water

MDPI

Article

Diversity and Biotechnological Potential of Xylan-Degrading Microorganisms from Orange Juice Processing Waste

Ioanna Zerva, Nikolaos Remmas and Spyridon Ntougias *

Laboratory of Wastewater Management and Treatment Technologies, Department of Environmental Engineering, Democritus University of Thrace, Vas. Sofias 12, 67132 Xanthi, Greece; izerva@env.duth.gr (I.Z.); nremmas@env.duth.gr (N.R.)

* Correspondence: sntougia@env.duth.gr; Tel.: +30-25410-79313

Received: 28 November 2018; Accepted: 31 January 2019; Published: 13 February 2019

Abstract: The orange juice processing sector produces worldwide massive amounts of waste, which is characterized by high lignin, cellulose and hemicellulose content, and which exceeds 40% of the fruit's dry weight (d.w.). In this work, the diversity and the biotechnological potential of xylan-degrading microbiota in orange juice processing waste were investigated through the implementation of an enrichment isolation strategy followed by enzyme assays for the determination of xylanolytic activities, and via next generation sequencing for microbial diversity identification. Intracellular rather than extracellular endo-1,4-β-xylanase activities were detected, indicating that peripheral cell-bound (surface) xylanases are involved in xylan hydrolysis by the examined microbial strains. Among the isolated microbial strains, bacterial isolates belonging to *Pseudomonas psychrotolerans*/*P. oryzihabitans* spectrum (99.9%/99.8% similarity, respectively) exhibited activities of 280 U/mg protein. In contrast, almost all microbial strains isolated exerted low extracellular 1,4-β-xylosidase activities (<5 U/mg protein), whereas no intracellular 1,4-β-xylosidase activities were detected for any of them. Illumina data showed the dominance of lactic and acetic acid bacteria and of the yeasts *Hanseniaspora* and *Zygosaccharomyces*. This is the first report on indigenous xylanolytic microbiota isolated from orange juice processing waste, possessing the biotechnological potential to serve as biocatalysts for citrus biomass valorization through the production of high-added value products and energy recovery.

Keywords: orange juice processing waste; biomass valorization; xylanolytic bacteria and yeasts; endo-1,4-β-xylanase activity; 1,4-β-xylosidase activity; hemicellulose hydrolysis; lactic acid bacteria (LAB); acetic acid bacteria (AAB)

1. Introduction

The cultivation of citrus is one of the major global agricultural sectors, which resulted in the production in 2015 of approximately 130 million tons worldwide, with China, the Mediterranean countries, Brazil, India, USA and Mexico being responsible for more than 80% of the world's citrus harvest [1]. Citrus utilization for processing reached globally 25 million tons, with 80% of the processed citrus being oranges [1]. During orange juice processing, more than 60% of the fruit is discarded as waste, used either for animal feed or depurated in conventional wastewater treatment plants in the cases of the solid and liquid fraction of the waste produced, respectively [2].

On the other hand, orange juice processing waste consists of high levels of lignin, cellulose and hemicellulose, which has been reported to be equal to 40% of the dry weight [3]. A range of biotechnological applications have recently been proposed for the valorization of orange juice

processing waste, including the recovery of high added-value products like natural antioxidants, essential oils and pectin [4,5], the manufacturing of absorbent material [6] and the production of bioethanol and biomethane [7–9]. Regarding enzyme technology, the use of citrus processing waste as bioresource for cellulases and pectinases has also been explored [10] for potential applications in food technology, solid-state fermentation and energy recovery [11,12]. In addition, the production of xylanases and the investigation of their potential uses in food and feed technologies, the pulp and paper industries, and biofuel production are of high interest [13].

Endo-1,4-β-xylanases (EC 3.2.1.8) belong to a class of catabolic enzymes which degrade xylan, a biopolymer containing β-1,4-glucosidic bonds, to D-xylose and xylooligosaccharides [14]. Despite the fact that xylan requires a range of enzymes for its complete depolymerization, endo-1,4-β-xylanase is considered as the key enzyme. 1,4-β-xylosidases (EC 3.2.1.37) catalyze the release of D-xylose from the non-reducing ends of xylooligosaccharides [15]. Indeed, hemicelluloses including xylan, together with celluloses, are considered as the most copious renewable biomass reserves on the globe [13]. Taking into account the high hemicellulose content of citrus waste, orange juice processing waste appears to be a suitable source for novel xylanolytic microorganisms and for the production of high-performance xylanases.

However, the study of xylanolytic microorganisms and their secreted xylanases in xylan-rich citrus processing wastes is limited to a few studies. In most cases, a single allochthonous fungal strain is applied to ferment orange wastes for solid-state fermentation purposes. For instance, a *Eupenicillium javanicum* strain that was isolated from forest rotten wood exhibited high xylanase activity when grown on such residues [16]. Moreover, the soil-isolated fungus *Aspergillus japonicus* PJ01 growing on orange waste powder exerted important xylanolytic activities [17]. To the best of our knowledge, there is no previous report on the isolation and molecular characterization of the main autochthonous xylanase-producing microbiota in citrus processing wastes.

Thus, this study attempts for the first time to reveal the xylanolytic potential of microorganisms obtained from orange processing waste by determining their endo-1,4-β-xylanase and 1,4-β-xylosidase activities and to proportionally assess the indigenous xylanolytic diversity identified in such waste by employing both conventional molecular and next-generation sequencing techniques.

2. Materials and Methods

2.1. Physicochemical Characterization of Orange Juice Processing Waste

Electrical conductivity (EC) and pH were recorded by a Crison CM35 and a Metrohm 632 meter respectively after 1:10 v/v sample: water dilution. A measurement of total organic matter and ash content was carried out after ashing at 550 °C for 4 h, whereas carbon content determination was based on the van Bemmelen transformation factor. Total nitrogen content was estimated by sulphuric acid Kjeldahl digestion of fine-grained sample and subsequent alkaline steam distillation in boric acid solution [18]. Total phosphorus was calculated spectrophotometrically according to Anderson and Ingram procedure [19]. Total soluble carbohydrate content was determined after potassium dichromate digestion in concentrated sulfuric acid and titration with acidified ferrous ammonium sulphate in the presence of o-phenantholine indicator [20]. Lastly, total soluble phenolics and anthrone-reactive carbon were measured according to the Singleton and Rossi [21] and Brink et al. [22] protocols, respectively.

2.2. Enrichment and Isolation of Xylan-Degraders

To isolate xylanolytic microorganisms from orange juice processing waste, an enrichment procedure was followed, where the orange processing waste was initially subjected to 1/10 dilution using a solution consisting of 2 g/L xylan from corn core (TCI Chemicals, Tokyo, Japan), 13 mM Na_2HPO_4, 87 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$ and 0.4 mM $MgSO_4$. After a 1-week incubation period at 28 °C, 1/100 of the exhausted medium was transferred to the sterile solution described above and was incubated for a period of 1 week. A third enrichment step was also included, transferring 1/100 of the second exhausted medium to fresh

sterile solution, following the above-mentioned incubation conditions. Growth plates for xylanolytic bacteria were made by mixing the above-mentioned solution with 1.7% w/v agar.

