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Biocatalyst Potential of Cellulose-Degrading Microorganisms Isolated 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

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Abstract: Cellulases can be applied as macerating and peeling enzymes in the orange juice processing industry. In this work, indigenous cellulose-degrading microorganisms were isolated from orange juice processing waste through successive enrichment procedures using carboxymethyl cellulose (CMC) as the sole carbon source. A total of 24 microbial isolates were screened for their ability to grow in CMC liquid medium, resulting in the selection of seven isolates. The latter were further assessed by determining their endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and β-1,4-D-glucosidase activities, of which their respective activities were as high as 3.89, 10.67, and 10.69 U/mg protein. All cellulose-degraders selected belonged to the genus *Paenibacillus*, although to distinct operational taxonomic units related to *P. xylanexedens*, *P. tundrae*, and *P. pabuli* (operational taxonomic unit—OTU#1) and to *P. wynnii*, *P. odorifer*, and *P. donghaensis* (OTU#2) spectrum. Regarding the cellulase activities of the orange juice processing waste, endo-1,4-β-D-glucanase activity (4.00 ± 0.11 U/g) was exerted only extracellularly, whereas exo-1,4-β-D-glucanase (2.60 ± 0.19 U/g) and β-1,4-D-glucosidase (5.69 ± 0.23 U/g) activities were exhibited both extracellularly and intracellularly. In conclusion, orange juice processing waste can be considered as a valuable source for the isolation of cellulose-degrading microbiota with potential uses in beverage industry, solid state fermentation and energy production.

Keywords: cellulolytic bacteria; cellulase; glucanase; glucosidase; beverage industry; solid state fermentation; *Paenibacillus*

1. Introduction

Oranges are one of the most cultivated and processed citrus fruits, with 23.89 million tons of orange juice being annually consumed worldwide [1]. Due to the high moisture content, which exceeds 80% of the fresh fruit biomass, oranges are considered to be easily spoiled crops. Thus, preservation approaches should be applied to expand their storage life. By valorizing orange fruits through conversion into various crop-processing products, mainly juice and sweet commodities, food lifetime duration is expanded, creating new business challenges [2].

In the recent years, the fruit juice industry has become one of the largest agro-industrial sectors worldwide. Indeed, an increased global interest has been sighted on juice production and commercialization [2,3]. Fruit juices mainly consist of long polymeric saccharides like cellulose, hemicellulose, pectin, and starch, which are considered the major constituents of orange dietary fibers [4]. Cellulose, a β-1,4-linked glucose-based biopolymeric carbohydrate, is the major plant structural polysaccharide and the most abundant biomolecule in the biosphere [5].

In beverage industries, orange crops are subjected to washing and thereafter to peeling, followed by chopping into pieces. At present, enzymatic methods for peeling are considered superior to chemical, thermal, and mechanical methods, due to the reduced flesh damage of the fruits and the lowered peeling losses, which increase the quantity and improve the quality of the produced juice [6]. Apart from the peeling of the fruits, cellulolytic enzymes can contribute to juice clarification and viscosity, which are crucial organoleptic characteristics of consumers' preference, reducing crop processing cost and increasing fruit juice yield. In addition, such enzymes significantly meliorate the consistency and the turbidity of cloudy juices and concentrates [7].

The applicability of enzymes depends upon the polysaccharides composition of the various fruit juices. For instance, Navel orange pulp contains a total dietary fiber of 35.4–36.9% dry weight (d.w.), with cellulose, hemicellulose, pectin, and lignin accounting for 25.3%, 5.4%, 15.7–16.3%, and 2.2–3.0% d.w., respectively [2]. Moreover, the total protein and fat content of orange dietary fiber concentrate is estimated to be 8.75% and 1.48% d.w. respectively, whereas the inorganic content is equal to 2.8% d.w. [8]. Regarding orange peels, the pectin, hemicellulose, cellulose, lignin, protein, fat, and ash content accounts for 42.5%, 10.5%, 9.21%, 0.84%, 6.5%, 3.5%, and 2.0% d.w., respectively [9]. Mixtures of enzymes, such as cellulases, hemicellulases, and pectinases, have been widely applied as macerating enzymes in fruit juice industries. During juice processing, maceration of fruits with such enzymes is performed after both crushing and juice extraction, hydrolyzing polysaccharides like cellulose and pectins into mono- and di-saccharides [6]. Apparently, macerating enzymes are key agents to ameliorate juice quality from a broad variety of fruits treated in juice processing agro-industries [7,10]. Apart from maceration, commercial mixtures of such enzymes, for example, Peelzyms series (NOVO Nordisk Ferment), are known as peeling agents that have been widely used in processing citrus fruits, including oranges [11].

