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NOTES AND SHORT COMMUNICATIONS

Metaproteomics Applied to Activated Sludge for Industrial Wastewater Treatment Revealed a Dominant Methylotrophic Metabolism of *Hyphomicrobium zavarzinii*

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Abstract In biological wastewater treatments, microbial populations of the so-called activated sludge work together in the abatement of pollutants. In this work, the metabolic behavior of the biomass of a lab-scale plant treating industrial pharmaceutical wastewater was investigated through a metaproteomic approach. The complete treatment process included a membrane biological reactor (MBR) coupled with an advanced oxidation process (AOP) for partial breakdown of non-biodegradable molecules. Proteins from biomass samples collected pre- and post-AOP application were investigated by two-dimensional gel electrophoresis (2DE), mass spectrometry (MS), and finally identified by database search. Results showed that most proteins remained constant between pre- and post-AOP. Methanol dehydrogenase (MDH) belonging to *Hyphomicrobium zavarzinii* appeared as the most constantly expressed protein in the studied consortium. Other identified proteins belonging to *Hyphomicrobium* spp. revealed a predominant methylotrophic metabolism, and *H. zavarzinii* appeared as a key actor in the studied microbial community.

Keywords Metaproteomics · Activated sludge · Wastewater · *Hyphomicrobium* · Methanol dehydrogenase

Introduction

Biological degradation processes are considered conventional treatments for sewage since almost a century. Several microbial populations cooperate in the degradation of wastewater pollutants and represent the main part of activated sludge. Over the years, different tools were developed to characterize these microbial consortia [1, 2]. As a first approach, microscopy can offer preliminary indications of the morphology and physiological status of the biomass, while standard physical determinations describe the main characteristics of sludge in terms of water/solid content, settleability, filterability, etc. Secondly, respirometry gives information about bacterial activity, cell growth, and decay through biomass oxygen consumption. Thirdly, molecular tools can characterize the biomass on the basis of gene sequences, like in fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR). Finally, metagenomics delivers data on the metabolic potential of microorganisms in wastewater treatment plants (WWTP). Improved understanding of interactions among microbial consortia in activated sludge requires profound investigation including functional analysis to optimize treatment processes. Valuable data on the physiological and metabolic activity of microorganisms can be obtained by targeting the entire protein set of a cell, such as in proteomics. Technical advances in mass spectrometry (MS) enabled the proteome analysis of complex microbial

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communities in environmental samples such as those of WWTP [3]. Metaproteomics was successfully applied to the study of numerous microbial communities from groundwater samples [4], while it was only recently applied to microbial consortia of biological WWTP, with interesting results [5, 6].

In this work, the metaproteomic approach was applied to investigate the metabolic behavior of the biomass of a lab-scale plant treating industrial pharmaceutical wastewater containing residues of nalidixic acid (NAL). The complete treatment process included a membrane biological reactor (MBR) coupled with an advanced oxidation process (AOP) for partial breakdown of non-biodegradable molecules. More details and results of the integrated process are described elsewhere [7, 8]. Biomass samples were collected before and after the AOP application, and proteins were extracted according to the phenol extraction protocol proposed by Kuhn and colleagues [9]. Proteins extracted in different days during steady operation of the system were first investigated by qualitative SDS-PAGE according to Laemmli [10], as shown in Fig. 1. No significant quality variations were pointed out in protein patterns as a result of AOP treatment, and interestingly a highly expressed band of about 65 kDa resulted always present. As better explained in the following, this band was related to the abundance of methanol dehydrogenase (MDH), which was found to belong to a specific bacterial strain (*Hyphomicrobium zavarzinii*). Samples collected on day 58 and day 108, called, respectively, pre- and post-AOP, were finally chosen for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): the first dimension was carried out through isoelectric focusing (IEF) by 18-cm IPG strips pH 4–7 (GE Healthcare), the second one through SDS-PAGE. Every analysis was performed in triplicate. The amount of total proteins in each extracted sample was evaluated through Total Protein Kit, Micro-Lowry (Sigma-Aldrich, Missouri, USA) in order to load the same protein content on each IPG strip. The software Delta2D™ (Decodon, Greifswald, Germany) was used for image analysis. 2D gels of pre- and post-AOP samples showed very limited changes in the protein pattern (Student's test, P values <0.05) so that most of the spots appeared unvaried (Fig. 2). For each 2D gel (pre and post AOP), a high number of 144 spots were manually excised, and 74 of these were subjected to tryptic digestion and analyzed by MALDI TOF MS/MS (Ultraflextreme, Bruker Daltonics, Massachusetts, USA) as described by Kluge and co-workers [11]. Proteins were identified by searching peptide spectra with MASCOT (http://www.matrixscience.com) against NCBInr (version 20140312). Each protein was associated to a specific spot when its Mowse score was at least 100, its number of peptides at least 2, and its molecular weight and isoelectric point were consistent with its position in 2D gels. If more proteins applied for a spot, the one with the best parameters was selected (Online Resource 1). Thirty-six spots were reliably identified by MALDI TOF MS/MS. In order to increase the number of identifications, 21 selected spots among those not identified were additionally analyzed by nanoHPLC-ESI MS/MS [12], resulting in the successful identification of four more proteins. Finally, 35 spots whose abundance seemed unvaried (Fig. 2, full arrows) and 5 spots with apparently changing abundance (dotted arrows and boxed IDs) were identified. Protein identifications are listed in Table 1, where all proteins were identified by at least two peptides and in many cases, this number was higher than 2. For each spot listed in Table 1, all candidate proteins were also evaluated (i.e., those with lower scores and number of peptides) as reported in