2.3. Determination of Endo-1,4-β-xylanase and 1,4-β-xylosidase Activities of Xylanolytic Isolates and Fermented Orange Juice Processing Waste

All xylan-degrading bacteria isolated from the orange juice processing waste examined were grown for a period of 1 week in the above described liquid medium, where xylan served as the sole carbon and energy source. The microbial biomass was obtained by centrifugation at 12,000 g for 5 min and the collected supernatant was transferred to a sterile tube to estimate extracellular activities. The pelleted biomass was rinsed twice in 0.02 M acetate buffer and then sonicated in an ice bath for 15 min under 0.6 s pulse/0.4 s interval (50% duty level) through the use of a UP200S sonicator (Hielscher, Germany) [23]. Cell debris was removed from the lysate through centrifugation at 4 °C and 15,000 g for 15 min and the clean lysate was used for the determination of the endocellular activities. In the case of the fermented orange juice processing waste, the waste was mixed thoroughly with 1/10 v/v 0.02 M acetate buffer for 30 min and subjected to centrifugation at 4000 g for 5 min to obtain the extract for determination of extracellular activities. Fermented orange juice processing waste mixed with 1/10 v/v 0.02 M acetate buffer and sonicated under the aforementioned conditions was used for the estimation of waste endocellular activities. The protein content of the collected supernatants and the cell-free lysates were measured by the Bradford method [24].

A mixture of 1.6 mL 4 mM p-nitrophenyl-β-D-xylopyranoside (Apollo Scientific Ltd., Manchester, UK) in 0.02 M acetate buffer and 0.4 mL cell-free lysate or culture supernatant/waste extract, as appropriate, was prepared for the determination of endocellular and extracellular 1,4-β-xylosidase activity, respectively. The mixture was incubated at 40 °C and the 1,4-β-xylosidase activity was measured spectrometrically at 410 nm by estimating the color change against blank at successive time intervals (up to 8 h) [25]. A standard curve was constructed by preparing dilutions from a 1 g/L p-nitrophenol solution in acetate buffer.

Endo-1,4-β-xylanase was estimated by mixing 1.6 mL 0.5% w/v xylan from corn core (TCI Chemicals, Tokyo, Japan) in 0.02 M acetate buffer and 0.4 mL lysate or culture supernatant/waste extract, as appropriate, and incubation of the mixture at 50 °C for a period of 12 h [26]. A color reaction was made by adding 2 mL dinitrosalicylic acid (DNS) solution, consisting of 1% w/v dinitrosalicylic acid, 0.2% w/v phenol, 0.05% w/v Na_2SO_3 and 1% w/v NaOH [27], and placing the mixture at 65 °C for a period of 6 min. Color stabilization was achieved by the addition of 0.67 mL 40% w/v potassium sodium tartate solution. The color developed against a blank was determined spectrometrically at 540 nm. The standard curve was constructed by preparing dilutions from 0.4% w/v xylose solution and determining the color developed after reaction of the mixture with DNS reagent.

In all enzymatic determinations, estimation of the incubation period was made in preliminary assays. Xylanolytic activities are expressed as U (Units)/mg protein, where 1 enzyme unit is defined as the amount of endo-1,4-β-xylanase or 1,4-β-xylosidase needed for the cleavage of 1 mole/min of the appropriate substrate.

2.4. Molecular Characterization of Xylanolytic Bacteria Isolated from Orange Juice Processing Waste

Genomic DNA from the xylan-degraders exhibiting the highest xylanolytic activities was extracted by scraping cells from agar plates and further processing the biomass through the "NucleoSpin Tissue" DNA extraction kit (Macherey-Nagel, Düren, Germany). PCR 16S rRNA gene amplicons were obtained in a TP600 PCR apparatus (TaKaRa, Shiga, Japan) by using the primer set 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). A 50 μL-containing mixture was prepared per each bacterial strain, which consisted of 30 ng DNA, 10× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP (Jena Bioscience, Jena, Germany) and 0.5 μM primer (both 27F and 1492R) as well as 2.5 U Taq polymerase (Minotech Biotechnology, Heraklion, Greece). The PCR program comprised of 2 min initial denaturation at 94 °C, and 35 thermocycles of 30 s denaturation at

94 °C, 30 s primers' annealing at 52 °C and 75 s at 72 °C. The thermocycling program was completed by a DNA extension period of 5 min at 72 °C. The 16S rRNA gene amplicons were ligated into T-Vector pMD20 (TaKaRa, Shiga, Japan) using T4 ligase (TaKaRa, Shiga, Japan) and the recombinant plasmids were TA-cloned into *Escherichia coli* DH5a cells (TaKaRa, Shiga, Japan). Plasmid DNA from cloned DH5a cells was obtained by using the "NucleoSpin Plasmid kit" (Macherey-Nagel, Düren, Germany) and sequenced at Eurofins Genomics (Germany). The amplified 16S rRNA gene products were merged in "CAP3 Sequence Assembly Program" [28]. The sequences of the most effective xylanolytic strains were deposited in GenBank under the code numbers MK212383-MK212384. The 16S rRNA gene amplicon and its closest relatives identified by using blastn were aligned through the use of "Clustal Omega" platform [29]. MEGA X was used for the construction of the phylogenetic tree [30] using the Jukes and Cantor algorithm [31], whereas the tree topology was based on the "neighbor-joining" method [32] and bootstrapping of 1000 trees.

2.5. Identification of Microbial Community Structure in Orange Juice Processing Waste by Illumina Sequencing

The Macherey-Nagel DNA extraction kit (Germany) was employed for crude DNA extraction from the orange juice processing waste, following the manufacturer's protocol. The V4 region of the 16S rRNA gene was amplified by the universal primers 515F (5′-GTGYCAGCMGCCGCGGTA-3′) and reverse primer 909R (5′-CCCCGYCAATTCMTTTRAGT-3′), while the ITS region was amplified by the primers ITS1Fgood (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′). The PCR mixture was prepared at "Mr DNA" (USA) by using the "HotStarTaq Plus Master Mix Kit" (QIAGEN, Germantown, MD, USA) and the amplified reads were obtained by applying an initial denaturation step of 3 min at 94 °C, and 28 thermocycles of 30 s at 94 °C, 40 s primers' annealing at 53 °C and 1 min DNA extension at 72 °C. The amplification procedure was completed by applying a DNA elongation step of 5 min at 72 °C. Ampure beads of Agencourt Bioscience (USA) were used for the purification of the amplified products. Illumina sequencing was carried out at "Mr DNA" (USA) in a MiSeq platform.

The amplified sequencing data was demultiplexed and trimmed by using the "Pipeline Initial Process" platform at RDP pipeline [33]. Sequencing data containing N(s), amplicons of inappropriate length and reads of low quality score (<20) were excluded by employing PandaSeq [34]. Chimeric sequences were discarded by using the UCHIME algorithm [35]. The bioinformatic tool of Nawrocki et al. [36] was employed to align non-chimeric reads, which were further grouped by the "Complete Linkage Clustering" option of RDP pipeline. The Illumina sequences of the three individual orange juice processing waste samples were deposited in SRA database at NCBI under the "Bioproject" code PRJNA506784 (BioSamples' accessions: SRX5058664-SRX5058669).

