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ORIGINAL PAPER

Isolation and characterization of onion degrading bacteria from onion waste produced in South Buenos Aires province, Argentina

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Abstract Onion production in Argentina generates a significant amount of waste. Finding an effective method to recycle it is a matter of environmental concern. Among organic waste reuse techniques, anaerobic digestion could be a valuable alternative to current practices. Substrate inoculation with appropriate bacterial strains enhances the rate-limiting step (hydrolysis) of anaerobic digestion of biomass wastes. Selection of indigenous bacteria with the ability to degrade onion waste could be a good approach to find a suitable bioaugmentation or pretreatment agent. We isolated bacterial strains from onion waste in different degradation stages and from different localities. In order to characterize and select the best candidates, we analyzed the growth patterns of the isolates in a medium prepared with onion juice as the main source of nutrients and we evaluated carbon source utilization. Nine strains were selected to test their ability to grow using onion tissue and the five most remarkable ones were identified by 16S rRNA gene sequencing. Strains belonged to the genera *Pseudoxanthomonas, Bacillus, Micrococcus* and *Pseudomonas*. Two strains, *Bacillus subtilis* subsp. *subtillis* MB2-62 and *Pseudomonas poae* VE-74 have characteristics that make them promising candidates for bioaugmentation or pretreatment purposes.

Keywords Onion waste · Growth patterns · Onion culture medium · Carbon sources · Hydrolysis

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Introduction

Annual world production of onions (*Allium cepa* L.) accounts for about 73 million tons and approximately 8 % of the world's production is discarded (FAO 2013). Onion is the most widely exported vegetable in Argentina and about 87 % is cultivated and packed in south Buenos Aires province. The production of onion in this region generates 12,000–20,000 tons of waste annually. The waste is composed of onion tops, dried leaves, roots and bulbs discarded on account of their shape, size or sanitary conditions. Crop wastes are produced from February to August, and the highest production is in April and May, i.e. fall in the southern hemisphere.

Generally, onion residues are collected in fields near pack-houses and incinerated after the harvest season. These arbitrary disposal methods cause serious problems to the neighbouring villages because they generate odours and attract insects. Globally, the current trend is to treat agro-industrial waste by processes that enable the organic part of the waste to be recycled, thus limiting the various problems that dumping can cause (Abouelwafa et al. 2008). Onions are considered to be a favorable source for biological utilization because they contain sugars and various nutrients (Horiuchi et al. 2004). However, the high concentration of organosulfur compounds in onion waste has a great influence on its degradation, because these compounds include allicin with antibacterial and antifungal activity (Benkeblia 2004).

Several utilization options have been proposed for onion waste: composting (Coventry et al. 2002; Horiuchi et al. 2004), vinegar production (Horiuchi et al. 1999) or use as an amendment in soil biofumigation (Mallek et al. 2007). Among organic waste reuse techniques, anaerobic digestion of onion waste could be a more valuable alternative.

Anaerobic digestion is the degradation of organic substrates to biogas (70 % methane and 30 % carbon dioxide) by a consortium of facultative anaerobic and strictly anaerobic bacteria and methanogenic archaea. In addition to producing methane, other major advantages of anaerobic digestion are reduction of organic waste, inactivation of pathogens and production of biofertilizer (Gerardi 2003). Biological hydrolysis is the rate-limiting step in anaerobic digestion of biomass wastes, as these tend to be high in cellulose or lignin. In order to improve solubilization of organic matter, a biological pretreatment can be used (Mata-Alvarez et al. 2000). It has been proven that substrate inoculation with aerobic or facultative anaerobic bacterial strains, like *Bacillus licheniformis*, *Bacillus subtilis* and *Paenibacillus macerans*, enhances hydrolysis and consequently, biogas production (Angelidaki and Ahring 2000; Singh et al. 2008; Sonakya et al. 2001; Yadvika et al. 2004).

Most organic substrates carry an indigenous population of microbes from the environment. Representatives of the three major microbial groups (Bacteria, Actinomycetes and Fungi) are normally present when the biodegradation process begins (Sylvia et al. 2005). Complete or partial organic matter degradation is performed by a consortium of microorganisms, the composition of which may vary with the type of substrate being degraded (Franke-Whittle et al. 2009). A high microbial diversity is a prerequisite for a satisfactory degradation process (Beffa et al. 1996). Results showed that bioaugmentation with native bacteria could be effective in wastewater treatment (Mongkolthanaruk and Dharmsthiti 2002).

It is essential to gain knowledge on the composition and growth of microorganisms responsible for the degradation of onions, in order to improve its degradation rate. Indigenous strains isolated from the substrate could be candidates for pretreatment or bioaugmentation purposes in anaerobic bioreactors. The aim of this work was to isolate and characterize native bacteria from onion waste in different degradation stages. We analyzed growth patterns of the isolates in onion medium in different temperature conditions and we evaluated their phenotypic diversity by carbon source (CS) utilization. Finally, the most remarkable strains were selected and their ability to grow using onion chops as sole source of nutrients was tested. Five potential onion degrading strains were identified by 16S rRNA gene sequencing.

Materials and methods

Sampling sites description

Wastes from three different production sites were collected. The localities Mayor Buratovich (MB; 39°15′40″S, 62°36′55″W), Villalonga (VL; 39°54′55″S, 62°37′10″W) and Viedma (VE; 40°48′30″S, 62°59′40″W) are from 80 to 200 km away from each other. Onion waste composition varies depending on the processing methods used at the different pack-houses and on the various pathogens that affect this crop among growing areas. In the region near the localities MB and VL the residue is composed mostly of bulbs, while in VE it consists mostly of onion tops, dried leaves and roots. These localities are situated in a semiarid region, with 400 mm annual average rainfall and characterized by high daily and seasonal temperature range. The average annual low temperature is 9 °C and high temperature is 22 °C. The low temperature record is −8 °C in July and the high is 43 °C in January. Frosts are recorded from mid-May till late September.

