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https://doi.org/10.5109/16104

出版情報：九州大学大学院農学研究院紀要. 54 (2), pp.279-284, 2009-10-29. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **54** (2), 279–284 (2009)

Biodegradation of Daunomycin Post–Production Wastes by *Bjerkandera Adusta* R59

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(*Received May 18, 2009 and accepted July 13, 2009*)

Wastes from industrial production of daunomycin belong to strongly toxic and recalcitrant substances. This study was investigated to the potential use of *Bjerkandera adusta* R59 as inoculum in process of daunomycin degradation in soil. Possible interactions between R59 strain and soil microorganisms during decay of wastes containing this toxic compound were also tested. Process of degradation of daunomycin post–production wastes in soil was described. Experiments were conducted in model systems. Degradation process proceeded the most efficiently in soil samples inoculated with *Bjerkandera adusta* R59 fungal strain, capable of decolorization of that cytostatic xenobiotic. Decrease of the waste biomass, carbon and daunomycin concentration (in daunomycin–rich pulp) as well as phenolics level (in post–production effluent) proved the effectiveness of this process, which was shown to depend on peroxidase biosynthesis in soil by *B. adusta* or other soil strains. Activity of this enzyme was the highest in version of experiment containing *Bjerkandera adusta* R59 fungal strain.

INTRODUCTION

Wastes after industrial production of anticancer anthracyclic antibiotics (daunomycin, adriamycin) belong to strongly toxic and recalcitrant substances. Anthracyclins are produced by *Actinomycetales*, including *Streptomyces peucetius* synthesizing daunomycin (Grein, 1987). Daunomycin is a glicoside composed of aminosugar, three anthracyclic rings (anthraquinonic compound) and metoxylic groups attached to first ring (Grein, 1987; Strauss, 1987). Anthraquinonic compound of daunomycin plays a role of chromophoric group – crucial in biological activity of this antibiotic. Cytostatic action of daunomycin is based on the drug–DNA linking during intercalation (Grein, 1987; Strauss, 1987). This antibiotic and products of its conversion possesses a wide anticancer spectrum, in particular in treatment of leukaemia (Strauss, 1987). Wastes from industrial production of daunomycin, after chemical inactivation by sodium subchlorate method, are directed to drain refineries. This leads to extreme chlorides concentrations in water reservoirs and to mismanagement of organic material (*Streptomyces peucetius* mycelium). Methods of microbial bioremediation of these wastes are commonly considered as less harmful for natural environment than chemical utilization.

Abilities for degradation of anthraquinones derivatives were proved for several bacterial strains e.g. *Bacillus subtilis*, *Pseudomonas* spp., *Alcaligenes* spp. (Bokhamy *et al*., 1994; Itoh *et al*., 1993; Nigam *et al*., 1996), yeasts e.g. *Pichia anomala* (Itoh *et al*., 1996) and fungi, including *Aspergillus terreus, A. niger, Geotrichum candidum* (Fujii *et al*., 1988; Fujitake *et al*., 1998; Kim *et al*., 1995). Special abilities in this field are presented by ligninolytic fungi representing basidiomycete, so called white–rot fungi: *Phanerochaete chrysosporium, Trametes* (*Coriolus*) *versicolor, Pleurotus cinnabarinus, Irpex lacteus* (De Jong *et al*., 1992; Glenn and Gold, 1983; Itoh *et al*., 1998; Jones *et al*., 1993; Kottermann *et al*., 1994; Nigam *et al*., 1996). Ligninolytic enzymes synthesized by these fungi – laccase and peroxidases – decay the mentioned xenobiotics on oxidative pathway (Jones *et al*., 1993; Novotny *et al*., 2000; Vyas *et al*., 1994).