2.6. Estimation of Ecological Indicators and Statistical Analysis

The Chao's richness, Shannon diversity and Pielou's evenness indices were determined as previously described [37]. ANOVA (Analysis of variance) carried out by Duncan's comparison tests ($p < 0.05$) were employed to identify statistically significant differences among microbial xylanolytic activities. Letters in common on the top of the chart bars denote non-statistically significant differences.

3. Results and Discussion

A combination of culture-dependent microbiological techniques and enzymatic assays, as well as next generation sequencing approaches, were employed to isolate the major xylan-degraders and to identify the entire microbial community structure in orange juice processing waste, which was mainly comprised of orange peels. Examination of physicochemical properties revealed that the orange processing waste had an acidic pH of 4.00 ± 0.01 and a high organic content, i.e., which exceeded 85% w/w of the dry weight. Moreover, an important part of the organic content, i.e., 28.16 ± 1.38% w/w, consisted of soluble carbohydrates, with anthrone-reactive C being equal to 9.63 ± 0.99% w/w (Table 1). Nitrogen content was estimated to be 1.21 ± 0.03% w/w, a fact that indicates a remarkable

high C/N ratio, whereas total phosphorus and soluble phenolics were determined to be equal as 2.38 ± 0.10 and 77.4 ± 4.0 mg/g d.w, respectively (Table 1).

Table 1. Physicochemical traits of orange juice processing waste.

Parameter	Mean ± SE (n = 3)
pH *	4.00 ± 0.01
EC (mS/cm) *	1.60 ± 1.02
Dry weight content (%)	14.18 ± 0.90
Total organic matter (% w/w)	85.95 ± 1.78
Carbon (% w/w)	49.85 ± 1.03
Ash content (% w/w)	14.05 ± 1.78
Total nitrogen (% w/w)	1.21 ± 0.03
Total soluble carbohydrates (% w/w)	28.16 ± 1.38
Anthrone-reactive C (% w/w)	9.63 ± 0.99
Total phosphorus (mg/g d.w.)	2.38 ± 0.10
Total soluble phenolics (mg/g d.w.)	77.4 ± 4.0

* 1/10 w/v, orange juice processing waste-to-water.

By following an enrichment strategy, a total of 24 microbial strains were isolated using agar plates where xylan from corn core served as the sole carbon and energy source. All microbial isolates were further screened through estimation of their xylanolytic activities. To evaluate their xylanolytic nature, both endo-1,4-β-xylanase and 1,4-β-xylosidase activities were determined in their lysate and exhausted growth medium. Regarding endo-1,4-β-xylanase, none of the microbial strains isolated exhibited extracellular activities, whereas certain microbial isolates exerted surface/intracellular endo-1,4-β-xylanase activities (Figure 1). In contrast, low extracellular 1,4-β-xylosidase activities were exhibited by almost all microbial strains isolated, except for isolates XYLA-14 and XYLA-18 (Figure 2), while no intracellular 1,4-β-xylosidase activities were detected for any of the examined isolates. Moreover, a total of 7 and 2 microbial isolates that grew on xylan-containing plates lacked endo-1,4-β-xylanase and 1,4-β-xylosidase activity, respectively (Figures 1 and 2).

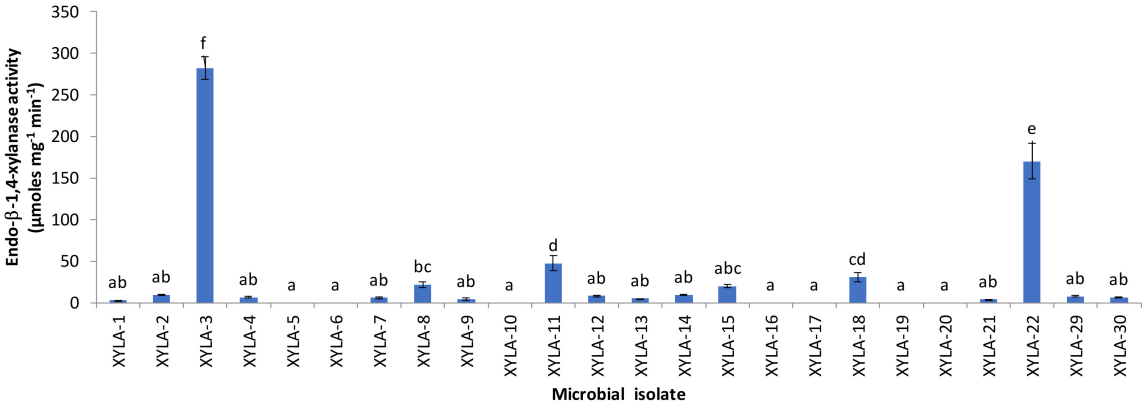

Figure 1. Endo-β-1,4-xylanase activities (surface/intracellular fraction) of the microbial strains isolated from orange juice processing waste.

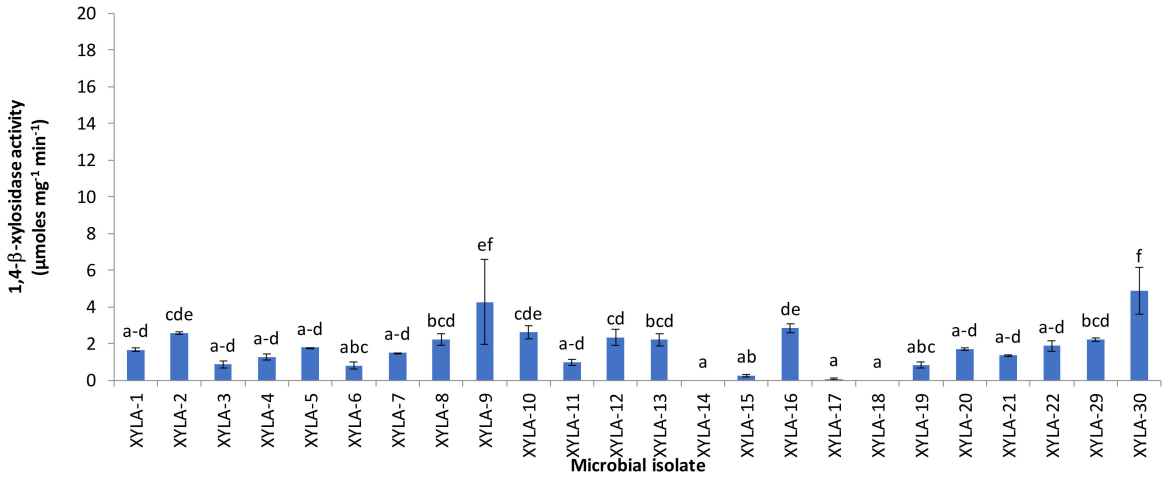

Figure 2. 1,4-β-xylosidase activities (extracellular fraction) of the microbial strains isolated from orange juice processing waste.