Enumeration of aerobic heterotrophic bacteria, fungi and yeast and strains isolation

Four different degradation stages of onion waste were analyzed: (a) onion waste stored at 4 °C for a month (MB1); (b) onion waste stored in 1 m^3 bin placed in open air for a month (MB2); (c) onion waste from field pile (bulbs and dried leaves that remain in the field after harvest) (VL); (d) onion waste collected from a packhouse (VE). Until VL and VE samples were obtained, we performed two conservation methods of MB waste in the laboratory because in this area the residue was burned.

Onion waste (10 ml wet volume) was placed in 250 ml glass flask containing 90 ml of distilled sterile water. Each treatment was made in duplicate. These suspensions were shaken at 200 rev/min for 30 min. Serial dilutions were prepared and aliquots (100 µl) were spread on triplicate Petri plates (90 mm × 15 mm) containing one of two culture media. Aerobic heterotrophic bacteria (AHB) were quantified on Nutrient Agar (NA, Merck). Filamentous fungi and yeasts were enumerated on Yeast Extract Glucose Chloramphenicol Agar (YGC, Merck).

All plates were incubated at 28 °C for 48 h and then AHB, filamentous fungi and yeast were enumerated. The number of cultivable AHB, filamentous fungi and yeast were expressed as $\log_{10}$ CFU g^{-1} onion waste. Differentiation between yeasts and filamentous fungi was based on cultural morphological characteristics (colony color, shape and texture). From the plates with NA, 83 bacterial colonies (approximately 20 from each sample) were picked randomly and subcultured until pure cultures were obtained. After purification, cultures were streaked in NA slants and stored at 4 °C for subsequent study. Isolates were coded according to their origin, as follows: MB, VL and VE.

Growth in culture medium prepared with onion juice

Ability to grow using onion nutrients was assessed by inoculating broth-grown pure cultures with optical density at 620 nm (OD_{620}) adjusted to 0.5. Each culture was inoculated (0.1 ml) in duplicate into tubes with 3 ml onion medium: 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 150 ml onion juice and 1 l distilled water, the pH of the medium was 7.4. Onion juice was obtained as follows: peeled yellow onions were cut and pressed in a mechanical juicer and the extract was centrifuged at 4,000 rev/min until a translucent juice was obtained. The medium was autoclaved for 15 min at 1 atm. Each culture was also inoculated in duplicate in Nutrient Broth (NB, Merck) as control medium. Test tubes were incubated horizontally on an orbital shaker at 150 rev/min. One set of tubes was incubated at 28 °C and the other at 4 °C for 7 days. In all cases a tube without inoculum was set under the same conditions as sterilization control. Cultures were homogenized by vortexing prior to measuring cell density. Microbial growth was assessed by OD_{620} with a spectrophotometer (Metrolab 1600DR, Wiener Lab, Argentina).

Carbon source utilization

Bacterial strains were prepared for substrate utilization assays by growing in NB, Merck at 28 °C until they reached $OD_{620} = 0.5$. The ability of the isolates to utilize various CS was evaluated in 96-well sterile microplates (Orange Scientific). Each well was filled with 225 µl minimal medium (MM) with the following composition: $NH_4H_2PO_4$ 1.25 g l^{-1}; KCl 0.25 g l^{-1}; $MgSO_4 \cdot 7H_2O$ 0.25 g l^{-1}; K_2HPO_4 0.50 g l^{-1}; in addition, the well contained 0.1 % CS and 0.005 % triphenyl tetrazolium chloride (TTC) as redox indicator according to Zabaloy and Gómez (2005). Thirty-one CS were tested: 14 carbohydrates (L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, sorbose, D-xylose, sucrose); 5 carboxylic acids (acetate, citrate, succinate, malonic acid, pyruvate); 4 amino acids (L-asparagine, L-cysteine, L-threonine, L-glutamate); 3 hydrosoluble vitamins (ascorbic acid, inositol, thiamine) 2 aromatic compounds (benzoate, catechol), 2 polymers (dextrin, inuline) and 1 alcohol (glycerol). A well containing MM and TTC without a CS was included as negative control (blank). Plates were prepared 24 h before use to verify that no contamination was present. Microplates were inoculated with 10 µl $well^{-1}$ of 53 isolates ($OD_{620} = 0.5$) and incubated at 28 °C in the dark. Optical density (OD_{592}) was measured every 24 h for 5 days with a Synergy HT microplate reader (Bio-Tek Instruments, VT, USA).

Onion chops as nutrient source in liquid medium

The ability of isolates to use onion tissue as the sole source of nutrients was assessed in liquid medium. Erlenmeyer flasks (125 ml) were prepared with 50 ml of saline solution (1 g K_2HPO_4 l^{-1}; 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}) and 1 g onion chops (0.5 × 0.3 mm); pH 7.4. Bacterial strains were added as 1 ml inoculums ($OD_{620} = 0.5$) and a flask without inoculation was included. Cultures were incubated at 28 °C for a week and aliquots of 1 ml were withdrawn every 24 h for OD_{620} measurements with a spectrophotometer (Metrolab 1600DR, Wiener Lab., Argentina). Onion chops were analyzed after the experiment with a stereomicroscope (Leica EZ4 HD, Leica Microsystems, Switzerland).

