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N-phosphonomethylglycine utilization by the psychrotolerant yeast *Solicoccozyma terricola* M 3.1.4.

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A B S T R A C T

Solicoccozyma terricola M 3.1.4., the yeast strain isolated from soil sample from blueberry cultivation in Miedzyrzec Podlaski in Poland, is capable to split of phosphorus to nitrogen and nitrogen to carbon bonds in *N*-phosphonomethylglycine (PMG, glyphosate). The biodegradation process proceeds in the phosphate-independent manner. It is the first example of a psychrotolerant yeast strain able to degrade PMG *via* C—N bond cleavage accompanied by AMPA formation and not like in most microorganisms *via* C—P bond disruption followed by the sarcosine pathway. Glyphosate oxidoreductase (GOX) type activity was detected in cell-free extracts prepared from *S. terricola* M 3.1.4. pregrown on 4 mM PMG as a sole phosphorus and nitrogen source in cultivation medium.

1. Introduction

Bioremediation of xenobiotics deposited in soils is an economical and friendly process for the natural environment [73]. So far, there are only few reports [49,70,71,77] pointing to the biodegradability of pollutants by yeast strains that actually have specific enzymes and emulsifiers that increase their ability of xenobiotics biodegradation [29,48]. Yeast is the dominant group of microorganisms in contaminated environments, but so far there is only one research article on the role of wild yeast in soil detoxification from phosphonates [72]. Moreover it is suggested that yeast better adopt to cold environments than bacteria [58]. Psychrotolerant yeasts grow under wide range of temperatures - from 5 °C to 20 °C or above and they are predominant in environments characterized by occurrence of periodic low temperatures. Such conditions are found in agricultural soil in temperate climate e.g. in Poland, the temperature of soil during the year varies from 0.4 to 25.2 °C on the soil surface and in its deeper parts (50 cm depth) from 3.2 to 20.7 °C [14]. Hence, in recent years, interest in research on the possibility of using psychrotolerant yeasts in the processes of soil and water bioremediation increased. There are number of reports about their ability for degradation of xenobiotics, including alkanes, petroleum hydrocarbons, phenol and phenol-related compounds, complex organic acids [21,35,57,60,59]. An important novelty was the recent research, which allowed the selection of several isolates, potentially degrading glyphosate in the soil [82].

Glyphosate is an active ingredient of the most common used in modern agriculture, non-selective herbicide Roundup® (Monsanto, 1974) and other glyphosate-based herbicides (abbr. GBHs) [63]. This synthetic organophosphate is the competitive inhibitor of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS; [EC 2.5.1.19]) [25,40], involved in the shikimate pathway, which is responsible for the biosynthesis of aromatic amino acids, auxin, phytoalexins, folic acid and other secondary metabolism products [40,75].

The GBHs have been used in agriculture for over 40 years. Currently, GBHs are used on a massive scale, due to the growing scale of GMO crops resistant to glyphosate. For example, in 2014, 825.8 million kg of glyphosate were used worldwide [6]. Initially, it was thought that PMG does not penetrate groundwater, does not accumulate in soil and have no toxic effects on humans or animals [51,88]. However, in 2015, the International Agency for Research On Cancer (IARC) classified glyphosate as potentially carcinogenic (Group A2) [8] and in recent

Abbreviations: 2-AEP, 2-aminoethylphosphonic acid ciliatine; AMPA, aminomethylphosphonic acid; CDM, Czapek Dox Medium; D_2O, deuterium oxide; DEAE-Sephacel, diethylaminoethyl-Sephacel; EPSPS, 5-*enol*-pyruvyl-shikimate-3-phosphate synthase; GOX, glyphosate oxidoreductase; PA, phosphonoacetic acid; PMG, *N*-phosphonomethylglycine glyphosate; Pi, inorganic phosphate; YPD, Yeast Peptone Dextrose medium; PCR, Polymerase Chain Reaction; ITS, Nuclear Ribosomal Internal Transcribed Spacer; dNTPs, deoxynucleoside triphosphates

years there are many reports regarding the negative impact of PMG and its direct decay product (AMPA) on all living organisms [2,5,20,24,26,31–33,54]. There are several possible chemical methods for soil remediation from pesticides [90] but in general chemical cleaning technologies require excavation at a site and moving the contaminated soil into storage area where it can be processed, what raise costs and make it inefficient [37]. In the case of glyphosate the possibility of use chemical methods of removing it from contaminated soils is even tougher due to presence in the PMG molecule of very stable C—P bond which makes it highly resistant to degradation by physico-chemical factors [80]. Therefore, microbial degradation of PMG seems to be the most convenient, economically feasible and effective method of removing glyphosate from contaminated soils. However, the half-life of this compound in the environment depends on the several factors, like climate conditions, type of soil and activity of soil microorganisms, but literature data indicates that it vary from 2 to 197 days [4]. Moreover, PMG degradation processes are inhibited by the presence of inorganic phosphate (Pi) in the environment [39,52,68,87].

To date, pro- and eukaryotic microorganisms have been identified that can use PMG for cellular purposes according to one of the three known mechanisms. The first biodegradation pathway is based on glyphosate oxidoreductase (GOX) activity which catalyzes oxidative decomposition of C—N bond in PMG. As a result of oxidative bond cleavage in the glyphosate molecule aminomethylphosphonic acid (AMPA) and glyoxylate are formed, followed by the AMPA conversion to methylamine and orthophosphate by C—P lyase complex. In the second pathway P—C bond cleavage occurs due to the C—P lyase complex activity resulting in formation of sarcosine and Pi. Sarcosine is then converted to glycine by sarcosine oxidase [EC 1.5.3.1] [36,68]. There is also an alternative degradation pathway known for the *Ochrobactrum anthropi* GPK3 [81]. Degradation of PMG begins with the production of AMPA through the GOX pathway, followed by the formation of formylphosphonate and alanine as a result of AMPA-specific transaminase activity. Formylphosphonate is a substrate for phosphonatase and due to the C—P bond cleavage, acetaldehyde and Pi are formed. Such degradation route is only a supposition, because no enzymes involved in this PMG degradation pathway have been isolated or characterized so far [79,81].