Regarding endo-1,4-β-xylanase, a range of 11 microbial isolates, i.e., XYLA-1, -2, -4, -7, -9, -12, -13, -14, -21, -29 and -30, marginally exhibited endo-1,4-β-xylanase activities, with their values being lower than 10 U/mg protein (Figure 1). In contrast, the endo-1,4-β-xylanase activities of the microbial strains XYLA-22 and XYLA-3 exceeded 170 and 280 U/mg protein, respectively, whereas endo-1,4-β-xylanase activities of 20–50 U/mg protein were determined for the microbial isolates XYLA-8, XYLA-11, XYLA-15 and XYLA-18 (Figure 1). Based on 1,4-β-xylosidase activity data, all microbial isolates (apart from XYLA-14 and XYLA-18) exhibited 1,4-β-xylosidase activities lower than 5 U/mg protein (Figure 2). The microbial isolates exhibiting the greatest xylanolytic activity, i.e., XYLA-3 and XYLA-22, were further characterized through 16S rRNA gene sequencing, both showing high phylogenetic similarity (99.9% and 99.8% respectively) to the type strains of the species *Pseudomonas psychrotolerans* and *P. oryzihabitans* (Figure 3). Even though certain *Pseudomonas* strains have been found to possess the ability to degrade xylan [38,39], no *P. psychrotolerans* or *P. oryzihabitans* isolates capable of degrading xylan have been reported so far.

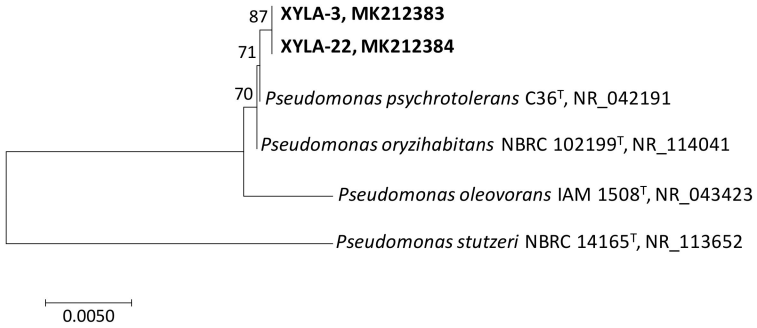

Figure 3. Phylogenetic position of the most effective xylanolytic micro-organisms isolated from orange juice processing waste, based on bootstrapping of 1000 trees. Scale bar represents substitutions per nucleotide site.

Reports on the induction of xylanases from allochthonous bacteria growing in orange processing (by)products are limited in number, while no work on indigenous xylanolytic microbiota isolated from orange juice processing waste exists in the international literature. *Streptomyces* [40–42] and *Bacillus*/*Geobacillus* [43,44] spp. isolated from soil are common bacterial strains that have found to exhibit endo-β-1,4-xylanase activities when tested on orange processing (by)products. In these studies, their endo-1,4-β-xylanase activities were in the same order with those determined in the microbial isolates XYLA-3 and XYLA-22. However, extracellular rather than intracellular endo-1,4-β-xylanase

activities were assessed in these studies. Even though the vast majority of xylanases are extracellularly secreted in order to breakdown this particular polysaccharide in the supernatant, the microbial strains in this work showed no detected extracellular activity. This means that either xylan is hydrolyzed to xylo-oligomers by an undetected negligible amount of extracellular endoxylanases, which can be then transported into the cells and broken down by intracellular endoxylanases [45], or more probably, peripheral cell-bound xylanases of the isolated microbial strains are involved in xylan hydrolysis [46,47], permitting the transport of oligomers and monomers into the cytoplasm. Interestingly, a *Pseudomonas putida* strain displayed surface endo-1,4-β-xylanase and 1,4-β-xylosidase activities [39]. In addition, no studies regarding 1,4-β-xylosidase activity of bacterial isolates growing in orange processing residues have been performed. The lower 1,4-β-xylosidase as compared to endo-1,4-β-xylanase activities could be attributed to the terminal-edge specificity of β-xylosidases in contrast to the internal multi-cleavage caused by xylanases during xylan breakdown.

On the other hand, allochthonous fungi, mainly of the genera *Penicillium* and *Aspergillus* spp., have been reported to exhibit both endo-1,4-β-xylanase and 1,4-β-xylosidase activities [48–50]. However, their orange processing (by)product-induced xylanase activity appeared to be lower in comparison to the respective activities expressed by terrestrial streptomycetes and bacilli.

A total of 245,457 and 391,508 bacterial and fungal non-chimeric reads respectively were obtained during triplicate analysis of the orange juice processing waste examined. At phylum/class level, the major bacterial taxa identified in this waste analyzed were mainly placed in *Firmicutes* and *Alphaproteobacteria* taxa, representing 74.34% and 25.54% of the total bacterial relative abundance (Figure 4).

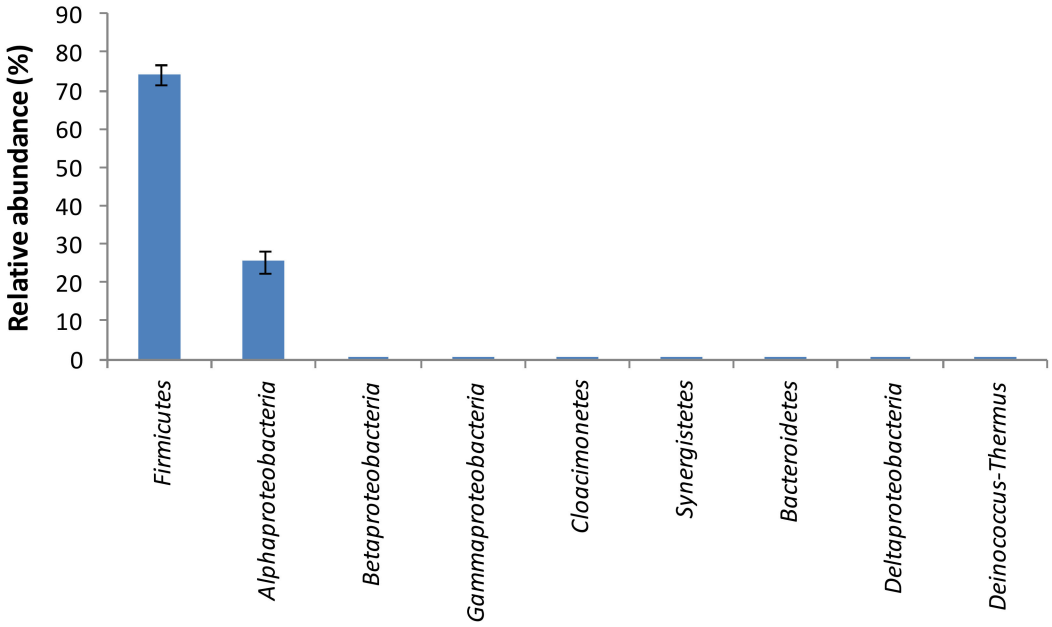

Figure 4. Bacterial diversity at phylum/class level in the orange juice processing waste examined by Illumina assay technology.

At the genus level, the main operational taxonomic units were related to members of the genera *Lactobacillus* and *Acetobacter*, corresponding to 74.19% and 25.52% of the relative bacterial abundance, i.e., 99.71% of the total reads (Figure 5). The remaining bacterial diversity consisted only of 31 taxa, as detected in the Illumina assay (Figure 5).

