Biodegradation of glyphosate by wild yeasts

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Degradación de glifosato por levaduras silvestres

Resumen. Se estudió la capacidad de detoxificación del glifosato por levaduras silvestres aisladas de suelos con y sin tratamiento con el herbicida. Se obtuvieron 77 colonias de los de suelos tratados y 38 de los no tratados, con mayor incidencia de las especie *Candida krusei* y *Yarrowia lipolytica* en cada grupo, respectivamente. *Y. lipolytica* presentó una fase lag prolongada cuando fue cultivada en el medio conteniendo glifosato como fuente de C, iniciando su consumo hasta el sexto día de incubación, mientras que *C. krusei* mostró una adaptación más breve (3 días de incubación) y un consumo mayor del organofosforado después de 20 días de cultivo (66% *vs.* 42.5%). Ambas especies fueron cultivadas en sustratos enriquecidos con fosfato, glifosato o ácido aminometilfosfórico (AMPA), para obtener cepas activas en medios conteniendo organofosforados como fuentes de C y N. Los medios enriquecidos incrementaron la densidad de las colonias, aunque el fosfato no favoreció la transformación del herbicida. Se confirmó la presencia de micoflora adaptada para degradar glifosato y sus metabolitos, lo que sugiere que las levaduras silvestres podrían contribuir eficazmente en los procesos de detoxificación naturales, especialmente en la biorremediación de áreas contaminadas con organofosforados.

Palabras clave: *Candida krusei*, *Yarrowia lipolytica*. biotecnologías ambientales, degradación de glifosato, fuentes de C N P.

Abstract. Glifosate detoxification by wild yeasts were studied from colonies isolated of treated and non treated soils with the herbicide. Seventy seven yeast colonies were obtained from treated soils and 38 from non treated samples, being *Candida kruseis* and *Yarrowia lipolytica* the dominant species in each group, respectively. *Y. lipolytica* showed a longer lag phase and degradation began 6 days from incubation, while in *C. kruseis* after 3 days from incubation with a higher total uptake of the herbicide (66 vs. 42.5%). Both species were cultivated in enriched media with phosphate, gliphosate or aminomethylphosphonic acid (AMPA) in order to obtain active strains cultivated on substrates with organophosphonates as C and P sources. Enriched cultures increased the mycelial density of the yeasts, although the phosphate presence inhibited the gliphosate breakdown. The presence of mycoflora adapted to degrade glyphosate and its metabolites was confirmed, therefore wild yeasts could be significant in natural detoxification processes, specially in the bioremediation of agricultural areas polluted with organophosphonates.

Key words: *Candida krusei, Yarrowia lipolytica,* environmental technologies, glyphosate degradation, C N P sources.

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Introduction

Pesticidal bioremediation minimizes machinery mobilization and materials cost, requiring only simple equipment, making biodetoxification attractive for reclamation of polluted areas [6]. In constrast to bacteria, fungi have certain advantages as efficient enzyme systems, metabolic flexibilities and greater biomass that enhance their potential to treat pesticide contaminated soils [2, 9]. Althought glyphosate N-(phosphonomethyl-)-glycine is resistant to breakdown, previous studies showed that environmental samples or bacteria were efective biodegraders [1, 4, 17], but among fungi only some white-rot and filamentous species utilize it [8, 13, 18].

Yeasts have been less widely exploited as biodegraders than other fungal groups [3], though they produce powerful emulsifyng agents and enzymes that enhance pollutant biotransformation [10, 14]. Up to now, *Debaryomyces vanrijiae* [15], *Trichosporun* spp. [24], *Pichia* spp., *Rhodotorula* spp. and *Candida* spp. [19, 21] had been studied as pollutant degrading species; howevere, these reports were oriented fowards the degradation of polycyclic aromatic hydrocarbons and biarilyc compounds.