Identification of isolates

Bacterial strains were identified by observation of colony morphology and by Gram staining. In addition, genomic DNA was used to amplify the spacer region between the 16S and 23S rRNA (ITS). Primers corresponding to conserved regions in 16S/23S rRNA sequences, 38r and 72f (Gürtler and Stanisich 1996), were used to amplify the ITS. PCR master mixture (25 µl) contained 0,125 µl Go Taq DNA Polymerase, 5 µl Green GoTaq Reaction Buffer (Promega, Madison, WI, USA), 2,5 µl dNTP (2 mM) (Inbio-Highway, Argentina), 0,625 µl of each primer (10 µM, Invitrogen, USA), 2 µl DNA template and 14,125 µl nuclease-free water. PCR amplifications were performed using a Life Express TC-96/G/H(b) thermal cycler (Bioer Technology Co. Ltd, Tokyo, Japan). Cycling conditions were as follows: initial denaturation at 95 °C for 3 min, 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min 30 s, a single final extension cycle at 72 °C for 10 min and a final soak at 4 °C. PCR products were checked by electrophoresis on a 2 % agarose gel and analyzed using Kodak Digital Science Image Analysis 3.0 software (Eastman Kodak Company, NY, USA).

Sequencing of 16S rRNA gene

Nearly full-length 16S rRNA gene sequences were obtained from five bacterial strains with universal primers 518f and 800r by Macrogen Inc. (Seoul, Korea). The sequences were edited using Chromas Lite v.2.1.1 (Technelysium Pty Ltd, Australia, http://www.technelysium.com.au/chromas_lite.html) and assembled with CAP3 (Huang and Madan 1999). All 16S rRNA sequences (of about 1,450 bp) were identified by comparison with sequences available in GenBank using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

The microbial counts obtained were analyzed by ANOVA considering the treatment as the independent variable. The means were separated by the Tukey's test, considering a significance level of $p < 0.05$ throughout the study. For analysis of CS utilization, raw OD data were corrected by blanking each response well against its own blank. Substrates with a final corrected OD <0.2 were omitted from the analysis (Lindstrom et al. 1998). The OD data for each substrate at 120 h were used for cluster analysis. Distance matrices were produced using the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) and represented in the form of a dendrogram. All statistical analysis was performed with NCSS software (Hintze 2013), version 9.0.13.

Results

Enumeration of aerobic heterotrophic bacteria, fungi and yeast

AHB counts were greater than filamentous fungi and yeasts in all treatments, reaching a maximum density of 7.4×10^9 - CFU g^{-1} in VE (Fig. 1). Statistical analysis indicated that the number of AHB in VE was significantly different from the numbers in MB1 and MB2. Furthermore, MB2 was also statistically different from VL. No significant differences were found between sampling sites according to the number of filamentous fungi. In contrast, the number of yeasts was statistically different among sites (Tukey's test, $p < 0.05$).

Growth in onion juice medium

From the 83 cultures grown in NB prior to the growth test, 30 cultures were not able to reach the required OD_{620} (0.5) during the incubation period, as a result they were not included in the experiment. Fifty-three cultures obtained from onion waste were able to develop in onion medium at 28 °C and only five of them grew in this medium at 4 °C. Bacterial strains showed different growth response to the nutrient content of the culture medium and the incubation temperature. For a better discussion, we arranged them into four groups considering the temperature and culture medium in which they grew better (Fig. 2). Most cultures developed better in NB (40.4 %, group 1). Strains included in group 2 reached almost the same turbidity in both media (34.6 %). On the other hand, 17.3 % of strains grew better in onion medium than in NB (group 3). Finally, 7.7 % strains were also able to develop in onion medium at 4 °C (group 4). Table 1 shows the distribution of the strains in each group according to their isolation source.

A particular case was presented by one bacteria isolate (Fig. 2, strain VE-74). This microorganism did not match with the groups described above. Strain VE-74 growth pattern changed with temperature; this isolate had greater turbidity in onion medium than in NB at 28 °C but the opposite occurred at 4 °C.

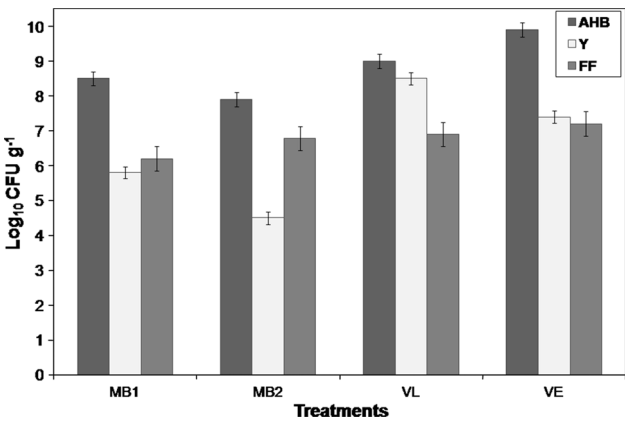

Fig. 1 Aerobic heterotrophic bacteria (AHB), filamentous fungi (FF) and yeast (Y) counts in onion waste from different localities. *MB* Mayor Buratovich, *VL* Villalonga, *VE* Viedma

Carbon source utilization patterns

The substrate utilization assay was performed in order to characterize the ability of the strains to metabolize thirty-one CS and to select the ones that could use the non-structural carbohydrates from onion bulbs as a screening of their potential for onion degradation. None of the strains were able to grow with lactose, raffinose, sorbose, ascorbic acid, thiamine and catechol as the sole CS, while three or fewer strains were able to use rhamnose, inuline and cysteine as sole source of carbon and energy. Additionally, twenty-four strains were incapable of using any CS during the test period. As a consequence, these strains and the CS mentioned before were not included in the analysis.