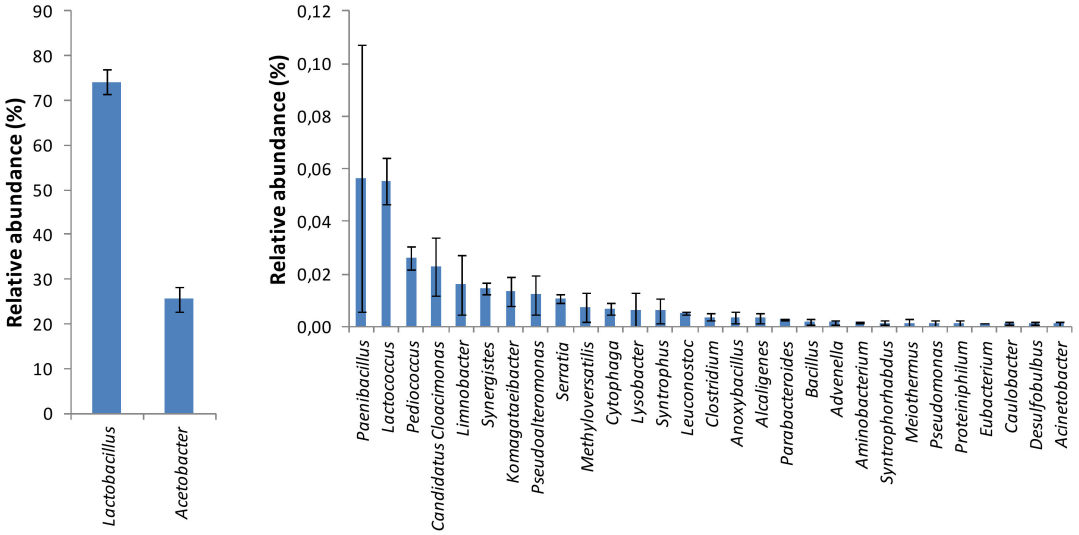

Figure 5. Bacterial diversity at genus level in the orange juice processing waste examined by Illumina assay technology.

Regarding fungal community structure, the majority of the taxa identified were classified into the classes Saccharomycetes and Tremellomycetes, representing 95.51% and 3.84% of the total fungal reads, respectively (Figure 6).

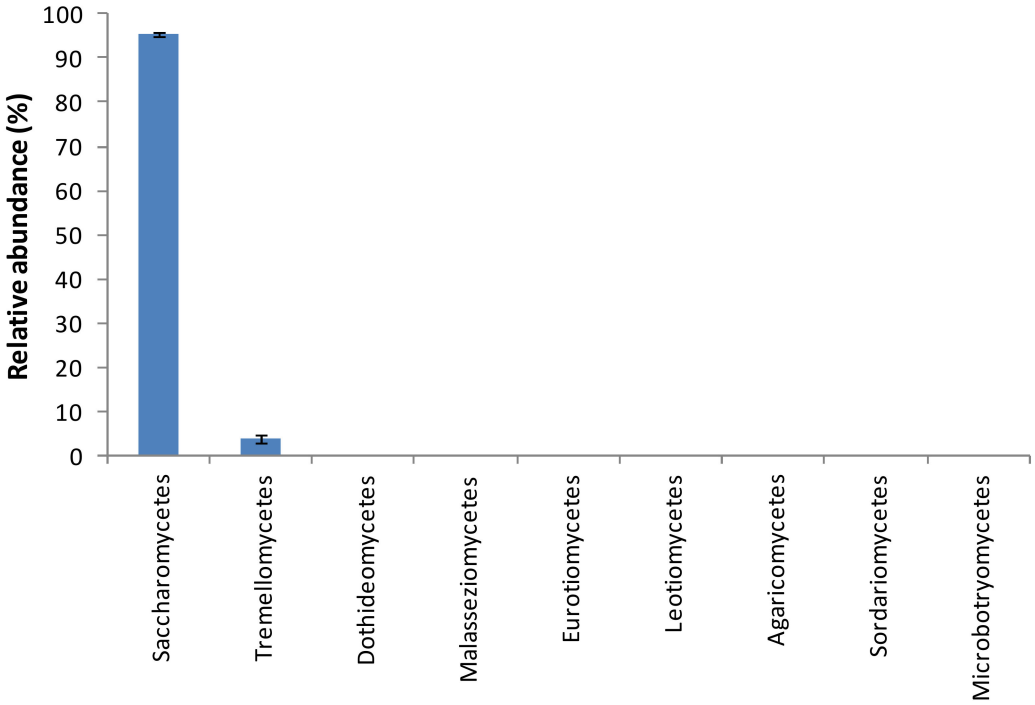

Figure 6. Fungal diversity at class level in the orange juice processing waste examined by Illumina assay technology.

Hanseniaspora Zygosaccharomyces, *Saccharomyces*, *Pichia* and *Cystofilobasidium* were the predominant taxa at genus level, corresponding to an overall proportion of 98.66%. In particular, *Hanseniaspora* and *Zygosaccharomyces* represented 59.66% and 21.97% of the total fungal reads, respectively (Figure 7). Ecological indicators of bacterial and fungal communities are presented in Table 2.

Table 2. Ecological indicator indices of bacterial and fungal communities in the orange juice processing waste examined.

Ecological Indicator Index	Bacterial Community	Fungal Community
Chao's richness index	29.44 ± 0.89	43.67 ± 2.73
Shannon diversity index	0.59 ± 0.02	1.15 ± 0.04
Pielou's Evenness index	0.18 ± 0.01	0.32 ± 0.01

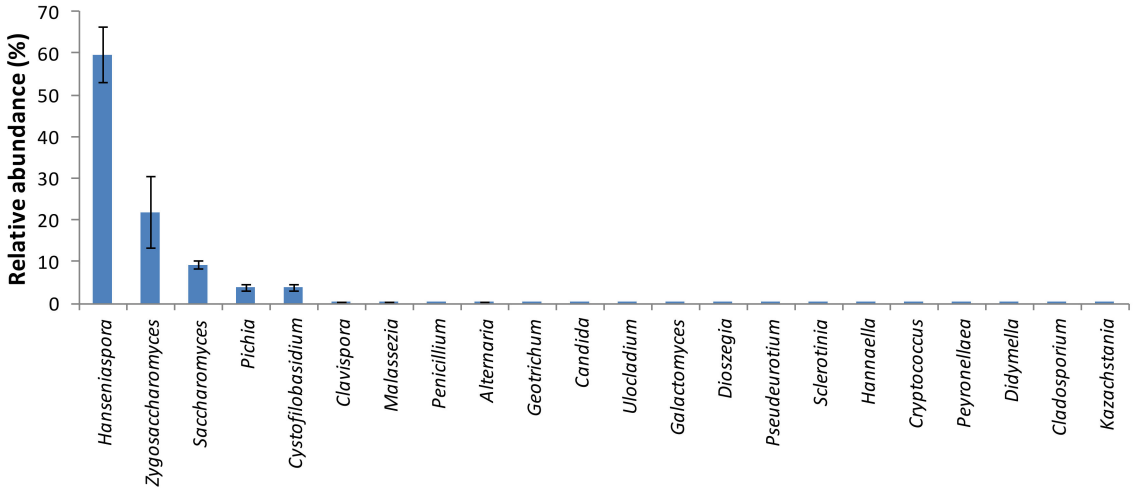

Figure 7. Fungal diversity in the orange juice processing waste examined by Illumina assay technology. Only fungal taxa with relative abundance above 0.01% are shown in the bar chart.