The hydrolytic cleavage of β-1,4-glycosidic bonds in cellulose is achieved by means of enzymes known as cellulase complexes. The latter is comprised of endo-1,4-β-D-glucanase (EC 3.2.1.4), which cleaves the internal bonds of cellulose moiety, exo-1,4-β-D-glucanase (EC 3.2.1.91), which cleaves the ends of the cellulose chain releasing cellobiose, and β-1,4-D-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose and cellulose-derived oligosaccharides from the non-reducing ends to glucose [10,12].

Considering orange peel microbiota, the dominant fungal taxa have been recently reported to be members of the genera *Penicillium*, *Meyerozyma*, *Paraphaeosphaeria*, and *Candida* [13]. In addition, *Pichia terricola* and *Hanseniaspora opuntiae* have been found to be the prevailing cultured yeast species during spontaneous fermentation of orange peel [14]. *Penicillium* sp. OP1 isolated from Japanese mandarin peels has been reported to be capable of degrading cellulose by 63% in a 2-week incubation period [15].

Paenibacillus spp. have also been reported to produce highly active and stable cellulases capable of degrading both soluble and microcrystalline cellulose at relatively low cost [16–18]. In addition, *P. xylanexedens* and *P. tundrae* are considered to be effective xylan- and cellulose-degrading bacteria [19]. Recent reports have also stated the biotechnological potential of cellulolytic enzymes from *Paenibacillus* spp. in juice clarification [20]. Despite the use of cellulases in fruit juice clarification [6], no indigenous cellulolytic bacteria, including *Paenibacillus* strains, have been isolated from orange juice processing waste until now. Only pathogenic to orange tree bacteria, (e.g., *Xanthomonas axonopodis* pv. *citri*, which causes citrus canker) have been found to exert low cellulase activities to penetrate their host (e.g., *Citrus sinensis*). Moreover, almost all the cellulose-degrading microorganisms used in the solid-state fermentation of orange peels/wastes are allochthonous fungi.

Thus, this work aims to uncover the cellulolytic potential of microbiota obtained from orange juice processing waste, by estimating their endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and β-1,4-D-glucosidase activities in order to find applications in food enzyme technology, in particular in the beverage industry as improvers of orange juice organoleptic properties, thus resulting in the valorization of the cellulose content of wastes generated by the orange juice processing industry.

2. Materials and Methods

2.1. Enrichment and Isolation of Cellulose-Degrading Microorganisms

An enrichment procedure was followed to isolate cellulolytic microorganisms from orange juice processing waste. This was obtained from an orange juice factory processing Navel variety fruits, where three independent bulk samples were combined. In particular, orange juice processing waste was subjected to ten-fold dilution (10 g to 100 mL) under aerobic conditions in a defined medium comprising of 2 g/L sodium carboxymethyl cellulose (CMC) (Sigma-Aldrich, St. Louis, MO, USA), 1.84 g/L Na_2HPO_4, 11.8 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, and 0.1 g/L $MgSO_4$ and placed in an incubation chamber at 28 °C. After 1 week of incubation, 1 mL of the exhausted medium was inoculated to 99 mL of the above described minimal medium and incubated under the same incubation conditions. The enrichment procedure was finalized by further inoculating 1 mL of the second exhausted medium to 99 mL of the above-mentioned fresh cellulose-containing medium, followed by 1 week of incubation at 28 °C. The third exhausted medium obtained was subjected to ten-fold dilution series in order to isolate cellulolytic microorganisms, where cellulose-based medium was solidified with 1.7% w/v agar. Determination of physicochemical composition was performed as previously described by Ntougias et al. [21].

2.2. Cellulase Activities of Orange Juice Processing Waste and Cellulose-Degrading Microorganisms Isolated from this Orange Juice Waste

The conducted enrichment procedure resulted in the isolation of 24 cellulose-growing microorganisms, which were further evaluated in terms of their cellulolytic potential by assessing their cellulases activities induced in defined growth medium consisting of carboxymethyl cellulose (CMC) as the sole carbon and energy source. Despite that the isolated strains grew on CMC agar, 17 out of 24 isolated strains failed to sustain growth on liquid cultures, probably due to their ability to catabolize agar without cellulose assimilation. The remaining seven microbial isolates were further assessed through estimation of their extracellular and intracellular cellulolytic activities. To estimate such potential, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and β-1,4-D-glucosidase activities were determined in their lysate and exhausted growth medium.

