

Res2ValHUM: Creation of Resource Management Tool and Microbial Consortia Isolation and Identification

A. Ribeiro, N. Valério, C. Vilarinho, J. Araujo, J. Carvalho

Abstract—Res2ValHUM project involves institutions from the Spanish Autonomous Region of Galicia and the north of Portugal (districts of Porto and Braga) and has as overall objectives of promotion of composting as an process for the correct managing of organic waste, valorization of compost in different fields or applications for the constitution of products with high added value, reducing of raw materials losses, and reduction of the amount of waste throw in landfills. Three main actions were designed to achieve the objectives: development of a management tool to improve collection and residue channeling for composting, sensibilization of the population for composting and characterization of the chemical and biological properties of compost and humic and fulvic substances to envisage high-value applications of compost. Here we present the cooperative activity of Galician and northern Portuguese institutions to valorize organic waste in both regions with common socio-economic characteristics and residue management problems. Results from the creation of the resource manage tool proved the existence of a large number of agricultural wastes that could be valorized. In the North of Portugal, the wastes from maize, oats, potato, apple, grape pomace, rye, and olive pomace can be highlighted. In the Autonomous Region of Galicia the wastes from maize, wheat, potato, apple, and chestnuts can be emphasized. Regarding the isolation and identification of microbial consortia from compost samples, results proved microorganisms belong mainly to the genus *Bacillus* spp. Among all the species identified in compost samples, *Bacillus licheniformis* can be highlighted in the production of humic and fulvic acids.

Keywords—Agricultural wastes, *Bacillus licheniformis*, *Bacillus* spp., Humic-acids, Fulvic-acids.

I. INTRODUCTION

THE northern region of Portugal (specifically the districts of Porto and Braga) and the Autonomous Community of Galicia (Spain) are characterized by dispersed populations in small localities. They also share similar population size: 2,500,000 inhabitants in the north of Portugal and around 2,700,000 in Galicia, but the areas are quite different, which makes the population density much higher in the north of Portugal (544 inhabit./km²) than in Galicia (93.3 inhabit./km²). However, residues production per capita is also quite similar:

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454 kg/inhabit./year in the north of Portugal (in 2012) and 409 kg/inhabit./year in Galicia [1]. The collection and treatment of wastes produced by the population thus become extremely costly and difficult, with most of it being destined for landfills and also for energy recovery. Accordingly, in 2016, in Portugal, 49% of the municipal waste (MW) was landfilling and in Spain, the rate was 57% [2]. Regarding waste management, the main challenge is the high proportion of organic waste production concomitant with the low transformation of these residues in organic compost. For instance, in Galicia, the organic fraction corresponds to 42% of the total mass of urban waste produced and in the intervention area of Lipor (greater Porto region) that fraction corresponds to 38% [2]. Therefore, there is a need for increasing the proportion of organic residues for transformation into compost rather than accumulation in landfill. The Res2ValHUM project was designed for two European regions across the border between Portugal and Spain, sharing similar problems in terms of organic waste management [3]. The main objective is to promote composting in order to decrease the amount of organic residues sent to landfill. To achieve this objective several actions were designed involving interaction with the population for sensibilization, management, technology, and basic scientific research. Sensibilization and management contribute to driving the organic wastes to home composting or to industrial composting, while research on technology and basic science will make possible new applications of humic and fulvic-enriched extracts from compost, based on chemical and biological properties. Increased composting with high-value applications to solve technological problems and to improve the human quality of living will valorize greatly composting and compost. Therefore, this project will contribute to valorize reutilization of organic waste. A consortium of Portuguese and Spanish companies and universities have joined in this project: Braval, Centro de Valorización Ambiental del Norte (CVAN), Centro para a Valorização de Resíduos (CVR), Lipor - Intermunicipal Waste Management of Greater Porto, Sogama, University of Minho (UMinho) and University of Santiago de Compostela (USC).