Ever-increasing pollution of natural environment with herbicides prompted researchers to look for microorganisms capable of organophosphonates biodegradation. Microbial enzymatic activities can serve as an entry point in the developing of novel biotechnological strategies of environment protection. Biodegradation of PMG and other organophosphonates was primarily studied using mesophilic strains of bacteria: *Enterobacter cloacae* [46], *Pseudomonas putida, Pseudomonas aeruginosa, Acetobacter faecalis* [66], *Flavobacterium* sp., *Acetobacter* sp., *Pseudomonas fluorescens* [61] and fungi: *Penicillium chrysogenum* [41], *Trichoderma viridae, Aspergillus niger*, *Fusarium oxysporum* [1].

Cold-adapted organisms of eukaryotic origin are capable of organophosphonates utilization. It was reported that *Geomycess pannorum* P11 can metabolize naturally occurring phosphonate (ciliatine) [44] and *Geomycess pannorum* P15 is able to utilize phosphonoacetic acid (PA) [43]. Both strains degraded phosphonates at 10 °C independently on the presence of Pi in the growth medium.

This paper describes for the first time the isolation and characterization of psychrotolerant yeast strain *Solicoccozyma terricola* M 3.1.4. able to utilize *N*-phosphonomethylglycine as a source of biogenic elements – phosphorous and nitrogen. PMG biodegradation process occurred independently on the presence of Pi in the environment *via* glyphosate oxidoreductase (GOX) pathway.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents of highest available purity, gradient grade HPLC solvents, ampicillin, chloramphenicol and phosphonates (*N*-phosphonomethylglycine and aminomethylphosphonic acid) were purchased from Merck (Poznan, Poland) unless otherwise stated. DEAE-Sephacel was purchased from Amersham Biosciences. Yeast extract and casein peptone were purchased from BTL (Lodz, Poland), bacteriological agar from Biocorp (Warsaw, Poland). Ampicillin was purchased from Polfa–Tarchomin (Warsaw, Poland), other culture media components were obtained from Avantor (Gliwice, Poland).

2.2. Environmental samples collection

Soil samples were collected from blueberry cultivation in Miedzyrzec Podlaski in lubelskie voivodeship and in two orchards in warmia-mazury voivodeship – in Wilamowo and Wojtowa Rola - in Poland. Roundup was there applied twice a year (spring and summer) for 5 years. Samples were collected in November 2015. Soil samples were gathered into sterile 50-ml conical centrifuge tubes from 0 to 10 cm depth. All samples were transported in ice box to the laboratory and kept at the +4 °C for further analysis.

2.3. Isolation of yeast strains

1 g of soil was transferred into sterile 50-ml conical centrifuge tubes and then suspended in 9 ml of 0.85% sterile saline solution. The suspensions were mixed vigorously for 15 s. After the sedimentation of the soil, 0.1 ml of dilution was spread on YPD agar (containing per liter: glucose 20 g, casein peptone 20 g, yeast extract 10 g and bacteriological agar 20 g) supplemented with ampicillin (final concentration 100 mg l^{-1}) and chloramphenicol (final concentration 34 mg l^{-1}) with the sterile glass rod and incubated at 18 °C for 4 days. Then colonies differing in morphological properties, followed by microscopic examination of cell morphology, were three times streak plating on YPD agar and incubated at 18 °C for 4 days to obtain pure cultures. Finally, the pure yeast cultures isolated during this study were deposited in Culture Collection of Department of Molecular Biotechnology and Microbiology (Chemical Faculty, Gdansk University of Technology).

2.4. Screening for yeast strains capable of glyphosate utilization as a sole nitrogen and/or phosphorus source

Pure cultures of all isolated yeast strains were subjected to starvation. This was achieved by inoculating of single yeast colony from YPD agar plate in 10 ml sterile test tube on 3 ml of CDM broth (containing per liter: glucose 15 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $NaNO_3$ 2.64 g, K_2HPO_4 1 g) with omitted phosphorus and/or nitrogen sources and incubated on rotary shaker at the speed of 140 rpm in 18 °C, for 4 days. After that period, 0.1 ml of each culture was transferred into 20 ml of CDM broth in 250 ml Erlenmeyer flasks, supplemented with filter-sterilized glyphosate solution as sole phosphorus and/or nitrogen source in final concentrations of 2 mM for phosphorus source and 4 mM for nitrogen or nitrogen and phosphorus source. Then, each culture was incubated on rotary shaker at speed 140 rpm at 18 °C for 7 days (first passage). Next the 0.1 ml of subculture was transferred to second passage, which was prepared according to the procedure described for the first passage. Yeast strains cultivated on 20 ml of CDM broth without any phosphorus and/or nitrogen source were used as negative control, which were cultivated under the same conditions as described for experimental cultures. The optical density (OD_{650}) was measured every 24 h of incubation (BioSpectrometer kinetic, Eppendorf). The PMG degradation by tested yeast strains in culture medium was analyzed by LC-MS.

2.5. Taxonomic identification of yeast strain M 3.1.4.

Apart from a few notable exceptions, identification of yeast isolate M 3.1.4 by sequencing of the partial sequences of D1/D2 region and ITS region have been conducted according the methods described by

Filipowicz et al. [21]. The PCR reaction performed for ITS region involved initial denaturation at 95 °C for 5 min, followed by 30 cycles in series of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1.5 min, with a final step of one cycle at 72 °C for 10 min to final extension. The PCR reaction performed for D1/D2 region involved initial denaturation at 95 °C for 5 min, followed by 30 cycles in series of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 45 s., with a final step of one cycle at 72 °C for 5 min to final extension. In both PCR reactions, 1 U of *Taq* thermostable DNA polymerase (A&A Biotechnology, Poland) was used.