The fermentation process proliferated various yeasts, since the latter covered 99.58% of the total fungal reads, whereas bacterial population was restricted only to lactic and acetic acid bacteria (Figure 5). However, the major yeast and bacterial taxa identified in the fermented orange juice processing waste, apart from *Cystofilobasidium* spp., appear to be common inhabitants of orange fruits [51–54].

Regarding the endo-1,4-β-xylanase activity in the fermented orange juice processing waste, non-statistically significant differences were detected between the lysed biomass and the unsonicated sample, indicating that endo-1,4-β-xylanase activity act exclusively extracellularly (Table 3). On the other hand, the statistical significant differences found in 1,4-β-xylosidase activity measurements with and without sonication ($p < 0.01$) can be attributed to the fact that part of such activity was exerted by intracellular or cell-bound enzymes. Despite the fact that *Pseudomonas* spp. expressed the highest endo-1,4-β-xylanase activities among the bacterial isolates obtained, they consisted only of a minor part of the bacterial diversity in the fermented orange juice processing waste examined. Thus, the proliferation of this particular taxon is due either to the enrichment procedure performed in order to isolate effective xylan-degraders and/or to the fact that the *Pseudomonas* species identified was among the taxa that its growth was self-sufficient and not reliant on interactions occurring in microbial food web. The fact that only a minor part of the microbiome is cultivated should not be excluded. Moreover, a range of microorganisms, such as the bacteria *Paenibacillus*, *Clostridium*, *Anoxybacillus*, *Acinetobacter* and *Caulobacter* as well as the fungi *Pichia*, *Penicillium*, *Cladosporium* and *Aureobasidium*, which constitute only a minor part of the microbial population, possess the genetic ability to degrade xylan, as was shown by MiSeq data comparisons against NCBI (Table 4). On the other hand, the fact that such microorganisms were not isolated indicates that either they are uncultured isolates, or they were not proliferated during the enrichment procedure.

Table 3. Xylanolytic activities of the naturally-fermented orange juice processing waste.

Xylanolytic Activity	Lysate	Bulk/Surface	*p*-Value
Endo-1,4-β-xylanase activity (U/g d.w.)	5.21 ± 0.59	4.77 ± 0.11	0.509
1,4-β-xylosidase activity (U/g d.w.)	3.41 ± 0.14	2.50 ± 0.13	0.009

Table 4. Potential xylan-degrading microbiota identified from MiSeq data.

Taxon	16S rRNA Gene Accession Number of the Closest Species	Closest Species	Identities	Reference
Potential xylan-degrading bacteria				
g__*Paenibacillus*	MF347934	*P. xylanexedens*	370/370 (100%)	[55]
g__*Clostridium*	NR_028611	*C. hiranonis*	350/396 (88.4%)	[56]
g__*Anoxybacillus*	CP012152	*A. gonensis*	392/396 (99.0%)	[57]
g__*Acinetobacter*	NR_113346	*A. lwoffii*	394/395 (99.7%)	[58]
g__*Caulobacter*	NR_074208	*C. segnis*	393/394 (99.7%)	[59]
Taxon	**ITS-5.8S rRNA Gene Accession Number of the Closest Species**	**Closest Species**	**Identities**	**Reference**
Potential xylan-degrading fungi				
g__*Pichia*	NR_130688	*P. fermentans*	173/174 (99.4%)	[60]
g__*Penicillium*	MH856375	*P. italicum*	267/267 (100%)	[61]
g__*Cladosporium*	NR_152286	*C. angustiherbarum*	250/250 (100%)	[62]
g__*Aureobasidium*	NR_156246	*A. leucospermi*	275/275 (100%)	[63]

It may be concluded that orange juice processing waste harbors a specialized xylanolytic community and should be considered as a source for the isolation of xylan-degraders and the recovery of xylanolytic enzymes with diverse environmental and industrial uses, such as in juice clarification, solid state fermentation and energy recovery from non-conventional organic resources.

Author Contributions: I.Z. performed the physicochemical analysis of orange juice processing waste and the isolation of xylan-degrading microbiota, I.Z. and N.R. performed the enzyme assays; I.Z. performed the identification of xylan-degrading microbiota; N.R. carried out the Illumina analysis; S.N. designed and supervised the work; S.N., N.R. and I.Z. drafted and edited the paper, read and approved the final manuscript.

Funding: This research is implemented through the Operational Program "Human Resources Development, Education and Lifelong Learning", code MIS (OPS) 5006203, and is co-financed by the European Union (European Social Fund) and Greek national funds.

Acknowledgments: This work was carried out in the frame of the research project "Optimizing the energy recovery of waste from oranges juice industries using specialized native microorganisms as a starting culture", code MIS (OPS) 5006203, which is implemented through the Operational Program "Human Resources Development, Education and Lifelong Learning" and is co-financed by the European Union (European Social Fund) and Greek national funds.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. FAO. *Citrus Fruit—Fresh and Processed, Statistical Bulletin, 2016*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2017.
2. Dwivedi, P.; Singh, M.; Sehra, N.; Pandey, N.; Sangwan, R.S.; Mishra, B.B. Processing of wet Kinnow mandarin (*Citrus reticulata*) fruit waste into novel Brønsted acidic ionic liquids and their application in hydrolysis of sucrose. *Bioresour. Technol.* **2018**, *250*, 621–624. [CrossRef]
3. de la Torre, I.; Ravelo, M.; Segarra, S.; Tortajada, M.; Santos, V.E.; Ladero, M. Study on the effects of several operational variables on the enzymatic batch saccharification of orange solid waste. *Bioresour. Technol.* **2017**, *245*, 906–915. [CrossRef]

