 |
| Phenol hydroxylase 2 | kPhe2-F-TGG GCC AGG TGG TTG AAC TC  kphe2-R-CG GTA AAG CGC TCG AAG GC | 304 | 57 |
| Catechol 1,2-dioxygenase | kCat-F-ATG GCT AAC ATT CTC GGC GG  kCat-R-TGG CCG AGT TTG TAA CAA CGG | 507 | 55 |
| Muconate isomerase | kMuc2-F- ATTCTCGAGATGCCGCATTTTATCGCC  kMuc2-R- ATTGCTCAGCTTATTTAAATAACGCGTGC | 494 | 62 |
| Dienelactone hydrolase | kDh-F-ATGACGCGATTAACGGCCAAAG  kDh-R-CCGTAGCAAAAACCGGTGATAC | 567 | 60 |

<…>

FIGURE CAPTIONS

**Fig. 1.** Phylogenetic trees of selected isolates *Pseudomonas* sp. KZNSA (a) and *K. pneumoniae Kp*KZNSA (b). The strains in this study are underlined and highlighted blue.

**Fig. 2.** Cell growth (1), 2, 4-DCP degradation (2) and Cl- release (3) by KZNSA (a) and *Kp*KZNSA (b). Standard deviation whiskers represent the errors of 3 means.

**Fig. 3**. Amplification of genes involved in the biodegradation of 2,4-DCP in *Pseudomonas* sp. KZNSA (a) and *K. pneumoniae Kp*KZNSA (b). M: 1 Kb DNA marker. Genes in (a): lane 1: catechol 1,2-dioxygenase (467 bp); lane 2: dienelactone hydrolase (491 bp); lane 3: dienelactone hydrolase 2 (429 bp); lane 4: phenol hydroxylase gene (600 bp); lane 5: phenol hydroxylase 2 (587 bp); lanes 6 and 7: positive control (750 bp). Genes in (b): lane 1: dienelactone hydrolase (567 bp); lane 2: phenol hydroxylase 1 (715 bp); lane 3: phenol hydroxylase 2 (304 bp); lane 4: catechol 1,2-dioxygenase (507 bp). (c) Detection of muconate cycloisomerase gene in *K. pneumoniae Kp*KZNSA (494 bp, lane 1), *Pseudomonas* sp. KZNSA (651 bp, lane 2) and positive controls (1500 bp, lanes 3 and 4). M: 1 Kb DNA marker.

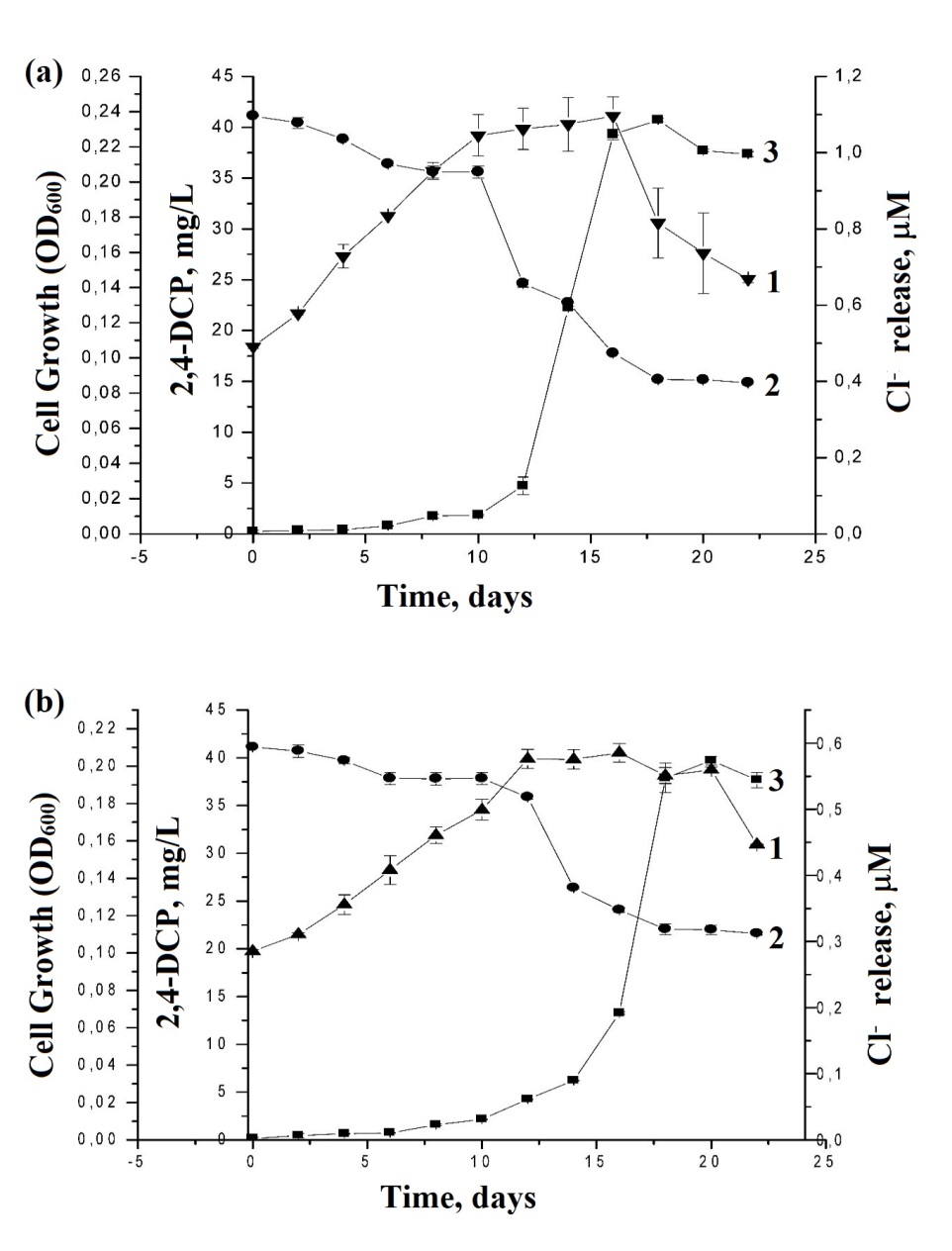
<…>  


Fig. 2.

<…>