2.6. Cultivation methods of Solicoccozyma terricola M 3.1.4.

2.6.1. General procedure

Solicoccozyma terricola was grown on modified C zapek liquid medium (CDM, pH 6.5) containing per liter: glucose 15 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $NaNO_3$ 2.64 g, K_2HPO_4 1 g. Depending on experiments assumptions, phosphorus and/or nitrogen sources were omitted and replaced with filter-sterilized *N*-phosphonomethylglycine at the final concentration of 2 mM (sole phosphorus source), 4 mM (sole nitrogen source) or 4 mM (sole phosphorus and nitrogen source). Liquid cultures (50 ml in 250-ml Erlenmeyer flasks) were inoculated with a yeast biomass (obtained from appropriate solid culture) suspension in 0.85% NaCl and incubated at 10 °C on a rotary shaker at 140 rpm. Initial turbidity (OD_{650}) of the inoculum was 0.05. Cultures were maintained for 6 days and 1 ml samples were taken each day which were then used to monitoring OD_{650} changes and concentration of substrate and products (Pi and/or AMPA) in post-culture fluid. Since the growth of the examined strain under certain culture conditions does not occur in the form of turbidity and it is not possible to measure the optical density, it was necessary to use two methods to control the biomass growth: measurement the amount of yeast dry matter and optical density measurement, depending on the form of growth. In order to compare these amounts, calibration curve to relate the absorbance with cell dry weight was generated, where a linear relationship between the number of cells and the absorbance was obtained. At the same time, control experiments were carried out on mineral medium containing K_2HPO_4 instead of PMG and uninoculated CDM medium supplemented with PMG. All experiments were performed at least in triplicate.

2.6.2. Culture conditions

Yeast strain was cultivated on modified CDM medium supplemented with 4 mM PMG served as the only phosphorus and nitrogen source as described above. The influence of aeration and temperature on growth were investigated. For this purpose, medium was inoculated with yeast and then the cultures were grown stationary or on a rotary shaker (140 rpm) at 4 and 10 °C. For 6 days 1 ml samples were taken and then they were analyzed for changes in OD_{650}, substrate loss and product increment (Pi and/or AMPA). In order to check whether the examined strain is able to decompose PMG independently on the presence of inorganic phosphate in the culture environment, the changes of phosphonate concentration in modified CDM culture broth were monitored for 6 days in the CDM medium containing combination of 2 mM Pi and various concentration of PMG (0, 0.5, 1, 2, 4 and 8 mM). 1 ml samples were taken daily and analyzed as above.

2.7. Preparation of cell-free extracts

Cultures were carried out on modified CDM liquid medium containing K_2HPO_4 as a sole phosphorus source until early mid-log phase as it has been described above. After incubation in 10 °C on a rotary shaker (140 rpm) for 5 days biomass was harvested by centrifugation (10,000 rpm, 10 min), washed twice with sterile distilled water and transferred to Erlenmeyer flasks (250 ml) containing 100 ml of distilled water in order to introduce a nutrient deficiency state. After 48 h of starvation biomass was transferred to the CDM liquid medium containing 4 mM PMG as a sole nitrogen and phosphorus source. Cultures were incubated on a rotary shaker (140 rpm) at 10 °C for 5 days. After this period microbial biomass, was harvested by centrifugation (8000 rpm, 15 min) and washed thrice with ice-cold 50 mM Tris-HCl buffer pH 7.5. Cell free extract was prepared as described previously [43]. Fractions containing the highest amount of proteins were combined and used to determine glyphosate oxidoreductase activity. The above steps of extract preparation, starting from the dialysis, were performed at 10 °C.

2.8. Cell-free extract activity assay. Glyphosate oxidoreductase activity determination

Carbon-nitrogen bond cleavage activity in cell-free extracts prepared from PMG utilizing strain was determined by modified Barry and Kishore method [91]. Glyoxylate was used as a standard to create a standard curve, performed for each freshly prepared stock of reagent mixture. Activity was expressed in nkat of glyoxylate liberated per second per milligram of protein. The analysis was performed in triplicate. Reported values are means ± SD over replicates.

2.9. Analytical methods

Inorganic phosphate was quantifed colorimetrically by means of the green malachite acid dye assay, as described previously [92], using potassium monophosphate as the standard. Protein concentration was measured by the method of Bradford [10], using bovine serum albumin as the standard. All determinations and treatments were carried out at least in triplicate; reported values are means ± SD over replicates.

2.10. LC-MS analysis of AMPA and glyphosate

Samples were analyzed by the HPLC system that consisted of a degasser, two binary pumps and thermostated auto sampler (Acquity UPLC I-Class, Waters, Ireland) connected to an Waters Synapt G2-Si HDMS QuanTOF mass spectrometry detector. Samples (0.5 µL) were applied to a reversed-phase column (HSS T3 2.1 × 50 mm, 1.8 µm; Waters, Ireland) thermostated at 40 °C. The system was operated at a flow rate of 0.45 ml/min with solvent A - water with 0.1% formic acid and solvent B - acetonitrile with 0.1% formic acid. The total analysis time lasted 7.5 min. The gradient started from 1% of B during the first 0.25 min. Next gradient was moved to 99% of B in 5 min and then reached 99% in 5.1 min. When reached 99%, it returned to starting conditions in 0.1 min, keeping the re-equilibration until 7.5 min. Data were collected in ESI negative (-) ion modes in separate runs on a QuanTOF operated in MS^E mode from 50 *m/z* to 600 *m/z* with a scan rate of 10 scans per second. Accurate mass measurements were obtained by means of an automated calibrant delivery system that continuously introduces a calibrating solution, which contains reference masses at *m/z* 120.0813 and *m/z* 556.2771 (Leucine-Enkephalin) in positive ion mode; and *m/z* 179.0821 and *m/z* 554.2615 in negative ion mode. The capillary voltage was set to 3000 V for negative ionization mode; the drying gas flow rate was 800 L/h. Samples were analyzed in a randomized order in separate runs. For better quality control, at the beginning and at the end of the analysis, a batch of samples for standard curve calculation were injected. The raw data collected by the analytical instrumentation was analyzed by QuanLynx. For quantification, *m/z* 110.0002 and *m/z* 168.0054 were used for AMPA and glyphosate, respectively.