This paper has two main objectives. The first one, is related to the creation of a resource management tool, especially for organic waste, bio-waste and residual biomass, which included information on the production of organic waste in the North of Portugal and the region of Galicia of Spain and the current production processes of (production/management of waste,

application of compost), as well as their specific needs. The second objective is related to the isolation and identification of microorganism consortia presented in compost samples. This identification will be crucial to classify the microorganisms responsible for producing acid and humic substances in compost.

II. MATERIALS AND METHODS

A. Materials and Reagents

Analytical grade reagents were supplied by Paralab, SA. (Valbom, Portugal). Culture media TSA (Trypticase soy agar), MacConkey, and Sabouraud were purchased from Enzymatic (Lisbon, Portugal). Genomic Identification of Microorganisms Species was executed from StabVida (Caparica, Portugal). Distilled water was used throughout.

B. Creation of Resource Management Tool

In this study were identified and quantified the main residues produced in the zone of validity of the Res2ValHUM project with potential for the composting process. This study will be only focusing the evaluation of agricultural wastes availability.

The assessments of agricultural residues availability (ARA) took into account the average annual production of crops (AAP), residue generation rate (RGR), sustainable removal rate (SSR) and other competitive uses (CU), according to (1):

$$AR_{CR} = (AAP)(RGR)(SSR)(1 - CU) \quad (1)$$

AAP of crops, as well as the selection of main crops, were obtained from FAO database and from the Portuguese and Spanish Institutes of Statistics.

The RGR can be understood as the ratio on a dry basis of the weight of residue produced to the total weight of the main crop product (e.g. the ratio of straw to wheat grain). RGR values may vary temporally and spatially according to several aspects, depending on the cultivation techniques, soil fertility and weather conditions [4]. Considering that RGR data used come from other studies targeted to different geographic areas, including those belonging to Natural Resource Module database, it is expected that these values encompass a technical, environmental and cultural variability so that their application meets only a rough estimate.

In order to meet environmental and economic sustainability associated with the agricultural activity, SRR of crop residues are needed. The main objectives of sustainable crop residues' removal consist in maintaining the natural soil fertility via incorporation of nutrients, promoting stability of upper soil layers and increase the organic matter content [5]. The percentage of crop residues that must be kept in the field depends on factors such as the soil structure and type, planting techniques (e.g. fertilizers usage, crop rotation and till or no-till farming) and conservation practices. The literature commonly suggested that a residue removal of up to 30% does not imply damage to the soil [6]-[8]. However, estimates for sustainable residue removal may vary greatly from crop to

crop, and it depends on another series of factors, such as geographical, climatic and technical/technological. For example, according to SoCo project team, regardless of the cultivation method, a maximum removal rate of straw around 70% is indicated [9]. Karkee et al. found that depending on soil characteristics, topography and farming practices (tillage, conventional and no-till), the percentage of available biomass to be removed may vary from 0% to 98%, without negative effects on the soil. In this study we adopted the SRR of 40% for wheat straw, maize residues and rice straw and husk [10].

In addition to SRR, agricultural residues may be used in a range of applications or CU, including energetic valorization, animal feed, and bedding, application in the construction sector, as well as starting materials for activated carbon production [11]. These applications represent CU with direct influence over the availability of residues and, therefore, must be taken into account. In general, very little information is available on industrial processing residues used for recycling/reuse purposes. Nonetheless, in order to proceed with conservative estimates, for those crops with no information on the percentage of residues turned to other valorization routes, it is assumed that only 50% is available [12], [13].

C. Microorganism Isolation from Compost Samples

Samples of compost were collected at different stages in composting process of LIPOR. It was collected samples from raw-material – OW (organic wastes), final of 1^o stage of active phase (1AP), final of 2^o stage of active phase (2AP) and final of maturation phase (MP).

Microorganisms were isolated through the utilization of specific culture media. In this work, TSA, MacConkey, and Sabouraud media were used.