It is important to point out that there are no reports showing that wild yeasts are able to metabolize organophosphonates, although yeasts were dominant in polluted habitats [20]. Thus, to assess the role of yeasts in natural detoxification processes, the objectives of this study were to isolate indigenous yeasts able to transform glyphosate, and to evaluate their abilities to grow on the herbicide as either carbon, nitrogen or phosphate sources.

Materials and methods

Isolation of glyphosate tolerant yeasts

Wild yeasts were obtained from glyphosate treated and non-treated soils, by adding 500 mg glyphosate to 1 kg soil and incubated for 30 days. Subsamples (1 g) of each treatment were shaked in 10 ml 0.85 % ClNa with 0.5 ml antibiotic solution (streptomycin 5.0 g, chloranphenicol 2.5 g and distilled water 1.0 l). Particles were removed by centrifugation and decimal dilutions of the supernatants were spread on agar-mineral medium (MM, [20]) with 20 mg glyphosate / l MM, in triplicate. The plates were incubated at 29 ºC, for 30 days. Simultaneously, sterile controls with glyphosate were incubated. The herbicide tolerant yeasts were selected from these plates on the basis of their prevailing growth on subsequent platings with increasing herbicide levels (40 and 60 mg gly / l), and they were identified by morphological and physiological features [14].

Culture conditions

The two dominant species were assayed in liquid culture with the herbicide as carbon source, in triplicate; 100 ml-erlenmeyers with 20 ml MM-liquid medium amended with 20 mg glyphosate / l MM, pH 6.8, were inoculated with 0.5 ml (2.0×10^6 cell / ml) of each yeast suspensions. The fungal suspensions were obtained from the glyphosate agar-plates, washed three times with 10 ml physiological solution, and used as inoculum. The yeast densities were periodically determined by microscopic direct counts in Thiefe Chamber (Fein-Optik, Bad Blankenbourg, Germany), by triplicate. The flasks were incubated at 29 ºC, for 20 days in a rotary shaker at 100 rpm, in darkness. A sterile control flasks without herbicide, were also implemented.

The degrading activities were measured in MM-liquid culture with 20 mg glyphosate and aliquots were periodically taken to determine glyphosate residual concentrations, by triplicate. The incubations were done at 100 rpm, 29 ºC for 20 days. Glyphosate separation from culture medium were achieved by Sigmacell type 100 cellulose thin-layer chromatography plates with ethanol / water / ammonium hydroxide / trichloroacetic acid / acetic acid (55 : 35 : 2.5 : 3.5 : 2, by vol), by triplicate. Amine-containing products were visualized with ninhydrin, its residues derivatised with 9-fluor enylmethoxy-carbomylchloride and detected at 280 nm by a Hewlett Packard 1090 Chemistation HPLC [23].

Enrichment procedures

Aliquots from non-treated and treated soils were also cultured in casein/peptone/starch (CPS) and in MM with 2 mM K_2HPO_4 or 2 mM glyphosate or 2 mM aminomethylphosphonic-acid (AMPA) as carbon or phosphorus source; in these cases 50 ml medium was inoculated with 0.5 ml yeast suspensions.

The nitrogen free medium was MM without NH_4Cl and 5.0 g potassium gluconate as C source, 0.5 ml inocula was added to 50 ml medium. As alternative C, N or C-N sources the following glyphosate degrading products or analogues were tested: acetate, glycolate and glyoxalate, sarcosine and glycine, dimethylglycine, betaine. After 2 weeks the cultures with 5.0 g analogues / l were plated on CPS-medium to isolate the yeasts. All the assays were done in triplicate.

Autoradiography

To study the ^{14}C-glyphosate accumulation, MM with 1.5 % bactoagar (Difco) was used; filter-sterilized carbon sources were added, prewarmed, once the agar cooled to 55 ºC. The solidified agar was partially dried by incubation of the inverted petri dishes at 45 ºC for 2-3 h. A 0.5 ml of 0.6 Ci ml^{-1} 3-^{14}Cglyphosate was spread over the agar-surface; the plates were overlaid with sterile nitrocellulose before being streaked with yeast isolates. The same procedure was repeated in MSM without NH_4Cl and 3-^{14}Cglyphosate as P source. After colonies development, the filters were removed, dried and coated in plastic foil.