3. Results and discussion

3.1. Yeast strains isolation

It has been suggested that the application of herbicides in agriculture may negatively affect the soil microflora including the change

in the structure of the microbial community [86]. The most active glyphosate-degrading microorganisms were isolated from soils contaminated with organophosphonates [64]. Due to these reports, several soil samples for yeast isolation were collected from glyphosate-contaminated soils. There were isolated 78 psychrotolerant yeast isolates - 55 from soil samples collected in lubelskie voivodeship and 23 from soil samples collected in warmia-mazury voivodeship.

3.2. Isolation of glyphosate-degrading yeast strains

The optical density (OD_{650}) for each yeast isolate was measured during the cultivation on second passage. Due to ability to grow on PMG as sole phosphorus, nitrogen and nitrogen and phosphorus source on CDM liquid medium, 23 yeast isolates were classified as probably able to degrade glyphosate as phosphorus source, 2 as nitrogen source and one as nitrogen and phosphorus source simultaneously. The PMG degradation ability of selected isolates was checked by LC-MS analysis of initial and final PMG concentration in post-culture liquid. The LC-MS analysis confirmed that four isolates were able to utilize PMG. Strains M 1.3.22. and M 3.1.9. were able to utilize PMG as sole source of phosphorus, M 1.1.18. as sole source of nitrogen and M 3.1.4 as sole sources of nitrogen and phosphorous. However, the literature study revealed, that research on PMG-degrading microorganisms was focused mainly on their potential of PMG mineralization as sole phosphorus source [38,62,78]. On the other hand, there are not many reports concerning the use of PMG as sole available nitrogen or nitrogen and phosphorus source [41,65,67] . Hence the attention has focused on further studies of strains M 1.1.18 and M 3.1.4. yeast strains.

Moreover, LC-MS analysis revealed that the highest decline of PMG concentration (66%) after 7 days of cultivation was detected for strain M 1.1.18 which utilize PMG as a sole source of nitrogen. For the strain M 3.1.4, the highest decline of PMG concentration (53%) was observed for PMG as a sole source of nitrogen. For comparison, for strains M 1.3.22., M 3.1.9, which utilize PMG as a sole source of phosphorous, the decline of PMG concentration was 48%, 64%, respectively. Although, on the basis of these results, strain M 1.1.18 seemed more promising than strain M 3.1.4 (preliminary comparison of PMG biodegradation potential), unfortunately, it will not meet all of set up criteria. During this study, soil yeast strains that are able to grow in temperature 10 °C (and not above 25 °C) were looked for. It should be noticed, that soil yeast strains growing in this temperatures range, could be useful for applications in agricultural soil bioremediation in temperate climate e.g. in Poland. As it was presented above, the temperature of agricultural soil in Poland, during the year varies from 0.4 to 25.2 °C on the soil surface and in its deeper parts (50 cm depth) from 3.2 to 20.7 °C [14]. Unfortunately, the strain M 1.1.18., in comparison to M 3.1.4, was not growing efficiently in lower temperatures. Therefore, in our opinion, the strain M 3.1.4. was better candidate for further applications in this field. On the base of both presented criteria, the strain M 3.1.4. was selected to further studies.

3.3. Taxonomic identification of yeast strain M 3.1.4.

The PCR products for D1/D2 region and ITS region of yeast strain M 3.1.4. were purified, sequenced and analyzed for nucleotide matching by nucleotide BLAST (high similar sequences- megablast). Partial sequence of large subunit ribosomal RNA gene from M 3.1.4. isolate (PCR product with the lenght 550 bp) showed the highest DNA sequence similarity (100%) and coverage (100%) to *Solicoccozyma terricola* CBS 4517 (GenBank accession number KY109683.1) which is deposited in CBS-KNAW Culture Collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). Simultaneously, partialITS1 5.8S rDNA ITS2 sequence from M 3.1.4. isolate (PCR product with length 563 bp) showed the highest DNA sequence similarity (100%) and coverage (100%) to *Solicoccozyma terricola* CBS 6435 (GenBank accession number KY105455.1) which is deposited in CBS-KNAW Culture

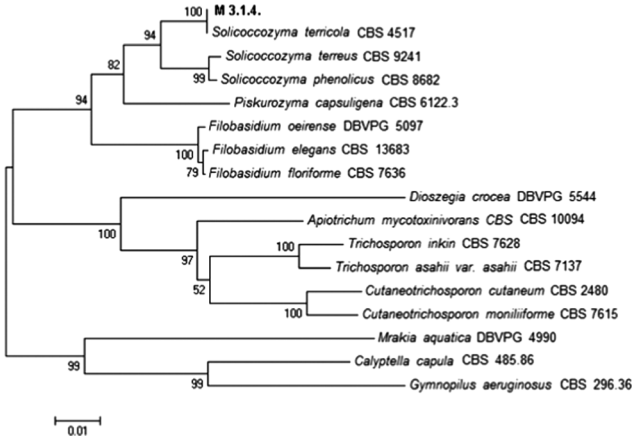

Fig. 1. The phylogenetic tree of yeast strain M 3.1.4. based on the analysis of the selected rDNA sequences (D1/D2 region).

Collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) The phylogenetic tree based on the analysis of DNA sequence of D1/D2 region and ITS region of yeast strain M 3.1.4. and the selected nucleotide sequences deposited in GenBank are presented on Figs. 1 and 2 respectively.

3.4. Biodegradation of PMG by Solicoccozyma terricola M 3.1.4.

Inorganic phosphate (Pi) has been demonstrated throughout the years that it is the preferred source of phosphorus (P) for microbial cells purposes what has been confirmed by the information included in Table 1, concerning examined psychrotolerant yeast strain.