TSA is rich in tryptone and peptone, carbohydrates, lipids, and proteins. This media is not selective, allowing the growth of all types of microorganisms. MacConkey media is selective for gram-negative microorganisms, due to the presence of bile salts and crystal violet that potentiates the inhibition of gram-positive microorganisms. It also makes the differentiation between lactose-fermenting and non-fermenting bacteria, because lactose-fermenting microorganisms stain pink, while non-fermenters have colorless colonies.

Finally, Sabouraud media is good for fungi and yeasts isolation because it has high glucose concentration which promotes fungal growth over bacteria that tolerate lower glucose concentrations. It can be also incorporated a broad-spectrum antibiotic, such as Chloramphenicol, that makes the medium selective, inhibiting the growth of gram-negative and gram-positive bacteria.

D. Genomic Identification of Microorganisms Species

A total of 40 samples were selected from each compost stage to represent the microbial diversity present in the compost samples.

These samples were sent to the company StabVida that was responsible for the genomic identification of the species.

III. RESULTS AND DISCUSSION

A. Resource Management Tool

Tables I and II show, respectively, the ARA in North of Portugal and Galicia region.

TABLE I
ARA IN NORTH OF PORTUGAL IN 2016

Crops	AAP 2016 (t)	RGR (residue/ terop)	PTR (t)	SRR (%)	CU (%)	ARA (t/y)
Temporary Cultures						
Wheat (straw)	8 083	1,25	10103	40	50	2021
Rye (straw)	11 208	1,61	18045	40	50	3609
Beans (Stem, Straw)	820	1,7	1394	40	50	279
Pear (pomace)	5 809	0,25	1452	30	50	218
Potato (bark, stem)	117 126	0,4	46850	50	50	11713
Corn						
Cob	1 872 880	0,33	618050	40	90	24722
leaf		0,22	412033	40	60	65925
Straw		1,05	1966524	40	60	314644
Oats (fodder)	291 582	1,42	414047	40	50	82809
Permanent Culture						
Apples (pomace)	105 340	0,3	31602	50	50	7901
Cherry (pomace)	3 707	0,25	926,74	30	50	139
Kiwi (pomace)	16 918	0,25	4229	30	50	381
Orange (bark, bagasse)	5 512	0,5	2755	30	50	248
Almond (shell)	5 470	0,477	2609	30	70	235
Chestnut	22 136	0,62	13724	30	70	1235
Walnut	1 259	0,8	1006	30	70	91
Olive (bagasse)	85 023	0,35	29757	30	70	2678
Grape (bagasse)	270 165	0,25	67541	30	70	6079
Total (Temporary Cultures + Permanent Culture)						524.925

TABLE II
ARA IN THE AUTONOMOUS REGION OF GALICIA IN 2016

Crops	AAP 2016 (t)	RGR (residue/terop)	PTR (t)	SRR (%)	CU (%)	ARA (t/y)
Temporary Culture						
Wheat (straw)	46808	1,25	58510	40	50	11702
Rye (straw)	13138	1,61	21152	40	50	4230
Potato (bark, stem)	505182	0,40	202073	50	50	50518
Maize (grain and fodder)	5050923					
Cob		0,33	1666805	40	60	266689
leaf		0,22	1111203	40	60	177792
Straw		1,05	5303469	40	60	848555
Permanent Culture						
Apples (pomace)	112242	0,30	33673	50	50	8418
Chestnut	142574	0,62	88396	30	70	7956
Grape (bagasse)	25228	0,25	6307	30	70	568
Total (Temporary Cultures + Permanent Culture)						1.376.428

Results plotted in Table I indicate that in 2016, 524.925 tons of agricultural or agricultural residues were available for compost production in the North of Portugal. Table I also shows that the principal crops in this region are: maize (405.000 tones/year), oats (82.809 tones/year), potato (11.713 tones/year), apple (7.901 tones/year), grape pomace (6.079 tones/year), rye (3.609 tones/year) and olive pomace (2.678

tones/year). In opposite, Table II indicates that in the Autonomous Region of Galicia, 1.376.428 tons of agricultural or agroindustry wastes are available annually for compost production, including maize (1.293.036 tones/year), wheat (11.702 tones/year), potato (50.518 tones/year), apple (8.418 tones/year) and chestnuts (7.956 tones/year).