Among the six preferentially utilized substrates, half of them were carbohydrates: glucose (72.4 %), mannitol (58.6 %) and maltose (48.3 %). Dextrin and asparagine enabled the growth of 58.6 % of the isolates. Among carboxylic acids, pyruvate was the most used (48.3 %), nevertheless citrate stimulated the highest average activity. Arabinose, xylose, malonic acid and benzoate were poorly utilized.

Cluster analysis of CS utilization divided twenty-two of the twenty-nine strains into four groups at Euclidean distance of 0.19 (Fig. 3). Cluster I was comprised of ten strains characterized by scant growth on CS (Table 2). Most strains (≥80 %) belonging to cluster II grew on seven CS: two hexoses (glucose, mannitol), three carboxylic acids (citrate, pyruvate, succinate), an amino acid

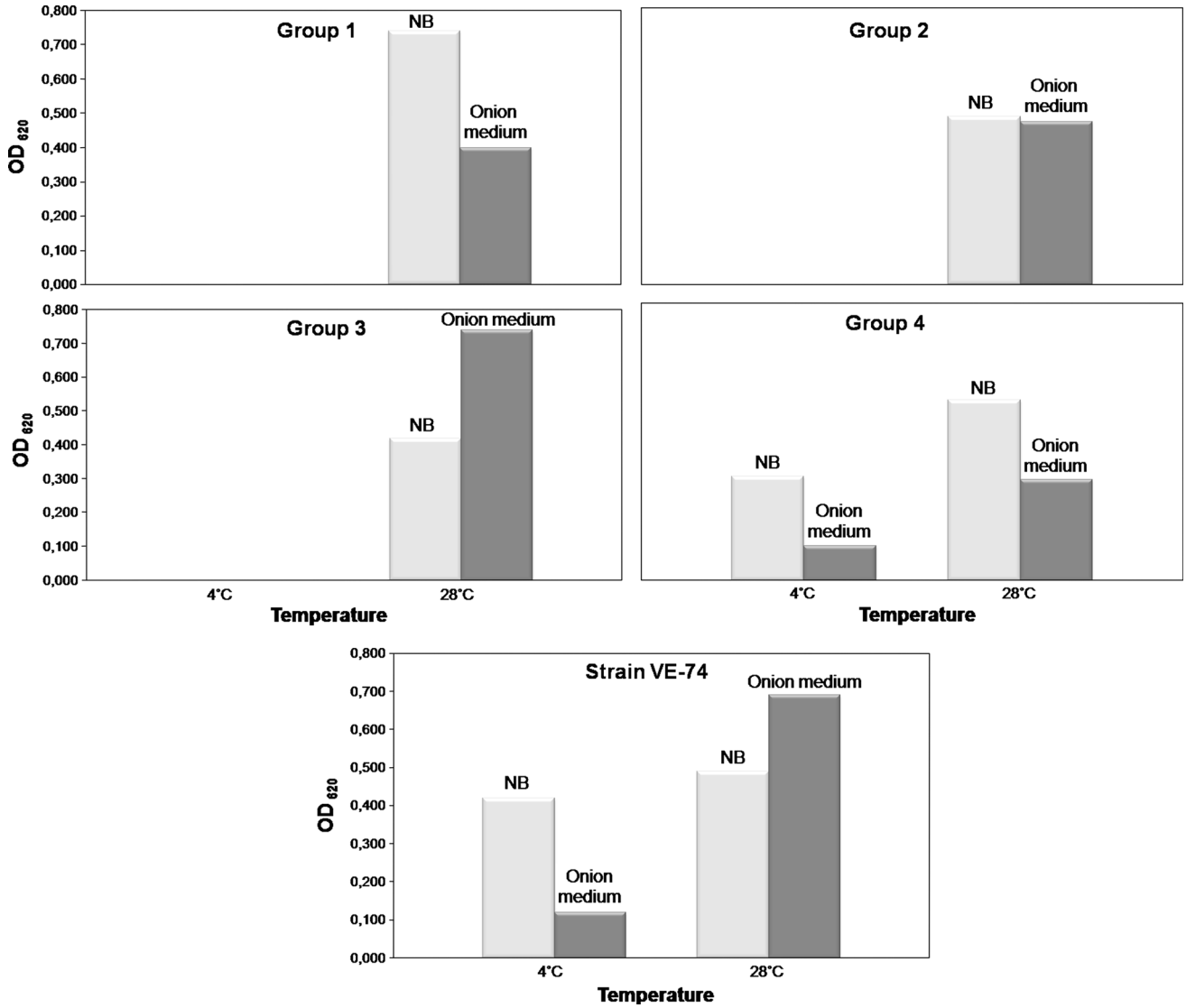

Fig. 2 Bacterial growth in nutrient broth (NB) and onion medium at 4 and 28 °C after 7 days of incubation. Graphs show the strain closer to the average value of each group. *Group 1* represents the bacteria that developed better in NB, *Group 2* correspond to bacteria that had the same turbidity in both media, *Group 3* depicts bacteria which grew better in onion medium, *Group 4* includes bacteria that were able to develop in both media at 4 and 28 °C. Strain VE-74 had a unique growth pattern

Table 1 Number of isolates by group and isolation locality

Group	Locality			Total group
	MB	VL	VE	
1	10	6	5	21
2	11	6	1	18
3	7	2	0	9
4	1	0	3	4
Total locality	29	14	9	52

(asparagine) and benzoate, solely consumed by this group. The two strains of cluster III consumed carboxylic acids (acetate, succinate, pyruvate) and amino acids (asparagine, glutamate). Finally, cluster IV consumed a polymer (dextrin), an alcohol (glycerol) and was the only group that grew on all the carbohydrates. The strains clustered at larger Euclidean distance (>0.2), namely outlier strains, showed a wide range of CS use.