However, xenobiotic compound – glyphosate was found to support quite efficiently yeast growth, when it was applied as the only, alternative P-source. Yeast causes a 60% reduction in PMG concentration in the cultivation medium (Table 1B) compared to the initial value and final biomass yield was comparable to that obtained in medium containing the same level of Pi (Table 1 A and B). The observed extension of mean generation time can indicate some additional adaptive mechanisms necessary for xenobiotic use for cell purposes. The phosphonate uptake has reached 1.12 mmol per g of yeast biomass what is an amount slightly lower than in the case of growth in the presence of inorganic phosphate (1.34 mmol per g of yeast biomass). Glyphosate can also serve as a source of nitrogen for microbial growth, but according to literature data, there are only few examples of microorganisms of such metabolic properties [41,30,27]. That is why, the effect of the availability of alternative source of nitrogen upon *Soliccozyma* growth and herbicide utilization were

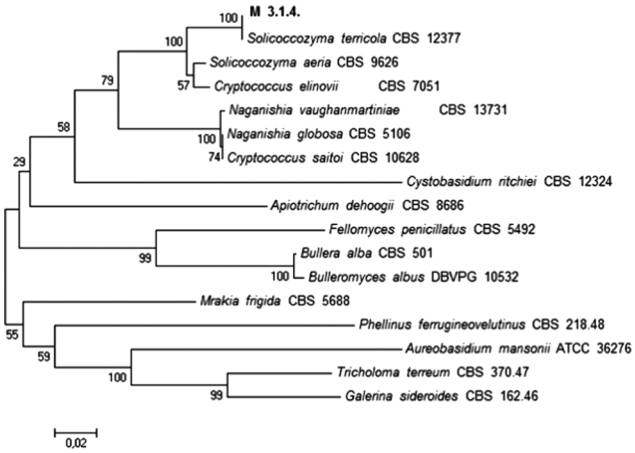

Fig. 2. The phylogenetic tree of yeast strain M 3.1.4. based on the analysis of the selected rDNA sequences (ITS region).

Table 1
Growth rates and biomass yields of *S. terricola M 3.1.4* in minimal media containing different concentration of PMG and/or Pi.

	Source of biogenic elements	Mean generation time [hours]	Yield of biomass [g/l]	PMG residual level [mM]	Pi residual level [mM]	Biomass [g/l]/milimolar equivalent of PMG/Pi assimilated
A	P: 2 mM Pi N: 2 mM $NaNO_3$	6.61 ± 0.51	1.219 ± 0.061	–	0.369 ± 0.008	0.75 ± 0.04
B	P: 2 mM PMG N: 2 mM $NaNO_3$	9.92 ± 0.44	1.078 ± 0.030	0.79 ± 0.01	0.025 ± 0.004	0.89 ± 0.02
C	P: 2 mM Pi N: 4 mM PMG	9.94 ± 0.49	1.097 ± 0.009	1.33 ± 0.01	0.792 ± 0.001	0.24 ± 0.01
D	P + N: 4 mM PMG	11.18 ± 0.23	0.494 ± 0.065	1.29 ± 0.02	0.035 ± 0.002	0.18 ± 0.02
	P + N: 8 mM PMG	10.71 ± 0.32	0.791 ± 0.031	3.98 ± 0.10	0.035 ± 0.004	0.20 ± 0.01
E	P: 2 mM Pi + 0.5 mM PMG N: 2 mM $NaNO_3$	8.11 ± 0.35	0.948 ± 0.011	0.26 ± 0.01	0.538 ± 0.006	0.42 ± 0.01
	P: 2 mM Pi + 1 mM PMG N: 2 mM $NaNO_3$	8.27 ± 0.23	0.689 ± 0.018	0.64 ± 0.02	0.606 ± 0.005	0.29 ± 0.04
	P: 2 mM Pi + 2 mM PMG N: 2 mM $NaNO_3$	9.29 ± 0.14	0.675 ± 0.073	0.67 ± 0.06	0.737 ± 0.003	0.20 ± 0.02

Final biomass was evaluated 6 days after inoculation. Generation time was calculated during logarytmic phase of growth. Data are means ± SD over three replications.

investigated (Table 1 C and D). Examined yeast strain has proved to possess the proper enzymatic potential to exploitation PMG used either as a sole source of nitrogen in the presence of inorganic phosphate or as a source of nitrogen and phosphorus at the same time. The flexibility of metabolism, manifested in the possibility of degradation of various compounds as nitrogen source seems to be very important adaptive mechanism for cold-adapted microorganisms in the context of competition for nitrogen sources in cold environments. The application of phosphonate instead of sodium nitrate in mineral medium composition resulted both in mean generation time extending as well as in the reduction of yeast growth, what indicates sodium nitrate as more preferable nitrogen source for growth. When PMG was used both as N and P source in cultivation medium, the biomass yield was much lower than in case of yeast cultivated in medium with this compound serving as N source, but unexpectedly herbicide degradation was substantially unaffected (Table 1 C and D). Raising the herbicide concentration to 8 mM did not change the amount of PMG used per gram of yeast biomass, when this compound served as the source of N and P at the same time (Table 1 D). Yeast adjusts their level of growth depending on the availability of nutrients by shortening or lengthening the cell cycle in response to the conditions prevailing in the environment. Growth may also be influenced by the availability of nutrients, where rapid growth is conditioned by the high availability of nutrients and slower with low availability of nutrients. Various resting states are generated that allow the cell to survive under conditions of nutrient limitation. In yeast, the availability of nutrients has two basic functions: they are metabolites for growth when cells can increase their mass and signalling factors which activate metabolic, transcriptional and developmental pathways. In order to understand the regulation significance of nutrients in yeast, it is necessary to understand the dual role of them. The speed and manner of growth in yeast is related to the quantity and quality of available nutrients [11,12]. The availability of nitrogen affects the regulation of metabolism, growth and transcription of proteins. Yeast cells are able to use many compounds, using them as a sole source of nitrogen for growth, but obviously they show some preferences. As demonstrated in previous studies, yeast strains preferentially use ammonia, glutamine, but are also able to use other nitrogen sources. However, the growth rate is then lower. In case of yeast, nitrogen catabolite repression also takes place, where the presence of preferred nitrogen sources inhibits the use of less favourable sources of nitrogen [55,12].