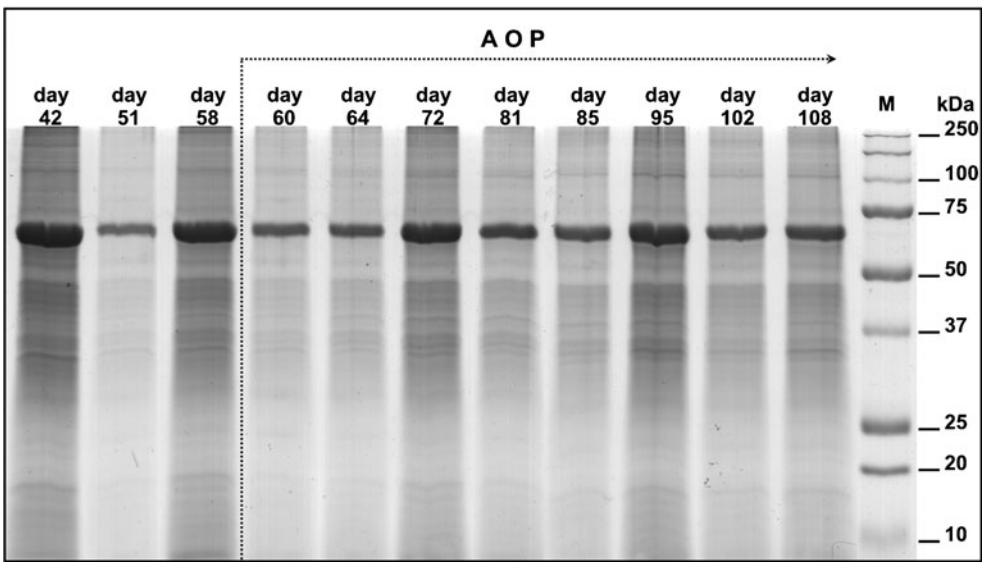

Fig. 1 SDS-PAGE. Steady-state pre-AOP condition is represented by days 42, 51, 58. Steady-state post-AOP condition by the following days