The strains included in cluster IV (MB2-5, MB1-7, MB1-42, MB1-60 and MB2-62) and three outlier strains (MB1-45, MB1-46 and VL-80) were capable of using the three sugars (glucose, fructose, sucrose) contained in onions. Among these isolates, VL-80 achieved the highest OD in these three carbohydrates. Interestingly, VL-80 was the only isolate able to grow using all the CS included in the analysis. MB1-42, MB2-5 and MB2-62 grew mostly on carbohydrates, like cellobiose, fructose, glucose, maltose,

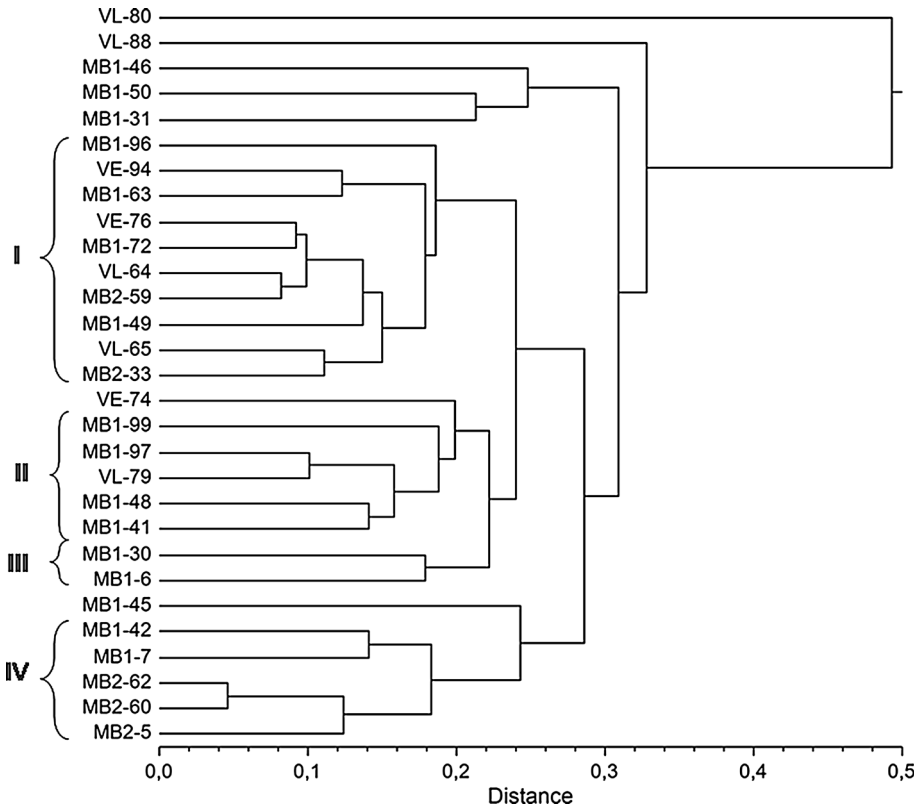

Fig. 3 Dendrogram showing phenotypic relatedness, based on utilization of 20 carbon source among 29 strains. Cluster analysis was performed using unweighted pair-group method with arithmetic average (UPGMA) and Euclidean distance. The correlation cophenetic value (r) was 0.867

mannitol and sucrose. The isolate that had a unique growth pattern in onion medium, VE-74, was capable of using only four carbohydrates (glucose, mannitol, galactose and arabinose). We selected these nine strains to study their ability to grow using onion tissue as the only source of nutrients, based on their CS utilization characteristics and their growth in onion medium.

Growth with onion chops as source of nutrients

We aimed to check if the selected strains were able to macerate onion tissue because the anaerobic digester would be fed with onion chops instead of onion juice. While all the tested isolates grew using onion chops as source of nutrients, different degradation characteristics were observed. Figure 4 shows that VE-74 grew poorly while strains MB2-5, MB1-42 and MB1-46 grew faster ($OD_{620} = 0.3$ in 24 h). Moreover, MB2-5 and MB1-42 attained the greatest OD during the experiment. Strain VL-80 developed on onion forming aggregates and strains MB1-60 and MB2-62 produced biofilm (Fig. 5). Owing to the growth characteristics of these three strains, their OD values were not considered to avoid underestimations. Strains MB1-60 and MB2-62 were the only two that could disrupt the onion tissue almost completely (Fig. 5).

Identification of the strains

Five strains (MB2-5, MB1-42, MB2-62, VE-74 and VL-80) were chosen for further characterization taking into account their different onion degradation performances. Cells of strain MB2-5 were Gram-negative rods and colonies were yellow on NA plates. Strains MB1-42 and MB2-62 were Gram-positive rods forming whitish colonies. Strain VE-74 had rod-shaped morphology, Gram-stain-negative and colonies were translucent. Finally, cells of strain VL-80 were Gram-positive cocci that occurred in tetrads and colonies were yellow pigmented. Agarose gel electrophoresis of ITS PCR products revealed different band patterns for each strain. A single band was obtained from isolates MB2-5 (630 bp) and VE-74 (576 bp), and two or more bands from strains MB1-42 (396, 500 bp), MB2-62 (348, 516 bp) and VL-80 (517, 643, 1,000 bp).