Further increasing concentration of PMG used as sole N and P source in the culture medium causes a decrease in the amount of biomass produced. However, even at 30 mM concentration, the growth of the strain was not completely inhibited (data not shown), which means that *S. terricola* M 3.1.4. is able to tolerate high concentrations of the herbicide. This observation is important, because examined yeast can possess PMG-insensitive EPSP synthase what can have implications regarding glyphosate-tolerant crops and weeds. On the other side, this feature and ability to run PMG degradation at low temperatures causes that the examined psychrotolerant yeast strain could be a pretender for bioremediation of glyphosate-contaminated soils especially in temperate climate.

The release of excess inorganic phosphate into the medium was observed when yeast was cultivated on modified medium containing PMG as the sole P or both N and P sources (Table 1 B and D), what suggested that *Soliccozyma* possesses enzymes cleaving the C—P bond as well as PMG catabolism seems to be independent on the presence of inorganic phosphate in the medium. This assumption was confirmed by the yeast capability of PMG degradation, when phosphonate served as the sole nitrogen source. Many studies investigating the influence of biogenic elements (mainly N and P) on yeast life cycle had focused mainly on their role as regulators of various metabolic processes [93–98]. It has been proven that yeast can obtain information about the environment in which they live not only through the activity of intracellular sensors, but also have nutrient detection systems located in the cell membrane [22]. In addition, recent studies have shown that there may be some dependencies and connections within individual metabolic and transcription pathways [74]. To date, available information proves that nutrients necessary for growth, give a common cell response to the deficiency of easily digestible biogenic elements through mechanisms that may contain common elements, but at the moment the exact way in which nutrients are distinguished by yeast has not been fully understood [15].

The next step of research was to investigate the rate of PMG used by *S. terricola* M 3.1.4. in the presence of inorganic phosphate (Table 1 E). For this purpose, three various concentrations of PMG were added to the culture medium containing 2 mM Pi as P source and 2 mM $NaNO_3$ as N source. The presence of two different sources of P in cultivation medium did not change the duration of the generation time but it caused the reduction of biomass production. Inorganic phosphate was preferentially utilized by yeast cells and the level of its utilization per gram of biomass remained quite similar with the main value of 1.8 mmol/g while the PMG partial degradation also was noticed. Yeasts are reported to possess two phosphate transport systems: a low affinity and high affinity transporter, which are independent on the concentration of inorganic phosphate in the culture medium, and depend on the amount of Pi present inside the cell [76]. Under given conditions, only one type of transport can take place in the cell, because both are mutually exclusive. Recent studies have also shown that yeasts have a feedback mechanism that allows the transporters to switch from one

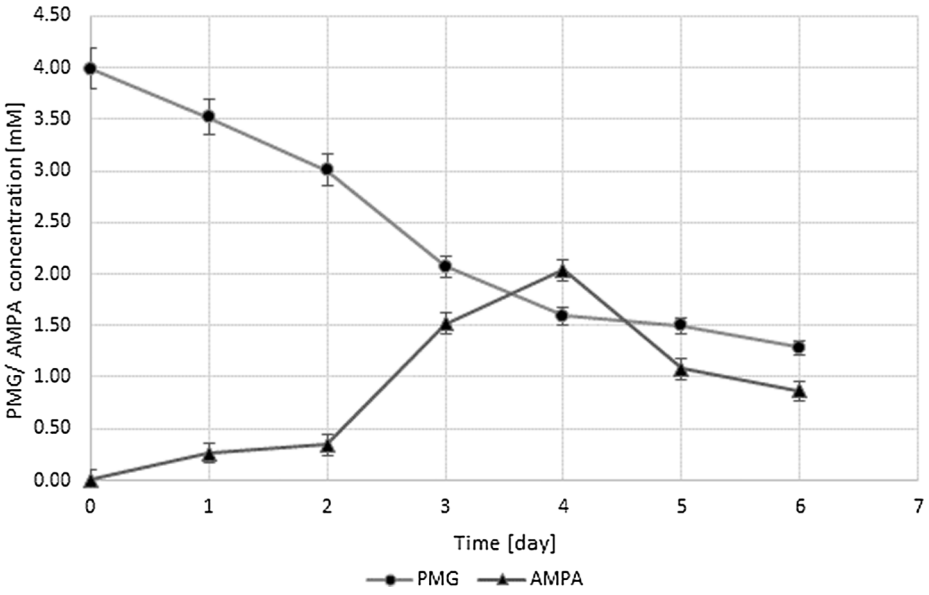

Fig. 3. Changes in glyphosate and AMPA concentration during *S. terricola* M 3.1.4. cultivation in mineral medium containing 4 mM PMG as the only NP-source. Data are mean ± SD of three replications.

to the other, depending on the availability of intracellular Pi [89]. Inorganic phosphate could inhibit either PMG uptake and/or enzyme activity expression, but in this case it seems that the competition for the low-affinity transporter occurred [85]. Above results are consistent with those obtained for other strains of microorganisms described in the literature [16,43] and indirectly confirms that the activity of PMG degrading enzymes are independent of the presence of Pi in cultivation medium. The exception is *Arthrobacter atrocyaneus* ATCC 13752, in which the presence of Pi in the medium inhibits the mineralization of herbicide [67]. On the other hand, *S. terricola* M 3.1.4 may have the pathway enabling the use of PMG under conditions of high availability of inorganic phosphate in the culture medium. This may be related to the activity of permeases that allow phosphonates to be transferred inside the cell *via* amino acids transport system, as previously proposed for two yeast strains: *Kluyveromyces fragilis* [83] and *Candida maltosa* [7]. Moreover, recent studies conducted on *Saccharomyces cerevisiae* have shown that at low temperatures, there is an increase in the expression of genes encoding permeases and a decrease in the expression of genes responsible for phosphate uptake [84]. Fungi form an extremely diverse group of eukaryotic organisms, whose representatives differ in their structural features and way of living. It is not surprising that there are also differences in transport systems, which indicates a wide range of physiological adaptations that have developed among them. In addition, low temperature causes changes in the cell, both physiological and biochemical (e.g. change in permeability and stiffness of the lipid bilayer), hence the transport of nutrients in such microorganisms must be adapted to environmental conditions [53].