4. Ayala, J.R.; Montero, G.; Campbell, H.E.; García, C.; Coronado, M.A.; León, J.A.; Sagaste, C.A.; Pérez, L.J. Extraction and characterization of orange peel essential oil from Mexico and United States of America. *J. Essent Oil Bear Plants* **2017**, *20*, 897–914. [CrossRef]
5. Barbosa, P.P.M.; Ruviaro, A.R.; Macedo, G.A. Comparison of different Brazilian citrus by-products as source of natural antioxidants. *Food Sci. Biotechnol.* **2018**, *27*, 1301–1309. [CrossRef]
6. Irem, S.; Islam, E.; Mahmood Khan, Q.; Ul Haq, M.A.; Jamal Hashmat, A. Adsorption of arsenic from drinking water using natural orange waste: Kinetics and fluidized bed column studies. *Water Sci. Technol.* **2017**, *17*, 1149–1159. [CrossRef]
7. John, I.; Muthukumar, K.; Arunagiri, A. A review on the potential of citrus waste for D-limonene, pectin, and bioethanol production. *Int. J. Green Energy* **2017**, *14*, 599–612. [CrossRef]
8. Muscolo, A.; Settineri, G.; Papalia, T.; Attinà, E.; Basile, C.; Panuccio, M.R. Anaerobic co-digestion of recalcitrant agricultural wastes: Characterizing of biochemical parameters of digestate and its impacts on soil ecosystem. *Sci. Total Environ.* **2017**, *586*, 746–752. [CrossRef] [PubMed]
9. Lotito, A.M.; De Sanctis, M.; Pastore, C.; Di Iaconi, C. Biomethanization of citrus waste: Effect of waste characteristics and of storage on treatability and evaluation of limonene degradation. *J. Environ. Manag.* **2018**, *215*, 366–376. [CrossRef] [PubMed]
10. Ibrahim, E.; Jones, K.D.; Taylor, K.E.; Hosseney, E.N.; Mills, P.L.; Escudero, J.M. Recombinant, *E. coli* cellulases, β-glucosidase, and polygalacturonase convert a citrus processing waste into biofuel precursors. *ACS Sustain. Chem. Eng.* **2018**, *6*, 7304–7312. [CrossRef]
11. Patidar, M.K.; Nighojkar, S.; Kumar, A.; Nighojkar, A. Pectinolytic enzymes-solid state fermentation, assay methods and applications in fruit juice industries: A review. *3 Biotech.* **2018**, *8*, 199. [CrossRef] [PubMed]
12. Wilkins, M.R.; Widmer, W.W.; Grohmann, K. Simultaneous saccharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol. *Process. Biochem.* **2007**, *42*, 1614–1619. [CrossRef]
13. Kumar, D.; Kumar, S.S.; Kumar, J.; Kumar, O.; Mishra, S.V.; Kumar, R.; Malyan, S.K. Xylanases and their industrial applications: A review. *Biochem. Cell Arch.* **2017**, *17*, 353–360.
14. Biely, P.; Singh, S.; Puchart, V. Towards enzymatic breakdown of complex plant xylan structures: State of the art. *Biotechnol. Adv.* **2016**, *34*, 1260–1274. [CrossRef] [PubMed]
15. Juturu, V.; Wu, J.C. Microbial exo-xylanases: A mini review. *Appl. Biochem. Biotechnol.* **2014**, *174*, 81–92. [CrossRef] [PubMed]
16. Tao, N.G.; Shi, W.Q.; Liu, Y.J.; Huang, S.R. Production of feed enzymes from citrus processing waste by solid-state fermentation with *Eupenicillium javanicum*. *Int. J. Food Sci. Technol.* **2011**, *46*, 1073–1079. [CrossRef]
17. Li, P.J.; Xia, J.L.; Shan, Y.; Nie, Z.Y.; Wang, F.R. Effects of surfactants and microwave-assisted pretreatment of orange peel on extracellular enzymes production by *Aspergillus japonicus* PJ01. *Appl. Biochem. Biotechnol.* **2015**, *176*, 758–771. [CrossRef] [PubMed]
18. Bremner, J.M.; Mulvaney, C.S. Nitrogen total. In *Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties*; Agronomy Monograph No. 9; Page, A.L., Miller, R.H., Keeney, D.R., Eds.; American Society of Agronomy and Soil Science Society of America: Madison, WI, USA, 1982; pp. 595–624, ISBN 0-89118-072-9.
19. Anderson, J.M.; Ingram, J.S.I. Microbial biomass. In *Tropical Soil Biology and Fertility: A Handbook of Methods*, 2nd ed.; Anderson, J.M., Ingram, J.S.I., Eds.; CAB International: Wallingford, UK, 1993; pp. 68–70, ISBN 0-85198-821-0.
20. Lowe, L.E. Total and labile polysaccharide analysis of soils. In *Soil Sampling and Methods of Analysis*; Carter, M.R., Ed.; Canadian Society of Soil Science, Lewis Publishers: Boca Raton, FL, USA, 1993; pp. 373–376, ISBN 0-87371-861-5.
21. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **1965**, *16*, 144–158.
22. Brink, R.H.; Dubach, P.; Lynch, D.L. Measurement of carbohydrates in soil hydrolyzates with anthrone. *Soil Sci.* **1960**, *89*, 157–166. [CrossRef]
23. Remmas, N.; Melidis, P.; Voltsi, C.; Athanasiou, D.; Ntougias, S. Novel hydrolytic extremely halotolerant alkaliphiles from mature landfill leachate with key involvement in maturation process. *J. Environ. Sci. Health A Tox Hazard. Subst. Environ. Eng.* **2017**, *52*, 64–73. [CrossRef]
24. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* **1976**, *72*, 248–254. [CrossRef]