Nucleotide sequences for isolates MB1-42, MB2-5, MB2-62, VE-74 and VL-80 will appear in the GenBank nucleotide database under accession numbers KJ843149 to KJ843153 respectively. The 16S rRNA gene sequences obtained showed that MB2-5 had 99 % identity to *Pseudoxanthomonas suwonensis* strain 11-1 (Accession No. NC 014924). Strains MB1-42 and MB2-62 had 100 % similitude values with *Bacillus megaterium* strain DSM319 (Accession No. CP 001982) and *B. subtilis* subsp. *subtilis* strain 168 (Accession No. NC 000964), respectively. Strain

Table 2 Differential utilization of substrates as sole carbon source by cluster and outlier strains[a]

Carbon source	% of strains utilizing substrate in cluster:				Utilization of substrates by outlier strain:[c]						
	I (n[b] = 10)	II (n = 5)	III (n = 2)	IV (n = 5)	MB1-45	VE-74	MB1-31	MB1-50	MB1-46	VL-88	VL-80
Carbohydrates											
L-arabinose	0	0	0	40	1	1	0	0	0	0	1
D-cellobiose	50	0	0	100	1	0	1	0	0	0	1
D-fructose	0	20	0	100	1	0	0	0	1	0	1
D-galactose	20	0	0	60	1	1	1	0	1	0	1
D-glucose	50	80	50	100	1	1	1	1	1	0	1
Maltose	40	0	50	100	1	0	0	1	1	0	1
D-mannitol	40	80	0	80	1	1	0	1	1	0	1
D-mannose	30	20	0	60	1	0	1	1	1	0	1
Sucrose	20	0	0	100	1	0	1	1	1	0	1
D-xylose	10	0	0	40	1	0	1	0	0	1	1
Carboxylic acids											
Acetate	20	60	100	0	1	0	1	1	0	1	1
Citrate	10	100	50	0	0	1	1	1	1	1	1
Succinate	10	80	100	0	1	0	1	1	1	1	1
Malonic acid	0	40	50	0	1	0	1	0	0	0	1
Pyruvate	20	80	100	0	1	0	1	1	1	1	1
Amino acids											
L-asparagine	40	100	100	0	1	1	1	1	1	0	1
L-glutamate	10	60	100	0	1	1	0	1	1	1	1
Aromatic compound											
Benzoate	0	80	0	0	1	0	0	0	0	0	1
Polymer											
Dextrin	50	20	50	100	1	0	1	1	1	0	1
Alcohol											
Glycerol	10	40	0	60	1	1	1	1	1	0	1

[a] Clusters and outlier strains according to carbon source dendrogram (see Fig. 3)

[b] Number of strains included in a cluster (see Fig. 3)

[c] 1 = utilization of carbon source (OD = 0.2–0.9); 0 = non-utilization (OD < 0.2)

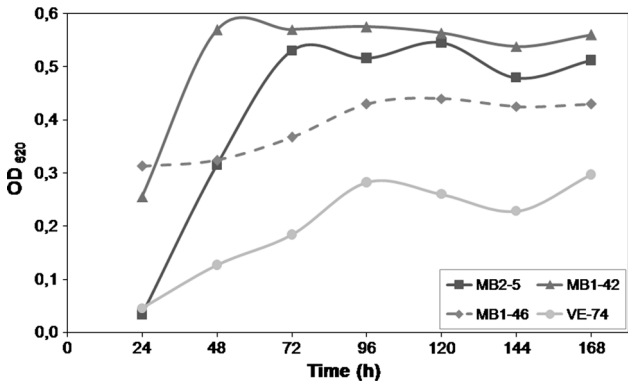

Fig. 4 Growth curves of strains MB2-5, MB1-42, MB1-46 and VE-74 using onion chops as sole source of nutrients

VL-80 exhibited a high nucleotide sequence similarity (99 %) with *Micrococcus luteus* strain NCTC 2665 (Accession No. NC 012803). Finally, VE-74 was closely related (99 %) to *Pseudomonas poae* strain RE 1-1-14 (Accession No. NC 020209).

Discussion

Selection of indigenous bacteria could be a good approach to find a suitable inoculum to enhance onion waste hydrolysis. In order to characterize bacterial strains isolated from onion waste and identify the best candidates, we analyzed the growth of the isolates in a medium prepared