The presence of inorganic phosphate in the culture medium in which PMG was used as the N and P sources prompted to look for the phosphonate degradation pathway under these conditions. Because so far, the suspicion of the occurrence of the GOX pathway has been discussed only for several fungal strains [13,47], and most of them use herbicide through the sarcosine pathway, it was important to determine whether the PMG degradation pathway in *S. terricola* M 3.1.4. is mediated by sarcosine oxidase [9] or one of the two described pathways using glyphosate oxidoreductase [80].

Because the pathway using *GOX* to degrade PMG is not very common in eukaryotes and the presence of this enzyme in examined psychrotolerant strain would be a remarkable novelty, first action was to investigate the presence of AMPA in post-culture fluid. The products of the PMG decomposition reaction *via* GOX activity are AMPA and glyoxylate. Glyoxylate supplies the anaplerotic reactions of the glyoxylate cycle, which provides precursors to the Krebs cycle [34]. Interestingly, AMPA is toxic to cells, causing inhibition of DNA repair processes in plants and animals [17,56], so most microorganisms are unable to mineralize this compound. Degradation of AMPA in the environment occurs as a result of the interaction of various groups of microorganisms [28]. Under laboratory conditions, it is difficult to reproduce such conditions and in most cases AMPA is excreted outside the cell [3,50] thus the change in the concentration of this product in the culture fluid was measured. As shown in Fig. 3, PMG supplied as the only source of N and P for yeast growth was degraded to final concentration of 1.28 mM and its degradation rate accelerated at 2–4 days of cultivation, while since then the utilization rate slowed down. Yeast growth was accompanied with AMPA accumulation in growth environment and this phosphonate concentration reached maximum value at 4th day of cultivation. After the 4th day of cultivation, a reduction in AMPA concentration in the culture medium can be observed. A similar course of the PMG degradation accompanied with AMPA formation was also observed in the case of *Aspergillus oryzae* A-F02 [23]. It can be assumed that the decrease in AMPA concentration during the *S. terricola* M 3.1.4 cultivation may be due to the induction of another pathway (e.g. C—P lyase pathway) as it is suspected for *A. oryzae* A-F02. Similarly to *S. terricola* M 3.1.4, PMG was not completely degraded by the *A. oryzae* A-F02 strain, which may indicate that one enzyme is not able to carry out complete herbicide degradation, and the PMG biodegradation includes a series of ordered processes involving different enzymatic pathways.

Regardless of the detailed course of PMG degradative pathway, the final product is inorganic phosphate [45]. The presence of Pi in the culture fluid may indicate an unprecedented ability of yeast to mineralize this compound as shown for *O. anthropi* GPK 3 [81]. Due to the fact that Pi is formed in each of described pathways and except AMPA, only the total level of inorganic phosphate in the culture medium was measured, the above statements are only suspicions and more research is needed to identify the real fate of AMPA and enzymes involved in this process.

The biomass formation under mentioned conditions was monitored (data not shown) and cultures entered the stationary phase after 4 days of incubation. It should be noticed, that PMG utilization and AMPA formation occurred in a non-stoichiometric manner, what could be explained on the basis of the analysis of ^{31}P NMR spectrum of yeast cell