Among already known microorganisms capable of biodegradation of anthraquinonic derivatives there are no strains which could decolorize and degrade the anthracyclic antibiotics. It is therefore worth noting that we have isolated a new ligninolytic fungal strain, initially classified as *Geotrichum*–like for its morphological similarity to this strain (Ginalska *et al*., 2004; Malarczyk *et al*., 1998). This fungus was characterized by decolorization and degradation potential against daunomycin in *in vitro* cultures, mainly due to peroxidase biosynthesis. Genetical analysis of this fungus revealed that it is an anamorphic stadium (*Geotrichum*–like) of *Bjerkandera adusta* (*Basidiomycota*) strain, representing white–rot fungi.

This research presents a detailed investigation into the potential use of *Bjerkandera adusta* R59 as inoculum in process of daunomycin degradation in soil. Possible interactions between R59 strain and soil microorganisms during decay of wastes containing this toxic

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compound were also tested.

MATERIALS AND METHODS

Daunomycin source

Wastes after pharmaceutical industrial production of daunomycin were used in our research. They were: the pulp (mycelium of *Streptomyces peucetius*, synthesizing the antibiotic) and effluent (culture liquid after daunomycin extraction). The wastes were kindly provided by Biotechnology and Antibiotics Institute (Warsaw, Poland). Table 1 presents some of chemical features of these materials.

Fungal strain

Samples of black earth soil (Phaeosols from a field near Lublin (South–Eastern Poland) were used for fungus isolation. The samples contained (in %): humus 3.95, N_{tot}. 0.210; pH_{KCl} 7.45. Fungi were isolated using a trapping method. The pulp after industrial production of daunomycin (*Streptomyces peucetius* mycelium) was used as trapping material. Detailed description of fungus isolation was given elsewhere (Ginalska *et al.*, 2004). Finally, on a base of morphological and genetic features, R59 strain was identified as an anamorphic stadium (*Geotrichum*–like) of *Bjerkandera adusta* (Willd. Ex. Fr.) P. Karst, representing white–rot fungi. Identification of nucleotide sequences (rRNA gene fragment) of the fungus are available in Gene Bank under a number AY 319191. The strain was also deposited in CCBAS culture collection under accession number CCBAS930.

Soil

The soil for our experiments contained only small quantities of organic substances and mineral colloids, in order to limit the sorption of daunomycin. The soil was classified as podzolic soil (Haplic Podzols, acc. FAO) developed from sand. Characteristics of basal chemical and physical features of this soil are summarized in Table 2.

Model of experiment

Fresh soil from cultivation field from the upper (up to 20 cm) layer. It was passed through a sieve (mesh: 0.2 cm), humidified to 60% of its total water capacity and placed into 1000 cm^3 vessels (1 kg of soil per one vessel; height of 15 cm). Daunomycin–rich material was put into the vessels in two experimental versions: with post–production pulp (I) and post–production effluent (II).

Version I: 15 g portions of daunomycin–rich pulp (20% of dry weight) in poliamide–6 bags (see 2.1) were put into the soil in vessels (5 cm under soil surface) in following combinations:

1 – without any additives (control)
2 – with 1 cm^3 of homogenized *Bjerkandera adusta* R59 mycelium (10^6 c.f.u.)
3 – with 1 cm^3 of homogenized *Bjerkandera adusta* R59 mycelium (10^6 c.f.u.) and 1% glucose

Version II: daunomycin–rich post–production effluent was used in following combinations:

1 – soil without additives (control 1)
2 – soil with 50% daunomycin effluent (50 cm^3)
3 – soil with 15 cm^3 of homogenized *Bjerkandera adusta* R59 mycelium (10^6 c.f.u.) (control 2)
4 – soil with 50% daunomycin effluent (50 cm^3) and 15 cm^3 of homogenized *Bjerkandera adusta* R59 mycelium (10^6 c.f.u.)