B. Microorganism Isolation from Compost Samples

Table III and Figs. 1-4 show the results of microorganism isolation from the different samples of compost analyzed. Results of microorganism isolation from different compost samples and culture media showed, in general, the presence of different species of fungi and yeasts, actinomycetes and non-fermentative bacteria.

TABLE III
RESULTS OF MICROORGANISM ISOLATION FROM DIFFERENT SAMPLES OF COMPOST ON DIFFERENT CULTURE MEDIUMS

Sample	Culture Medium Isolation
OW (Fig. 1)	TSA: Heterogeneous and abundant microflora; MacConkey: Predominance of non-fermenting bacteria of lactose with rare lactose fermenting colonies; Sabouraud: Abundant microflora with indication of the presence of yeasts.
1AP (Fig. 2)	TSA: Abundant microflora with little differentiation; MacConkey: Presence of non-fermenting colonies of lactose only in the 1st quadrant; Sabouraud: Absence of microbial flora.
2AP (Fig. 3)	TSA: Abundant and heterogeneous microflora; MacConkey: Presence of rare mixed colonies distributed by the 1st quadrant; Sabouraud: Presence of some colonies suggestive of the presence of yeasts and 1 filamentous fungus.
MP (Fig. 4)	TSA: Heterogeneous microflora with colonies suggestive of the presence of actinomycetes; MacConkey: Presence of rare colorless colonies, dispersed by the 1st quadrant of the plaque; Sabouraud: Total absence of colonies.

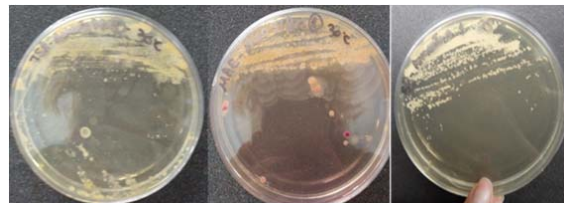


Fig. 1 Microorganisms from Organic Wastes (OW): TSA, MacConkey and SB medium

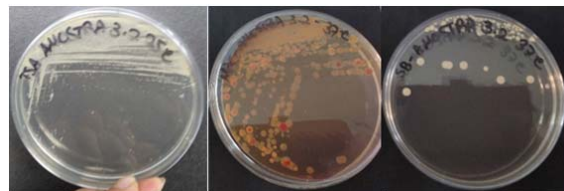


Fig. 2 Microorganisms after 1° stage of Active Phase (1AP) from the compost samples: TSA, MacConkey and SB medium



Fig. 3 Microorganisms after 2^o stage of Active Phase (1AP) from the compost samples: TSA, MacConkey and SB medium

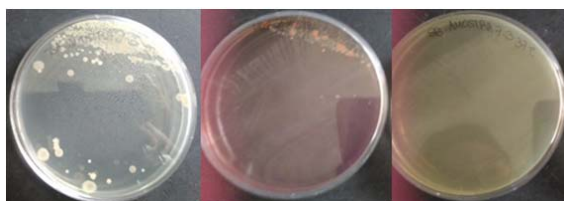


Fig. 4 Microorganisms after MP from the compost samples: TSA, MacConkey and SB medium