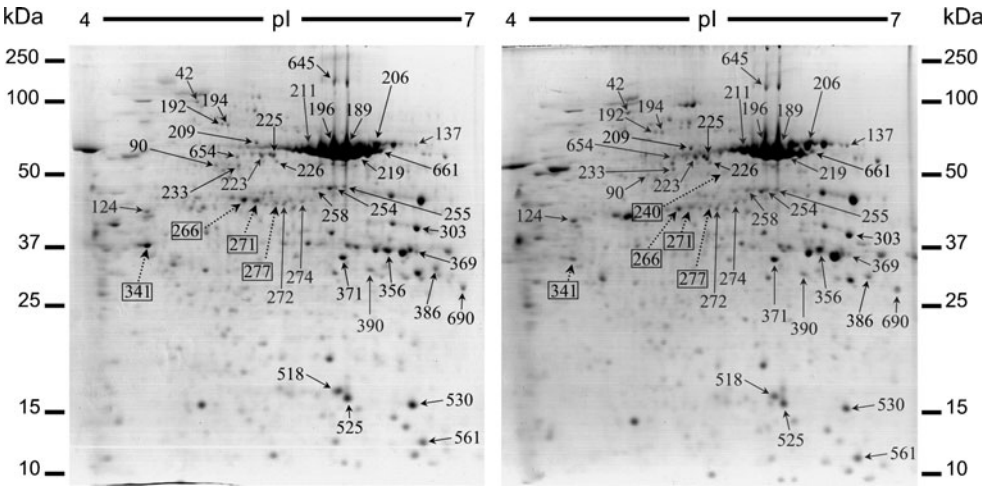

Fig. 2 Spots of identified proteins in 2DE gels. Pre-AOP gel (day 58) is on the *left*, post-AOP is on the *right* (day 108). Spots resulted constant between both conditions are indicated by *full arrows*, variable ones by *dotted arrows* and *boxed IDs*

supplementary Online Resource 1. Moreover, the "Unipept metaproteomics analysis pipeline" (Unipept 3.1) was applied to all peptides, and it confirmed the *Hyphomicrobium* genus as the most prevalent in the consortium, as shown in Online Resource 2 [13].

Metaproteome analysis showed that 31 database hits of identified proteins were taxonomically affiliated to the genus *Hyphomicrobium*, of whom 17 referred to *H. zavarzinii*, 9 to *Hyphomicrobium nitrativorans*, and 3 to *Hyphomicrobium denitrificans*. The 65-kDa band with high expression in SDS-PAGE corresponded to the larger and constantly expressed spots in 2D-PAGE identified as methanol dehydrogenase (MDH) of *H. zavarzinii*. Also ethanol dehydrogenase (EDH) belonging to *H. zavarzinii* was interestingly found at constant expression levels. These two important and constant identifications suggest that the incoming wastewater contained relevant amounts of methanol and ethanol, so that *H. zavarzinii* was well adapted to these substrates and appeared well represented in the biomass, confirming previous findings for methanol-fed biological reactors [14]. The aldehyde-activating protein (AAP) belonging to *H. nitrativorans*, which presents the same domain of the formaldehyde-activating enzyme (FAE) belonging to *Methylobacterium extorquens* AM1 (KEGG entry W911_13190), was found constant too. Vorholt and coworkers [15] showed the strong relationship between the MDH and the FAE describing the reversible reactions of the FAE pathways as an option to achieve the anabolism of one carbon compound entering the serine cycle, useful for biosynthesis or acetyl-CoA production. The identification of the aldehyde-activating protein (AAP) belonging to *H. nitrativorans* also leads to the hypothesis of a metabolic cooperation with the MDH of *H. zavarzinii* in the oxidation of methanol.

Furthermore, no differences in the expression of the chaperones GroEL and DnaK were observed in pre- and post-AOP gels. This suggests that the microbial communities did not experience severe stress, appearing well adapted to process conditions including the new by-products formed in the AOP treatment of effluents. In addition, diverse ATP-binding cassette (ABC) transporter permeases and binding proteins indicated an active transport of nutrients or metabolites. In particular, many studies attributed a primary role to ABC transporters in the bacterial capability to develop drug resistance, as it may happen for NAL [16]. As for the DNA polymerase III subunit beta, despite the well-known toxic effects of NAL on DNA replication, no significant difference in expression was observed before and after AOP application, suggesting possible adaptation of *H. zavarzinii*. Moreover, a glutamate/glutamine/aspartate/asparagine ABC transporter of *Roseovarius* sp. TM1035 resulted as downregulated at the end of the AOP process. The amino acids transported by this permease are key molecules in nitrogen metabolism. This correlates with the increased availability of alternative nitrogen compounds following modifications of NAL (which structurally contains nitrogen) due to the AOP.