Extraction of phenolic compounds was performed according to Hruszka (1982): 15 g of dry soil material was extracted twice with 50 cm^3 of mixture containing: C_2H_5OH:redistilled water:CH_3COOH (70:28:2) for 8 h. The filtrate was dried and residues were dissolved in 25 cm^3 of redistilled water. Both versions in each combinations were prepared in duplicates. Vessels were incubated for 5 months at 20 °C (±2 °C) in conditions of controlled humidity. Chemical and biochemical assays were performed after 3, 30, 60, 90, 120 and 150 days of exper-

Table 1. Chemical composition of daunomycin wastes (g . kg^{-1} of dry weight)

Waste	Dry weight	Daunomycin Content	Carbon	Hydrogen	Nitrogen	pH
Daunomycin pulp	200	$5 \cdot 10^{-4}$	408.9	57.5	51.7	5.8
Daunomycin effluent	30	$2.67 \cdot 10^{-3}$	295.3	39.2	11.9	3.5

Table 2. Some physical and chemical features of soil (g . kg^{-1} of dry weight)

N tot.	C org.	Humus	Mg	$CaCO_3$	P_2O_5	K_2O	pH_{KCl}
0.55	6.1	10.5	0.038	3.4	0.325	0.119	5.92
Particle size distribution (diameters in mm)							
1.0–0.5	0.5–0.25	0.25–0.1	0.1–0.05	0.05–0.02	0.02–0.006	0.006–0.002	<0.002
%							
8	30	28	10	14	7	2	1

iment.

Analytical procedures

Periodical assays of daunomycin–rich pulp (for version I) introduced into soil included the estimations of:

– decrease of dry biomass (after complete drying at 105 ˚C)
– daunomycin content (A_{480}nm) after extraction of 100 mg of dry biomass of daunomycin pulp with 10 cm^3 of ethanol. Amount of the antibiotic was calculated using standard curve for pure daunomycin (ICN)
– carbon content in dry biomass of pulp (using Perkin Elmer CHN 2400)

Assays of daunomycin–rich effluent (for version II) included estimation of:

– phenolics level (A_{725}nm) according to Swain and Hillis (1959).
– peroxidase activity: 10 g samples of soil were dizintegrated with alumina and 5 cm^3 of Tris/HCl buffer pH 7.5, centrifuged at 3000 rpm. Activity of enzyme was estimated in supernatant according to Maehly and Chance method with o–dianisidine as a substrate (Maehly and Chance, 1954).

In both experiment the viability of *Bjerkandera adusta* R59 was tested by material layout method (for version I) or by dilution plating method (for version II) on selective medium for fungi (Martin, 1950). Identification of *B. adusta* R59 strain was performed on a base of microscopic observations (*Geotrichum*–type sporulation) and confirmed by decolorization of daunomycin on Petri dishes containing agarized medium. All assays were performed in triplicates (except of estimation of carbon content – in duplicates). Results were analyzed by the statistical method of variance analysis with Tukeya confidence intervals defined in our work as LSD (lowest significant difference) and by correlation method.

RESULTS AND DISCUSSION

Investigations of biodegradation of daunomycin–rich post–production pulp

Biomass of the pulp and its carbon content decreased regularly for the whole time of experiment duration. Decreases of biomass of pulp were considerably high only up to third month of the test – during this period the biomass of pulp reached 53.2% of its initial weight. Drop of carbon content was the most significant during first month of investigation – afterwards it was not so noticeable (Table 3 and Table 4). Considering a decrease of biomass and carbon content as criteria for estimation of pulp decay it was found that biodegradation was performed with the lowest rate in control soil samples (without *B. adusta* R59). The presence of this fungus in soil induced an increasing drop of biomass and carbon content, in comparison with control samples (Table 3 and Table 4). Specially visible decrease of pulp biomass was observed in comparison with the combination with the sole fungal inoculum (after 5 months of experiment).