Chemicals

Glyphosate was obtained from ICI Agrochemicals; 3-^{14}Cglyphosate was purchased from Amersham International. All the other chemicals were analytical grade from Sigma.

Statistical analyses

Data from the biotransformation experiments, yeast densities and colony types numbers were evaluated by analysis of variance with comparisons against the sterile controls by ANOVA [26]. Significance level were set at $P = 0.01$.

Results and discussion

Two different yeast populations were obtained from glyphosate treated and untreated soils and 115 environmental yeasts were isolated by both strategies; 77 and 38 different colony types grew in treated and untreated soils, respectively. The other selection criterion, such as CPS, phosphate, glyphosate and AMPA, gave significant different colony numbers, being the herbicide the minor uptake substrate. However, the glyphosate treated soils contained 33 % more degrading yeasts than the untreated one no matter the second isolated medium was, but they had less diversity of colony types (Fig. 1).

Yarrowia lipolytica and *Candida krusei* were the dominant isolates from untreated and treated soils, respectively. Althought both species biotransformed the herbicide, *Y. lipolytica* showed a longer lag phase, the uptake began at the 6th day and no increment in the degradation was obtained after 12th day incubation time. On the contrary, *C. krusei* increased the glyphosate uptake at the 3th. day and the

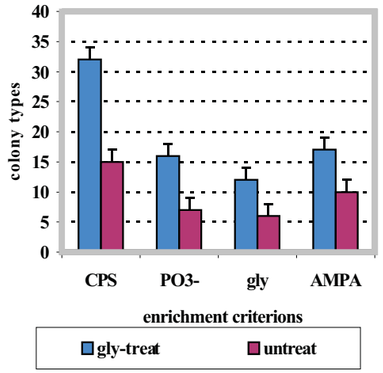

Figure 1. Yeast colony types obtained under different enrichment criterions (gly: glyphosate treatment; untreat.: without glyphosate; CPS: caseín/peptone/starch; PO3-: phosphate; gly: glyphosate).

exponential phase lasted till the 15th days, being the degradation rate signifcant higher than the *Y. lipolytica* one. While *C. krusei* up took the 66.0 % of the herbicide, *Y. lipolytica* transformed 42.5 % in 20 days; this both species were active biodegraders (Fig. 2).

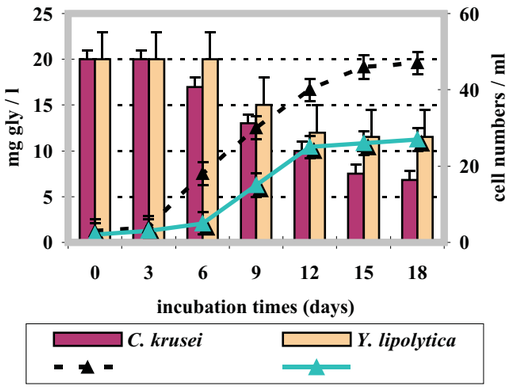

Figure 2: *Y. lipolytica* and *C. krusei* growth curves and glyphosate residual concentration, in the assay with the herbicide as carbon source.

To confirm the yeast ability to grow with organophosphonates as P source, the isolated yeasts were subsequently tested with phosphate, glyphosate or AMPA. In phosphate presence, 24 and 14 isolates were obtained from untreated and treated soils, respectively. The yeasts adapted to the herbicide produced 24 distintive colony types with phosphate, 2 with glyphosate, and 6 with AMPA as P source. On the other hand, when CPS was used as grown medium for untreated soils, only 14 isolates subsequently grew with phosphate, 3 and 6 with glyphosate and AMPA as P-source; so, the previous exposure of soils to the pollutan revealed an increase in the strains able to degrade the toxicant as a consequence of their enzymatic adaptation due to previous exposure (Fig. 3).