In conclusion, the investigation of the microbial consortium through metaproteomics allowed the identification of the methylotroph *H. zavarzinii* with its high expression of MDH as the most important player in the studied system. Furthermore, the metaproteomic approach also provides indications that are useful for understanding the metabolic pathways of microbial communities. The consequent identification of critical biochemical steps could lead to relevant improvements in the efficiency of industrial wastewater treatments. Further studies should address different types of wastewater treatment and different analytical approaches (including the less labor-intensive gel-free methodologies and new algorithms for the analysis of peptide sequences [13, 17, 18]) and could open new perspectives in the functional characterization of complex microbial consortia.

Table 1 Protein identifications

Constant spots both for pre-AOP and post-AOP gels									
Spot	gi of best hit	MW [kDa]	pI	Mowse sc.	No. of peptides	SC [%]	P value	Functional group	Protein
90	563686738	50.9	4.80	423.90	6	18.0	0.41	Metabolism	FOF1 ATP synthase subunit beta [*Hyphomicrobium nitrativorans* NL23]
233	254501162	50.7	4.62	554.36	6	19.0	0.33	Metabolism	ATP synthase F1, beta subunit [*Labrenzia alexandrii* DFL-11]
356	*300024658*	*34.5*	*6.00*	*922.80*	*31*	*53.1*	*0.53*	Metabolism	Citryl-CoA lyase [*Hyphomicrobium denitrificans ATCC 51888*]
369	488578310	34.4	6.01	334.17	4	22.5	0.48	Metabolism	Citryl-CoA lyase [*Hyphomicrobium denitrificans* 1NES1]
226	518930061	51.8	5.49	273.73	2	5.2	0.06	Metabolism	Peptidase S41 [*Hyphomicrobium zavarzinii*]
137	518930489	69.8	6.41	436.82	5	10.2	0.32	Metabolism	Quinoprotein ethanol dehydrogenase [*Hyphomicrobium zavarzinii*]
206	518930489	69.8	6.41	295.25	4	8.2	0.52	Metabolism	Quinoprotein ethanol dehydrogenase [*Hyphomicrobium zavarzinii*]
189	*338737333*	*69.5*	*5.80*	*629.00*	*9*	*18.1*	*0.88*	Metabolism	Methanol dehydrogenase subunit alpha [*Hyphomicrobium* sp. MC1]
196	518931602	69.0	6.07	405.02	8	15.1	0.69	Metabolism	Methanol dehydrogenase [*Hyphomicrobium zavarzinii*]
211	518931602	69.0	6.07	566.66	7	14.6	0.10	Metabolism	Methanol dehydrogenase [*Hyphomicrobium zavarzinii*]
219	518931602	69.0	6.07	605.55	10	17.2	0.45	Metabolism	Methanol dehydrogenase [*Hyphomicrobium zavarzinii*]
645	518931602	69.0	6.07	577.20	7	14.5	0.42	Metabolism	Methanol dehydrogenase [*Hyphomicrobium zavarzinii*]
661	518931602	69.0	6.07	313.37	7	10.5	0.90	Metabolism	Methanol dehydrogenase [*Hyphomicrobium zavarzinii*]
518	563688372	17.9	5.50	295.54	2	17.6	0.38	Metabolism	Aldehyde-activating protein [*Hyphomicrobium nitrativorans* NL23]
525	563688372	17.9	5.50	479.65	4	36.5	0.87	Metabolism	Aldehyde-activating protein [*Hyphomicrobium nitrativorans* NL23]
530	563688372	17.9	5.50	556.55	5	45.3	0.48	Metabolism	Aldehyde-activating protein [*Hyphomicrobium nitrativorans* NL23]
561	563688372	17.9	5.50	224.79	3	27.1	0.22	Metabolism	Aldehyde-activating protein [*Hyphomicrobium nitrativorans* NL23]
690	*338741566*	*30.6*	*5.60*	*395.10*	*14*	*16.3*	*0.11*	Metabolism	NADH-dependent enoyl-[acyl-carrier-protein] reductase [*Hyphomicrobium* sp. MC1]