Results obtained in described experiment revealed that highly toxic daunomycin post–production wastes (pulp – *S. peucetius* mycelium and effluent) undergo a slow biodegradation when introduced into soil. Inoculating the soil samples with *Bjerkandera adusta* R59 strain, capable of effective decolorization of this antibiotic, increased the rate of daunomycin degradation in both pulp and effluent after industrial production of this substance. It was confirmed by higher decrease of biomass, carbon and daunomycin content (pulp decay) and phenolics level (effluent degradation) in comparison with samples without the fungus. Process of biodegradation of these wastes was substantially correlated with time of their contact with soil. After 3 month of experiment, biomass of waste and daunomycin content in presence of R59 decreased to 50% and 40% of their initial values, respectively. In analogical combination with daunomycin effluent, phenolics level dropped to 20% of its initial concentration.

The results show that pulp biomass degradation was accompanied by a decrease of daunomycin content. Significant decrease of daunomycin concentration in control combination was observed only during first month of contact with soil. In other combinations, drop of antibi-

Table 3. Average values of pulp biomass (in % of its initial weight)

Time (days)	Version of experiment *		
	1	2	3
0	100.0	100.0	100.0
3	83.3	79.5	76.5
30	67.4	63.6	64.4
60	60.0	57.3	57.7
90	57.2	52.4	50.0
120	56.5	46.8	45.8
150	54.1	45.4	38.9

* Explanations: 1 – daunomycin pulp in soil (control); 2 – daunomycin pulp in soil with *B. adusta* R59; 3 – daunomycin pulp in soil with *B. adusta* R59 and 1% of glucose; $LSD_{0.05}$ for time=4.7; $LSD_{0.05}$ for version of experimen=2.4

Table 4. Average values of carbon content in pulp (in % of its initial content)

Time (days)	Version of experiment		
	1	2	3
0	100.0	100.0	100.0
3	96.2	91.5	92.8
30	92.9	84.2	88.3
60	88.7	82.9	84.2
90	86.5	79.7	80.4
120	88.5	78.6	79.1
150	83.7	79.8	75.0

Explanations: 1 – daunomycin pulp in soil (control); 2 – daunomycin pulp in soil with *B. adusta* R59; 3 – daunomycin pulp in soil with *B. adusta* R59 and 1% of glucose; $LSD_{0.05}$ for time=5.7; $LSD_{0.05}$ for version of experiment=2.4

otic content was fast and intensive. Final amount of daunomycin was the lowest in version I containing both R59 strain and glucose. The influence of the sugar presence was the most visible between 30th and 60th day of experiment (Table 5). Statistical estimations showed that changes in pulp biomass, carbon and daunomycin content were strongly correlated with the time of experiment (Table 6). It is possible that some daunomycin from the pulp introduced into soil could be removed from the system in non–enzymatic way – eluted with water – for its solubility in this liquid (Grein, 1987). Significant drop of daunomycin content during the first month of experiment in sample non treated with R59 seems to confirm this hypothesis.

Table 5. Average values of daunomycin content in pulp (in % of its initial content)

Time (days)	Version of experiment		
	1	2	3
0	100.0	100.0	100.0
3	92.8	83.8	81.7
30	65.9	68.0	52.7
60	63.4	49.9	37.3
90	57.9	36.9	30.5
120	52.6	32.4	28.3
150	51.2	32.9	28.6

Explanations: 1 – daunomycin pulp in soil (control); 2 – daunomycin pulp in soil with *B. adusta* R59; 3 – daunomycin pulp in soil with *B. adusta* R59 and 1% of glucose; $LSD_{0.05}$ for time=3.5; $LSD_{0.05}$ for version of experiment=1.8

Table 6. Correlation coefficients (r) and significance levels (α) between the duration of experiment and pulp weight, daunomycin content or carbon content for particular versions of experiment

Experiment version	Pulp weight		Daunomycin content		Carbon content	
	r	A	r	α	r	α
1	–0.8464	0.05	–0.8916	0.01	–0.9204	0.01
2	–0.8803	0.01	–0.9320	0.01	–0.8300	0.05
3	–0.9056	0.01	–0.8732	0.05	–0.9486	0.01

Explanations: 1 – daunomycin pulp in soil (control); 2 – daunomycin pulp in soil with *B. adusta* R59; 3 – daunomycin pulp in soil with *B. adusta* R59 and 1% of glucose.