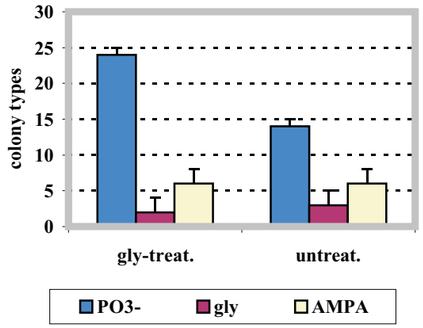

Figure 3: Yeast colony types that uptake different phosphorus sources (PO3-: phosphate; gly: glyphosate; AMPA: aminomethylphosphonic acid).

The herbicide degradation was confirmed in the *Y. lipolytica* and *C. krusei* culture supernatants when the substrate was tested as C source; on the contrary, in the parallel assays in phosphate presence, neither potential intermediates nor a reduction in the glyphosate quantity was obtained.

The ainomethylphosphonic-acid (AMPA) was also assayed and 26 % of the isolates uptook this metabolite as C (Fig. 1) or P source (Fig. 3) in untreated soils; a minor percentage were obtained from glyphosate treated samples. Any way, the AMPA degrading yeasts were more frequent in both soils than the herbicide degrading ones; being the AMPA metabolic isolates 25 and 50 % more numerous in the C or P sources assays, respectively.

The use of structurally related compounds to the herbicide, such as cleavage products, was an alternative strategy to obtain enrichment cultures, thus, the yeasts assayed on these analogues might degrade glyphosate. Higher populations resulted in analogue presence, obtaining 19 and 48 % more colony types when they were used as C or N sources, respectively. This populations showed 10 colony types with acetate, 12 with glycolate and glyoxalate, 6 with sarcosine and glycine; but no growth was observed with dimethylglycine and betaine as C source. In the case of sole source of nitrogen, the yeast populations increased up to 9 colonies with acetate, 7 with glycolate and glyoxalate, 6 with sarcosine and glycine and 3 with dimethylglycine and betaine.

To screen the isolates able to accumulate glyphosate, autoradiographic detection of colonies grown on the radiolabelled herbicide was done. The ^{14}C-glyphosate accumulation into the cells was observed in 32 and 13 isolates from treated and untreated soils, respectively; thus nearly 50% of the isolates were unable to use the herbicide, suggesting that these yeasts transported the glyphosate but did not degradi it. Even more, the phosphate addition inhibited the herbicide uptake by most of the isolates.

Pesticidal detoxification had been widely studied, but mostly with bacteria, other fungal groups or soil communities [11-12, 13]. We remarked that scarce research work with yeasts in relation to herbicides had been realized; the only previous report studied the glyphosate as inhibition factor of pigment synthesis in yeasts [16].

The herbicidal pretreatments favoured the increment of effective yeast populations with detoxification potential of glyphosate and glyphosate metabolites in the untreated soils, although they were more frequent in the treated ones; this fact confirmed that the preexposure is frequently the reason for microbial enzymatic adaptation to most of the pollutants, not only in the organophosphonate presence [1, 22].

Overall, the ability to cleave easily the AMPA molecule was more widespread than the glyphosate uptake, and this is ratified by the development of a mycoflora adapted to degrade this metabolite [5]. Moreover, the addition of related analogues increased the uptaking communities, no matter the substrates were used as C or N sources and similar densities of colony types were obtained in both cases. The AMPA and analogues biodegradation rates (data not provided), confirmed that the intermediates were easily transformed by the wild micoflora; this might be in relation to its more simple chemical structure or due to the adaptation of the strains exposured to glyphosate in agricultural soils [7, 25]. Therefore, wild yeasts could be useful in natural detoxification processes, specially in the bioremediation of agricultural areas polluted with organophosphonates.

The failure of most of the yeasts to uptake glyphosate in phosphate presence suggests the occurrence of organophosphonate inhibition transport by inorganic-P. Moreover, the P presence in the growth medium affected the degrading activity being the herbicide transport or degradation diminished, probably by repression at gene level [12].

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