124	495558445	42.3	4.48	580.98	6	21.8	0.27	Active transport	ABC transporter permease [*Roseovarius* sp. TM1035]
303	565410210	42.6	6.38	176.09	2	6.0	0.96	Active transport	ABC transporter permease [*Advenella kashmirensis* W13003]
371	496113034	36.7	4.84	244.25	2	4.7	0.27	Active transport	amino acid ABC transporter substrate-binding protein [*Mesorhizobium alhagi*]
386	518231422	36.0	5.58	101.60	2	4.2	0.36	Active transport	ABC transporter substrate-binding protein [*Chelatococcus* sp. GW1]
390	563688570	32.5	6.56	123.35	2	5.8	0.57	Active transport	ABC transporter substrate-binding protein [*Hyphomicrobium nitrativorans* NL23]
272	518929690	40.3	5.48	256.53	4	13.2	0.34	DNA replication	DNA polymerase III subunit beta [*Hyphomicrobium zavarzinii*]
274	518929690	40.3	5.48	445.93	7	19.4	0.72	DNA replication	DNA polymerase III subunit beta [*Hyphomicrobium zavarzinii*]
192	518928257	67.9	4.94	790.51	8	18.5	0.29	Chaperone	Molecular chaperone DnaK [*Hyphomicrobium zavarzinii*]
194	518928257	67.9	4.94	864.42	9	22.2	0.95	Chaperone	Molecular chaperone DnaK [*Hyphomicrobium zavarzinii*]
223	488577786	57.9	5.26	502.33	4	11.5	0.49	Chaperone	Chaperonin GroEL [*Hyphomicrobium denitrificans* 1NES1]
225	518929419	57.8	5.11	546.18	7	13.9	0.32	Chaperone	Molecular chaperone GroEL [*Hyphomicrobium zavarzinii*]
209	563688966	63.5	5.04	751.76	10	19.9	0.10	Protein synthesis	30S ribosomal protein S1 [*Hyphomicrobium nitrativorans* NL23]
254	518931496	43.9	5.61	750.93	9	29.6	0.30	Protein synthesis	Elongation factor Tu [*Hyphomicrobium zavarzinii*]
255	563688521	43.8	5.72	107.71	2	7.0	0.96	Protein synthesis	Elongation factor Tu [*Hyphomicrobium nitrativorans* NL23]
258	160895952	43.0	5.39	134.22	2	7.6	0.69	Protein synthesis	Elongation factor Tu [*Delftia acidovorans* SPH-1]
42	518930248	81.9	4.93	421.66	4	7.3	0.63	–	Hypothetical protein [*Hyphomicrobium zavarzinii*]
654	518930545	52.8	5.91	380.91	5	14.0	0.59	–	Hypothetical protein [*Hyphomicrobium zavarzinii*]
Changing spots: more expressed in pre-AOP gel									
Spot	gi of best hit	MW [kDa]	pI	Mowse sc	No. of peptide	SC [%]	*P* value	Functional group	Protein
266	7245498	40.8	5.04	838.49	8	32.3	<0.01	Structure and motility	Actin, chain A, crystal structure of CaATP-actin in complex with gelsolin segment 1 [*Dictyostelium* spp.]
271	940010	28.9	4.78	219.11	2	14.2	<0.01	Structure and motility	Actin [*Dictyostelium discoideum*]
277	563688758	41.7	5.66	457.87	6	18.5	<0.01	Metabolism	Malate-CoA ligase subunit beta [*Hyphomicrobium nitrativorans* NL23]
341	*149203333*	*35.6*	*4.40*	*185.90*	*8*	*14.2*	*<0.01*	Amino acid transport	Glutamate/glutamine/aspartate/asparagine ABC transporter, periplasmic substrate-binding protein [*Roseovarius* sp. TM1035]
Changing spots: more expressed in post-AOP gel									
Spot	gi of best hit	MW [kDa]	pI	Mowse sc.	No. of peptide	SC [%]	*P* value	Functional group	Protein
240	518931328	51.8	5.59	220.94	4	9.8	<0.01	Metabolism	Glutamine synthetase [*Hyphomicrobium zavarzinii*]

NanoHPLC-MS/MS results are in italics. Spots with *P* value <0.05 resulted changing in abundance

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