Biodegradation of daunomycin–rich post–production effluent

Results obtained previously showed that *B. adusta* R59 strain plays a significant role in biodegradation of daunomycin in soil milieu. Assuming the drop of phenolic compounds as a indicator of daunomycin decay its was found that phenolics level in control soil without R59 (control I) remained practically unchanged during experiment time. In soil samples inoculated with fungus but without daunomycin effluent (control II) a slight drop of these substances was found only between 30th and 60th day of experiment. In both combinations enriched with daunomycin effluent decreased intensively until 90th day of test; then it remained stable (Table 7). It was estimated that at the end of experiment phenolics content in samples only with daunomycin dropped to 35% of its initial value, while in samples with daunomycin and inoculated with *B. adusta* R59 – to 20%. Changes in peroxidase activity level showed generally the presence of two maxima in first an third month of experiment (except of in control 1) (Table 8).

The smallest variability in peroxidase activity appeared in soil samples without any additives. The highest variability and higher value of the enzyme activity was measured for soil containing R59 without antibiotic, while the highest peroxidase activity occurred in combination with both R59 and daunomycin in soil (Table 8). Similarly as in previous experiment, analysis of correlation revealed that only phenolics level correlates with time in significant extent. There was no correlation between time of experiment and peroxidase activity (Table 9).

Autochthonic microorganisms colonizing soil used in our experiment synthesized small amounts of peroxidase. However, the presence of daunomycin–rich effluent in the soil systems brought about a significant increase

Table 7. Average values of phenolics content (in mg . kg^{-1} of soil dry weight)

Time (days)	Version of experiment			
	1	2	3	4
3	0.901	1.439	0.911	1.412
30	0.880	1.407	0.882	0.860
60	0.880	1.090	0.730	0.523
90	0.784	0.509	0.605	0.264
120	0.880	0.510	0.600	0.266
150	0.890	0.515	0.600	0.280

Explanations: 1 – soil (control 1); 2 – soil with daunomycin effluent; 3 – soil inoculated with *B. adusta* R59 (control 2); 4 – soil with daunomycin effluent and inoculated with *B. adusta* R59; $LSD_{0.05}$ for time=0.042; $LSD_{0.05}$ for version of experiment=0.031

Table 8. Average values of peroxidase activity (in $U \cdot 10^{-1} \cdot kg^{-1}$ of soil dry weight)

Time (days)	Version of experiment			
	1	2	3	4
3	0.000	0.000	0.000	0.000
30	10.000	19.463	17.700	36.320
60	7.380	19.543	16.951	23.910
90	6.622	33.789	20.475	31.350
120	5.768	14.481	3.758	14.481
150	0.000	12.812	0.000	11.369

Explanations: 1 – soil (control 1); 2 – soil with daunomycin effluent; 3 – soil inoculated with *B. adusta* R59 (control 2); 4 – soil with daunomycin effluent and inoculated with *B. adusta* R59; $LSD_{0.05}$ for time=3.425; $LSD_{0.05}$ for version of experiment=2.507

Table 9. Correlation coefficients (r) and significance levels (α) between the incubation time and phenolics level or peroxidase activity for particular versions of experiment

Version of experiment	Phenolics level		Peroxidase activity	
	R	α	*R*	α
1	–0.1843	no	–0.1922	–
2	–0.9275	0.01	0.2954	–
3	–0.9316	0.01	–0.2285	–
4	–0.8879	0.05	–0.0206	–