TABLE IV
IDENTIFICATION OF ISOLATED MICROBIAL SPECIES

Sample	Culture Media	Result of Microbial Identification	Access Number (NCBI)	Result (%)
OW	TSA	<i>Proteus vulgaris</i>	CP033736.1	99.85
OW	MacConkey	<i>Lampropedia puyangensis</i>	NR_148315.1	96.33
OW	Sabouraud	<i>Candida rugosa</i>	KT336717.1	97.34
OW	Sabouraud	<i>Candida catenulata</i>	KT336721.1	100.00
1AP	MacConkey	<i>Proteus mirabilis</i>	CP034090.1	99.92
1AP	MacConkey	<i>Pseudomonas thermotolerans</i>	NR_028008.1	99.54
1AP	TSA	<i>Proteus mirabilis</i>	CP034090.1	100.00
1AP	MacConkey	<i>Pseudomonas thermotolerans</i>	NR_028008.1	99.76
1AP	MacConkey	<i>Staphylococcus hominis</i>	CP033732.1	100
1AP	TSA	<i>Bacillus licheniformis</i>	CP025226.1	99.92
1AP	MacConkey	<i>Pseudomonas formosensis</i>	NR_118141.1	99.92
1AP	MacConkey	<i>Pusillimonas noertemannii</i>	JX966649.1	98.84
2AP	TSA	<i>Bacillus halosaccharovorans</i>	MH497631.1	100.00
2AP	TSA	<i>Pseudomonas pseudoalcaligenes</i>	JF911373.1	100.00
2AP	TSA	<i>Bacillus licheniformis</i>	CP025226.1	100.00
2AP	MacConkey	<i>Enterobacter cloacae</i>	KY524293.1	100.00
2AP	TSA	<i>Idiomarina indica</i>	KM407769.1	98.17
2AP	TSA	<i>Bacillus licheniformis</i>	CP025226.1	99.92
2AP	TSA	<i>Solibacillus sp.</i>	MH712078.1	100.00
2AP	MacConkey	<i>Pseudomonas thermotolerans</i>	NR_028008.1	99.29
MP	MacConkey	<i>Halomonas huangheensis</i>	MF928371.1	99.77
MP	MacConkey	<i>Pseudomonas mendocina</i>	KY021743.1	99.68
MP	MacConkey	<i>Pseudomonas formosensis</i>	NR_118141.1	99.93
MP	TSA	<i>Alcanivorax dieselolei</i>	MF139136.1	100.00
MP	MacConkey	<i>Brevibacillus borstelensis</i>	MK088265.1	100.00
MP	MacConkey	<i>Bordetella petrii</i>	MH410499.1	100.00

C. Genomic Identification of Microorganisms Species

Regarding to the identified microbial consortia, it can be concluded that they belong mainly to the genus *Bacillus* spp. This information is in agreement with several studies carried out, in which it was verified that the main groups of microorganisms present during the composting process belonged to the genus *Thermus*, *Bacillus* and *Hydrogenobacter* spp. [3]. Some species of *Bacillus* spp. were also identified, mainly from the first stage of Active Phase treatment [14].

Regarding to humic and fulvic substances, these results proved that a large microbial consortium could be responsible for its production in compost. Among them, *Bacillus licheniformis* can be highlighted in the production of humic and fulvic acids. Gao et al. also isolated this bacterium from organic wastes. These authors concluded that *B. licheniformis* is capable of Leonardite degradation into humic-acids [15].

IV. CONCLUSIONS

The multi-approach strategy of this project, involving sensibilization of the population, creation of management tools and characterization of the chemical and biological properties of compost and humic and fulvic extracts allows the design of innovative applications in engineering, agriculture, namely new agrochemicals, and cosmetics. Circular economy can efficiently be achieved by acting at the different levels of the waste chain. Within the activities of the Res2ValHUM project, a wide variety of publics were sensibilized for the need of composting in both regions.

Additionally, identification and quantification of organic wastes production with as adequate management tool allows to improve the efficiency of waste collecting and re-direction of organic waste to composting but also to map potential markets for compost acquisition. Results from the Resource Management Tool proved that are available for composting process 524.925 tons of agricultural wastes in the North of Portugal and 1.376.428 tons of agricultural wastes in the Autonomous Region of Galicia in Spain.

Results on chemical characterization of different composts allow to conclude that the content of humic and fulvic acids depends on the origin of the organic waste. Results from isolation of microbial consortia proved the existence of different species of microorganisms, being the most common species, fungi and yeasts, actinomycetes and non-fermentative bacteria. A total of 40 samples were sent to identification. Results of microbial consortia identification showed the existence of more than 20 species of microorganisms in compost samples. Among them, *Bacillus licheniformis* could be the responsible of humic and fulvic substances production in compost process.

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