25. Baldrian, P.; Valášková, V.; Merhautová, V.; Gabriel, J. Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead and zinc. *Res. Microbiol* **2005**, *156*, 670–676. [CrossRef] [PubMed]
26. Camassola, M.; Dillon, A.J.P. Effect of different pretreatment of sugar cane bagasse on cellulase and xylanases production by the mutant *Penicillium echinulatum* 9A02S1 grown in submerged culture. *Biomed. Res. Int.* **2014**, *2014*, 720740. [CrossRef] [PubMed]
27. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
28. Huang, X.; Madan, A. CAP3: A DNA sequence assembly program. *Genome Res.* **1999**, *9*, 868–877. [CrossRef] [PubMed]
29. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Söding, J.; et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **2011**, *7*, 539. [CrossRef] [PubMed]
30. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
31. Jukes, T.H.; Cantor, C.R. Evolution of protein molecules. In *Mammalian Protein Metabolism*; Munro, H.N., Ed.; Academic Press: New York, NY, USA, 1969; pp. 21–132, ISBN 0125106017.
32. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [CrossRef]
33. Cole, J.R.; Wang, Q.; Fish, J.A.; Chai, B.; McGarrell, D.M.; Sun, Y.; Brown, C.T.; Porras-Alfaro, A.; Kuske, C.R.; Tiedje, J.M. Ribosomal Database Project: Data and tools for high throughput rRNA analysis. *Nucl. Acids Res.* **2014**, *42*, D633–D642. [CrossRef]
34. Masella, A.P.; Bartram, A.K.; Truszkowski, J.M.; Brown, D.G.; Neufeld, J.D. PANDAseq: Paired-end assembler for Illumina sequences. *BMC Bioinform.* **2012**, *13*, 31. [CrossRef]
35. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]
36. Nawrocki, E.P.; Kolbe, D.L.; Eddy, S.R. Infernal 10: Inference of RNA alignments. *Bioinformatics* **2009**, *25*, 1335–1337. [CrossRef] [PubMed]
37. Magurran, A.E. *Ecological Diversity and Its Measurement*, 1st ed.; Princeton University Press: Princeton, NJ, USA, 1988; ISBN 978-94-015-7358-0.
38. Maki, M.L.; Idrees, A.; Leung, K.T.; Qin, W. Newly isolated and characterized bacteria with great application potential for decomposition of lignocellulosic biomass. *J. Mol. Microbiol. Biotechnol.* **2012**, *22*, 156–166. [CrossRef] [PubMed]
39. Schulte, M.F.; Tozakidis, I.E.; Jose, J. Autotransporter-based surface display of hemicellulases on *Pseudomonas putida*: Whole-cell biocatalysts for the degradation of biomass. *ChemCatChem* **2017**, *9*, 3955–3964. [CrossRef]
40. Wang, S.L.; Yen, Y.H.; Shih, L.; Chang, A.C.; Chang, W.T.; Wu, W.C.; Chai, Y.D. Production of xylanases from rice bran by *Streptomyces actuosus* A-151. *Enzyme Microb Technol.* **2003**, *33*, 917–925. [CrossRef]
41. Adigüzel, A.O.; Tunçer, M. Production, characterization and application of a xylanase from *Streptomyces* sp. AOA40 in fruit juice and bakery industries. *Food Biotechnol.* **2016**, *30*, 189–218. [CrossRef]
42. Rosmine, E.; Sainjan, N.C.; Silvester, R.; Alikkunju, A.; Varghese, S.A. Statistical optimisation of xylanase production by estuarine *Streptomyces* sp. and its application in clarification of fruit juice. *J. Gen. Eng. Biotechnol.* **2017**, *15*, 393–401. [CrossRef]
43. Bibi, Z.; Ansari, A.; Zohra, R.R.; Aman, A.; Qader, S.A.U. Production of xylan degrading endo-1,4-β-xylanase from thermophilic *Geobacillus stearothermophilus* KIBGE-IB29. *J. Radiat. Res. Appl. Sci.* **2014**, *7*, 478–485. [CrossRef]
44. Kumar, L.; Nagar, S.; Mittal, A.; Garg, N.; Gupta, V.K. Immobilization of xylanase purified from *Bacillus pumilus* VLK-1 and its application in enrichment of orange and grape juices. *J. Food Sci. Technol.* **2014**, *51*, 1737–1749. [CrossRef]
45. Collins, T.; Gerday, C.; Feller, G. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* **2005**, *29*, 3–23. [CrossRef]
46. Tanaka, T.; Shimomura, Y.; Himejima, M.; Taniguchi, M.; Oi, S. Characterization of xylan-utilizing anaerobes from mesophilic and thermophilic methane sludge and their xylan degrading enzymes. *Agric. Biol. Chem.* **1986**, *50*, 2185–2192. [CrossRef]

47. Murty, M.V.S.; Chandra, T.S. Expression of xylanase and cellulase enzymes in a newly isolated *Clostridium* sp. SAIV. *Enzyme Microb. Technol.* **1991**, *13*, 430–435. [CrossRef]
48. Mamma, D.; Kourtoglou, E.; Christakopoulos, P. Fungal multienzyme production on industrial by-products of the citrus-processing industry. *Bioresour. Technol.* **2008**, *99*, 2373–2383. [CrossRef] [PubMed]
49. Terrasan, C.R.F.; Temer, B.; Duarte, M.C.T.; Carmona, E.C. Production of xylanolytic enzymes by *Penicillium janczewskii*. *Bioresour. Technol.* **2010**, *101*, 4139–4143. [CrossRef] [PubMed]
50. Facchini, F.D.A.; Vici, A.C.; Reis, V.R.A.; Jorge, J.A.; Terenzi, H.F.; Reis, R.A.; de Moraes, M.D.L.T. Production of fibrolytic enzymes by *Aspergillus japonicus* C03 using agro-industrial residues with potential application as additives in animal feed. *Bioprocess. Biosyst. Eng.* **2011**, *34*, 347–355. [CrossRef] [PubMed]
51. Rezzadori, K.; Benedetti, S.; Amante, E.R. Proposals for the residues recovery: Orange waste as raw material for new products. *Food Bioprod. Process.* **2012**, *90*, 606–614. [CrossRef]
52. Aneja, K.R.; Dhiman, R.; Aggarwal, N.K.; Kumar, V.; Kaur, M. Microbes associated with freshly prepared juices of citrus and carrots. *Int. J. Food Sci. Technol.* **2014**, *2014*, 408085. [CrossRef] [PubMed]
53. Liang, S.; Gliniewicz, K.; Gerritsen, A.T.; McDonald, A.G. Analysis of microbial community variation during the mixed culture fermentation of agricultural peel wastes to produce lactic acid. *Bioresour. Technol.* **2016**, *208*, 7–12. [CrossRef]
54. El Sheikha, A.F.; Levin, R.; Xu, J. *Molecular Techniques in Food Biology*, 1st ed.; John Wiley & Sons Ltd.: New York, NY, USA, 2018; ISBN 9781119374633.
55. Nelson, D.M.; Glawe, A.J.; Labeda, D.P.; Cann, I.K.; Mackie, R.I. *Paenibacillus tundrae* sp. nov. and *Paenibacillus xylanexedens* sp. nov., psychrotolerant, xylan-degrading bacteria from Alaskan tundra. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1708–1714. [CrossRef]
56. Kitahara, M.; Takamine, F.; Imamura, T.; Benno, Y. *Clostridium hiranonis* sp. nov., a human intestinal bacterium with bile acid 7alpha-dehydroxylating activity. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 39–44. [CrossRef]
57. Belduz, A.O.; Dulger, S.; Demirbag, Z. *Anoxybacillus gonensis* sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 1315–1320. [CrossRef]
58. Bouvet, P.J.M.; Grimont, P.A.D. Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int. J. Syst. Bacteriol.* **1986**, *36*, 228–240. [CrossRef]
59. Abraham, W.R.; Strompl, C.; Meyer, H.; Lindholst, S.; Moore, E.R.; Christ, R.; Vancanneyt, M.; Tindall, B.J.; Bennasar, A.; Smit, J.; et al. Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*. *Int. J. Syst. Bacteriol.* **1999**, *49*, 1053–1073. [CrossRef] [PubMed]
60. Arteau, M.; Labrie, S.; Roy, D. Terminal-restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis profiling of fungal communities in Camembert cheese. *Int Dairy J.* **2010**, *20*, 545–554. [CrossRef]
61. Vu, D.; Groenewald, M.; de Vries, M.; Gehrmann, T.; Stielow, B.; Eberhardt, U.; Al-Hatmi, A.; Groenewald, J.Z.; Cardinali, G.; Houbraken, J.; et al. Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Stud. Mycol.* **2019**, *92*, 135–154. [CrossRef] [PubMed]
62. Bensch, K.; Groenewald, J.Z.; Braun, U.; Dijksterhuis, J.; de Jesus Yanez-Morales, M.; Crous, P.W. Common but different: The expanding realm of *Cladosporium*. *Stud. Mycol.* **2015**, *82*, 23–74. [CrossRef]
63. Crous, P.W.; Summerell, B.A.; Swart, L.; Denman, S.; Taylor, J.E.; Bezuidenhout, C.M.; Palm, M.E.; Marincowitz, S.; Groenewald, J.Z. Fungal pathogens of *Proteaceae*. *Persoonia* **2011**, *27*, 20–45. [CrossRef]