Explanations: 1 – soil (control 1); 2 – soil with daunomycin effluent; 3 – soil inoculated with *B. adusta* R59 (control 2); 4 – soil with daunomycin effluent and inoculated with *B. adusta* R59; "no" – no significance of the correlation coefficient; " – " impossible to estimate

of peroxidase activity, specially in combinations containing *B. adusta* mycelium. This phenomenon proves the crucial role of this enzyme in biodegradation of tested xenobiotic. These results are confirmed by research performed by other authors (De Jong *et al*., 1992; Field *et al*., 1995; Heinfling *et al*., 1998a; Heinfling *et al*., 1998b; Kottermann *et al*., 1994), stating that peroxidases synthesized by *Bjerkandera* sp. including *B. adusta* are effective in process of degradation of aromatic xenobiotics, including anthraquinonic dyes Poly R–478 containing methoxyl groups and therefore structurally closely related to daunomycin.

Increase of peroxidase activity in soil containing daunomycin was accompanied by drop of phenolic compounds level, specially after inoculation with *B. adusta* strain. This effect confirms the contribution of fungal peroxidases in biodegradation of aromatic compound of daunomycin and phenolics transformation in the system applied in described experiments. Interestingly, the phenolics level dropped also in combination of soil with fungus but without daunomycin; the peroxidase activity in this system was also higher in comparison with pure soil without any additives. Probably *B. adusta* shows the degradation abilities also against natural phenolic compound present in soil, showing the high possible adaptation abilities of this fungus. Therefore, *B. adusta* possesses a high value for its potential in biotechnological applications. Rate of daunomycin removal from post–production pulp inoculated with *B. adusta* R59 increased significantly in presence of glucose. Similar observations were reported by Martens and Zadrazil (Martens and Zadrazil, 1996; Martens and Zadrazil, 1998). Glucose was also a main energy source in experiment on decolorizing of anthraquinonic dyes by *Bjerkandera* sp. BOS55 strain (Kottermann *et al*., 1994) and *Geotrichum candidum* Dec1 (Kim *et al*., 1995; Kim and Shoda, 1999).

Considering the above results, it is highly probable that *B. adusta* R59 decays daunomycin on cometabolic way rather that during its primary metabolic pathways. It is in agreement with present hypothesis on biodegradation of aromatic compounds by fungi. These processes are performed by fungal enzymes but degradation products are rarely incorporated into fungal material. For this reasons, aromatic compounds undergo degradation only in presence of other easily assimilated substrates. Addition of glucose stimulated daunomycin degradation but simultaneously decreases the rate of biomass decay and mineralization of organic carbon of post–production pulp. Most probably this effect was a result of catabolic repression of enzymes participating in decay of tested daunomycin wastes. The addition of glucose induced a decrease of waste biomass just after 5 months of its contact with soil therefore when this sugar has been already metabolized. These speculations are supported by numerous reports on glucose repression of fungal enzymes degrading polysaccharides and proteins (Carline and Watkinson, 1994; Kornillowicz–Kowalska, 1997). The results presented in this work show the usefulness of *B. adusta* R59 strain in remediation of environment polluted by toxic anthracyclins. This strain reveals a long viability in daunomycin–enriched soils which makes it additionally more valuable for biotechnological purposes. *Bjerkandera adusta* R59 covers 5 – 15% of total fungal population settling the antibiotic–enriched soil mentioned above.

Our results allows for a hypothesis that autochthonic soil microorganisms participate in biodegradation of tested daunomycin wastes. The daunomycin pulp content decreased to 58% after 3 months of culturing in soil sample without *B. adusta* inoculation; a significant drop of phenolics level (to 65% of its initial content) and an increase of peroxidase activity was observed in analogical combination but with daunomycin effluent. These observations suggest that self–selection of a team of microorganisms capable of daunomycin biotransformation took place. *Streptomyces peucetius* mycelium, left after industrial daunomycin production, contained not only this toxic antibiotic but also a significant amount of nitrogen (Table 1). According to our results, the cooperation of *B. adusta* action (daunomycin decay) and autochthonic soil microorganisms activity (the degradation of remaining biomass of *S. peucetius* mycelium) could be useful in composting method decay of recalcitrant compounds (e.i. ligninocellulose).

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