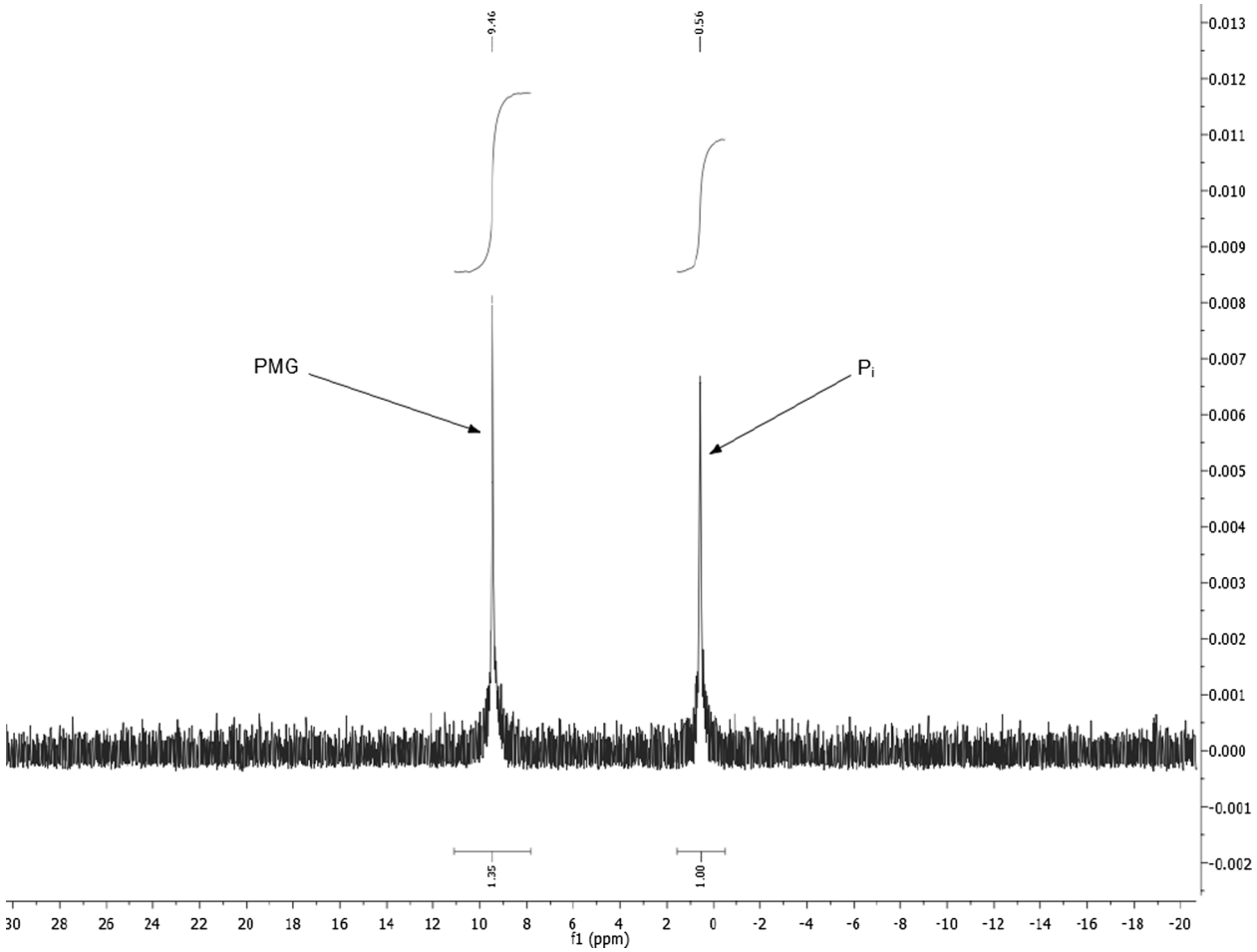

Fig. 4. ^{31}P NMR analysis of the *S. terricola* M 3.1.4 cell free extract after cultivation on 4 mM PMG as the sole phosphorus and nitrogen source for growth.

free extract (Fig. 4). Such analysis of fungal cell-free extract showed the presence of PMG (δ = 9.46 ppm), proving the uptake and accumulation of the phosphonic substrate in the yeast cell (Fig. 4).

Interestingly, when supplied as both P and N source, glyphosate was gradually removed from the culture broth with accompanying release of inorganic phosphate into the culture medium (data not shown). However, the final concentration of Pi in culture broth did not exceeded 0.035 mM (Table 1D). The release of Pi into the growth medium was observed also for *Penicillium oxalicum* strain utilizing the phosphonoacetate applied as the only P source for growth as well as in case of *Geomycess pannorum* with the capability of ciliatine degradation applied as the only NP source in culture medium [42,43].

The obtained results allowed to hypothesize that examined yeast is able to degrade PMG *via* glyphosate oxidoreductase pathway. In order to obtain the appropriate amount of biomass necessary for cell-free extract activity assay, the strain was cultured under stress conditions. The ultrasonically method for cell disruption allowed to obtain a crude extract containing 54 µg/ml of released protein. For the first time glyphosate oxidoreductase activity from eukaryotic strain belonging to the psychrotolerants was successfully detected. Cell-free extract prepared from psychrotolerant *S. terricola* M 3.1.4. biomass pregrown on 4 mM PMG (sole phosphorus and nitrogen source) contained detectable *GOX* activity. Specific activity amounted to 0.031 ± 0.003 nkat of glyoxylate released per second per milligram of protein. Above studies prove that this examined strain, apart from the ability to decompose C—P bond, can also use PMG as a sole phosphorus and nitrogen source and thus carry out degradation of C—N bond in *N*-phosphonomethylglycine molecule. Activity of *S. terricola* M 3.1.4 crude extract was lower than those getting from bacterial strains [3,91]. Low enzyme activity may be due to the presence of two parallel pathways involved in the phosphonate breakdown: GP-specific C—P lyase and glyphosate oxidoreductase. The occurrence of the two degradation paths was also observed for other strains [34,80]. The share of individual distribution ways varied depending on the strain what may indicate that GP-dependent C—P lyases from various microorganisms may differ in sensitivity to inhibition by AMPA and methylphosphonic acid. Literature data indicate that GOX path is characteristic for microbes isolated from PMG polluted environments, what is reaffirmed in this case. It is worthy to emphasize that the most active PMG-degrading microorganisms were usually isolated from PMG-contaminated soils, such as *Ochrobactrum anthropi* GPK 3 and *Achromobacter* sp. Kg 16 [18,19]. The path of herbicide decomposition through the *via* GOX is considered as a result of the adaptation of microorganisms to the long presence of PMG in the environment. So far there were only two microorganisms capable to degrade PMG using the AMPA pathway that were isolated from other sources than industrial PMG-degrading effluents [16,69]. However, obtaining activity of cell-free extract and confirming the biodegradation process *via* AMPA pathway is an interesting novelty and can become an inspiration for further exploration of such activities in psychrotolerant microbes isolated from PMG-contaminated environments.

Obtaining *GOX* activity and tolerance for high concentrations of PMG in cultivation medium by *S. terricola* M 3.1.4. may have potential applications in the bioremediation of glyphosate-contaminated soils, and the isolation of relevant genes may be used to build herbicide-resistant plants in the future. As it was achieved by insertion of microbial origin glyphosate oxidoreductase gene into different plant genomes which led to construction of Roundup Ready™ crops [91].

4. Conclusions

The aim of the study was isolation a new psychrotolerant yeast strain from soils treated with PMG and to highlight the possible role of this yeast strain in glyphosate degradation. This is the first report on PMG degradation as a phosphorus and nitrogen source by psychrotolerant yeast strain isolated from glyphosate contaminated soil. This study fulfils a gap in PMG biodegradation research between bacteria and filamentous fungi. The yeast ability to use PMG as NP source for growth is a novelty, and the obtained results provide a valuable extension of knowledge about the diversity of microorganisms involved in the C—P bond compounds biodegradation. Release of inorganic phosphate to the culture medium confirmed the progress of xenobiotic biodegradation suggesting that the tested strain degrade this compound in an independent manner of the phosphate cell status. For the first time it was managed to obtain an active cell-free extract towards glyphosate oxidoreductase from psychrotolerant eukaryotic organism. Decomposition of the herbicide relies upon *GOX* activity what strongly suggests the release of AMPA into the culture medium. Understanding the mechanisms of phosphonate derivatives degradation by various eukaryotes broadens knowledge in the field of degradation of phosphonic compounds and may contribute to the development of effective methods of soil and water bioremediation.

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