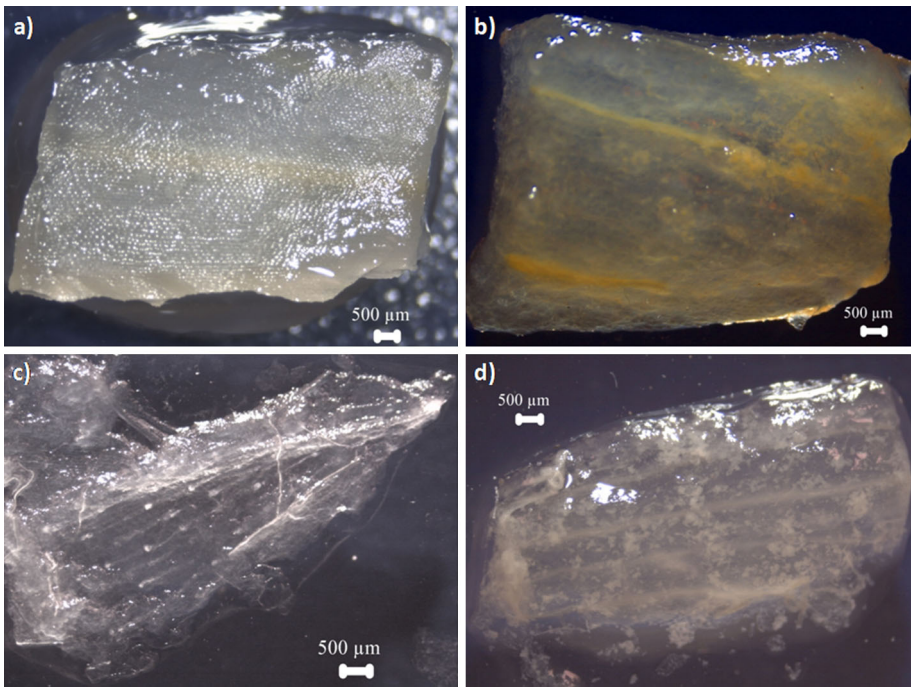

Fig. 5 Stereomicroscope images of onion chops with different levels of tissue maceration after the degradation assay: **a** control, **b** VE-74, **c** MB2-62 and **d** VL-80

with onion juice and we evaluated CS utilization. Finally, the ability to grow using onion tissue was tested on nine selected strains. The most remarkable cultures were identified by 16S rRNA gene sequencing. Two strains, *B. subtilis* subsp. *subtillis* MB2-62 and *P. poae* VE-74, isolated in the present investigation have characteristics that make them promising candidates for future bioaugmentation tests in anaerobic digestion.

Culturable aerobic heterotrophic bacteria, fungi and yeast

Despite the high concentration of organosulfur compounds, antibacterial and antifungal properties of onions, microbial counts were similar to those found by Ryckeboer et al. (2003a) and by Chroni et al. (2009) in the initial degradation of a mixture of food and garden wastes. Those mixtures had more variety of nutrients and did not represent a restrictive environment for microorganisms as onion does (Fig. 2). This suggests the existence of a microbiota that is adapted to the characteristics of onion waste. As expected, bacterial counts in all treatments were higher than numbers of filamentous fungi and yeast. The number of AHB and yeast proliferating during the degradation process differed among localities and it is reasonable to suppose that these microbial populations not only come from the starting material (onion bulbs) but also from the field where onion is grown (Jay 2000a). The large microbial counts found suggest that onion waste supports a rich microbial community. According to Ishii and Takii (2003), the microbes proliferating in organic matter degradation processes would be adapted to each environment, and selected by the chemical characteristics of the substrate being degraded.

In MB1 the microbial load was similar to the other treatments despite being stored for a month in the refrigerator. This suggests that keeping onions at 4 °C does not cause a negative effect on the subsequent degradation. It has been widely reported that the agents responsible for fruit and vegetable spoilage are bacteria and yeast, even under refrigeration temperatures (Ancasi et al. 2006; Brocklehurst and Lund 1981; Jay 2000b). Warade and Kadam (2004) recommended storing onions at 0 °C to preserve them as higher temperatures (4 °C) do not prevent microbial proliferation.

Growth in culture medium prepared with onion juice extract

We assumed that nine bacteria, included in group 3 (Fig. 2), were better adapted to the onion environment than the other isolates because they showed a better performance in onion juice than in the control medium. The behavior of microorganisms included in group 4 demonstrates its ability to grow on onions under refrigeration temperatures.

Generally, microorganisms from different sampling sites showed dissimilar growth patterns. This suggests a difference in microbial physiological diversity among onion wastes collected from different localities. Also, the unique

growth pattern of strain VE-74 suggests that there is probably more metabolic versatility in the onion-associated bacterial communities that were not isolated in this study. In addition, waste composition has also influenced microbial diversity. For example, MB waste was composed mainly of bulbs affected by bacterial diseases while VE waste was mainly composed of dried leaves, roots and onion tops. All this caused different selection pressures on microorganisms resulting in metabolically versatile growth strategies.

The fact that onions have allicin and sulphur-containing compounds (Horiuchi et al. 2000) with anti-bacterial function may explain why most cultures grew better in NB than in onion medium. Nevertheless, there were isolates that grew better in onion medium (group 3 and strain VE-74) that are clearly adapted to onion juice and are presumed to endure the chemical characteristics of onion waste.

To the best of our knowledge, there is only one study in which onion medium was used for selective isolation of onion-pathogenic and onion-associated bacteria (Zaid et al. 2012). Although these authors used onion extract as the main source of nutrients for their medium, they autoclaved it for 35 min at 120 °C twice. It is well known that autoclave sterilization changes soluble sugars concentration (Singleton 2004), which are precisely one of the principal onion constituents. As a consequence, onion extract nutrients were probably different from raw onion. In our opinion, the onion medium prepared in our work could be closer to the properties of onion because we utilized pure onion juice and a mild sterilization in autoclave. Therefore, the medium we have developed may be more adequate for the isolation of indigenous onion microorganisms. Our medium allowed the growth of fifty-three cultures of onion waste bacteria and as a result, we could identify four different growth groups. Among the isolated strains there were representatives of the phylum *Proteobacteria*, *Firmicutes* and *Actinobacteria*.

Carbon source utilization profiles

In environmental bacterial communities, glucose supports considerable bacterial growth. This monosaccharide was used by most bacterial strains but fructose stimulated higher activity in those strains that could use both hexoses. Almost all strains could degrade at least one carbohydrate, but utilization of substrates with more complex molecular structures (e.g. benzoate) was mediated by few strains. In general, strains that had a high OD in onion culture medium also had high carbohydrate consumption in CS test.

Cluster analysis showed that there were notable differences in CS consumption among strains isolated from onion waste. It is known that substrate degradation depends strongly on species or even strain-specific abilities (Verniere et al. 1993). Cluster II and III were constituted of strains distinguished by carboxylic acids consumption. Bacteria of groups I and IV were characterized by growing on carbohydrates. The identified strains of Cluster IV, *P. suwonensis, B. megaterium and B. subtilis* subsp. *subtilis* belong to distantly related phyla (*Proteobacteria* and *Firmicutes*). However, these bacteria share the ability to grow with several carbohydrates (Goodfellow 2012). This could be an indication that similar substrate profiles do not necessarily involve a close phylogenetic relationship. Environmental microbial communities' are characterized by a redundancy of functions, i.e. various capabilities are often distributed over taxonomically different populations (Nannipieri et al. 2003). A high level of redundancy is the most important factor that controls decomposition rate of organic matter (Andrén et al. 1995). As a result, the dendrogram presented in this study is most likely reflecting functional groups.

Outlier strain VE-74, identified as *P. poae*, consumed preferentially carbohydrates and aminoacids. This strain shared the properties given in the species description (Behrendt et al. 2003) with the exception that strain VE-74 was unable to utilize sucrose. The major difference in substrate utilization pattern was observed in strain VL-80. This isolate, identified as *M. luteus*, clustered at the highest distance in the dendrogram. The broader capacity for CS utilization of strain VL-80 is probably due to the fact that it belongs to a group (*Actinobacteria*) characterized by microorganisms that exhibit diverse physiological and metabolic properties (Ventura et al. 2007). Morphological and cultural characteristics of strain VL-80 were compatible with the typical characteristics of the species *M. luteus* (Schleifer 2009). Conversely, strain VL-80 was differentiated from *M. luteus* strain NCTC 2665 in utilization of various CSs such as maltose, xylose, acetate and citrate (Wieser et al. 2002). However, the substrates consumed by VL-80 as sole CS corresponded with the physiological characteristics described for the species *M. luteus* (Schleifer 2009) and closely related strains (Wieser et al. 2002).

Throughout the different analyses performed, it could be assumed that sample MB1 had the highest functional diversity. Strains isolated from this sample were distributed in all cluster groups and most of the strains that were not included in the clusters belonged to MB1. The large functional diversity of MB1 may have been stimulated by the selective pressure imposed by the storage of the sample at 4 °C for a month. It is known that this type of conservation causes a decrease in most abundant groups, allowing other microorganisms to develop (Goberna et al. 2005).

In summary, the heterogeneity observed in CS utilization may be a consequence of the changing environmental conditions and nutrients availability in the different onion waste

degradation state. Strains that can utilize diverse CS may have more chances of survival and substrate exploitation.

Onion waste degrading strains selection

The presence of multiple ITS bands agreed with the fact that some of our isolates possessed multiple rRNA operons (Gürtler and Stanisich 1996; Klappenbach et al. 2000). The 16S rRNA gene sequences obtained from the onion degrading cultures exhibited broad bacterial diversity, with strains belonging to three different phyla: *Proteobacteria*, *Firmicutes* and *Actinobacteria*. All the sequenced strains belonged to genera that are commonly associated with degradation of different biomass wastes. Representatives of *Bacillus* and *Pseudomonas* are frequently detected in the hydrolysis stage of anaerobic digestion (Bolarinwa and Ugoji 2010; Cardinali-Rezende et al. 2011; Chachkhiani et al. 2004). Particularly, *B. megaterium, B. subtilis, M. luteus* and *P. suwonensis* have been found in compost and the former three have also been detected in kitchen and agricultural wastes (Ryckeboer et al. 2003b; Weon et al. 2006). Conversely, *P. poae* RE*1-1-14 has only been reported as an endophytic bacterium from sugar beet (Müller et al. 2013). To our knowledge, only *B. megaterium* and *B. subtilis* have previously been described in association with onion (Obi and Umezurike 1981; Zaid et al. 2012).

In our study, strain MB2-62 identified as *B. subtilis* subsp. *subtilis* had the best degradation performance. This isolate was included in group 3, which comprised the bacteria putatively adapted to onion waste. Also, VE-74 identified as *P. poae* had a distinctive growth pattern which suggested that this strain was adapted to grow using onion nutrients in a broad temperature range (4–28 °C). Therefore, these two strains could be used as inoculums in onion waste biodegradation in order to enhance the hydrolysis stage. It has been proved that a commercial product containing strains from genera *Bacillus* and *Pseudomonas* enhances methanogenesis and odor control in anaerobic digestion (Duran et al. 2006). In addition, strain MB2-62 and VE-74 could potentially be used as a biocontrol agent for onion phytopathogens, like *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotium cepivorum*, as antagonistic activity against these fungi has been reported for different *B. subtilis* strains and for *P. poae* strain RE*1-1-14 (Asaka and Shoda 1996; Müller et al. 2013; Obi and Umezurike 1981; Phae et al. 1990; Utkhede and Rahe 1983).

In view of the above-mentioned arguments, we propose that strains MB2-62 and VE-74 (*B. subtilis* subsp. *subtilis* and *P. poae* respectively) isolated in this present study could be favorite candidates for bioaugmentation or pretreatment purposes. These strains could facilitate efficient degradation of onion and shortening of start-up times in anaerobic digestion. In addition, they could be studied as possible biocontrol agents for onion crop phytopathogens. Consequently, the identified bacteria could be of great biotechnological interest.

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