All cellulose-degrading microbiota obtained from orange juice processing waste were incubated in the aforementioned cellulose-based liquid medium for a period of 1 week. The biomass from each cellulolytic isolate was centrifuged at 12,000× *g* for 5 min and the exhausted broth was obtained in order to measure endo- and exo-cellulase activities. On the other hand, the centrifuged biomass was rinsed twice in 20 mM sodium acetate and further resuspended in the same buffer. The resuspended cells were sonicated for 15 min under 0.6 s pulse/0.4 s intervals, at 50% power level of a UP200S device (24 kHz frequency/200 W input power, Hielscher, Germany), as previously described in Remmas et al. [22]. Disrupted cells were removed by centrifugation at 15,000× *g* for 15 min (4 °C) and the cell-free lysate was collected for estimating intracellular cellulolytic activities. The protein content of both exhausted broth and cell-free lysate was determined according to the methods of Bradford [23]. To estimate the cellulolytic activities of orange juice processing waste, 10 g of this waste was mixed with 20 mL of 20 mM sodium acetate for 20 min at 200 rpm and then subjected to sonication under the previously mentioned conditions. Following centrifugation at 15,000× *g* for 15 min (4 °C), the biomass residues were removed and the lysate was used for downstream analysis. The same mixture was also prepared (although was not sonicated) in order to assess the extracellular cellulolytic activities of orange juice processing waste.

An aliquot of 1.6 mL 4 mM p-nitrophenyl-β-D-cellobioside (Apollo Scientific, Stockport, UK) dissolved in 0.02 M acetate buffer was mixed with 0.4 mL cell-free lysate or exhausted broth (orange juice processing waste extract, as appropriate) in order to assess the intracellular and extracellular exo-1,4-β-D-glucanase activities of the cellulolytic isolates (or orange juice processing waste), respectively. The enzymatic reaction was carried out at 40 °C by determining the absorbance change at

410 nm against blank [24]. The β-1,4-D-glucosidase activities were determined by following the same protocol as for the exo-1,4-β-glucanase activities, but using 4-nitrophenyl β-D-glucopyranoside as the cleavage substrate. A standard curve was constructed by measuring the color change of reference standards derived from 1 g/L p-nitrophenol solution, which was dissolved in 20 mM acetate buffer.

Endo-1,4-β-D-glucanase activity was assessed by adding 0.4 mL lysate or culture broth (orange juice processing waste extract), as appropriate, in 1.6 mL 0.5% w/v CMC (dissolved in 20 mM acetate buffer) and placed at 50 °C for 12 h [25]. A color reaction was achieved by boiling the mixture for a period of 15 min in the presence of 2 mL dinitrosalicylic acid (DNS) reagent, which was prepared by adding 10 g/L DNS, 2 g/L phenol, 0.5 g/L w/v Na_2SO_3, and 10 g/L NaOH [26]. The color developed was stabilized by adding 0.67 mL 1.42 M potassium sodium tartrate solution. The color change against blank was assessed at 540 nm and the standard curve was made by performing serial dilutions from 0.4% w/v glucose, which were further reacted and stabilized with DNS and potassium sodium tartrate solution, respectively.

Endo-1,4-β-D-glucanase as well as exo-1,4-β-D-glucanase and β-1,4-D-glucosidase activities are expressed as U/mg protein or U/g d.w., where 1 enzyme unit represents the amount of cellulase required for the release of 1 μmole/min of glucose or p-nitrophenol, respectively. In all enzyme assays, blanks containing cell-free lysate/exhausted broth (as appropriate) in the absence of substrate and substrate in the absence of cell-free lysate/exhausted broth (as appropriate) were included and samples' estimated values were corrected for any background activity determined.

2.3. Phylogenetic Identification of Cellulose-Degrading Bacteria

Extraction of genomic DNA from cellulose-degrading microorganisms was carried out by employing the Macherey-Nagel's "NucleoSpin Tissue" (Düren, Germany). PCR products were amplified in a TP600 thermocycler (TaKaRa, Shiga, Japan) through the use of the forward primer 341f (5′-CCT ACG GGA GGC AGC AG-3′) and the reverse primer 907r (5′-CCG TCA ATT CMT TTG AGT TT-3′) [27]. A reaction mixture of 50 μL was made by adding 1 μL of 40 ng genomic DNA, 10× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM each of the forward and reverse primer and 5 U Taq polymerase (Minotech Biotechnology, Heraklion, Greece). PCR amplification was achieved by placing the PCR mixture for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. The PCR reaction was completed by keeping the temperature at 72 °C for 5 min. The amplified PCR sequences were inserted into pMD20 vector (TaKaRa, Shiga, Japan) by TA-ligation and the ligated products were cloned into *Escherichia coli* DH5a cells provided by TaKaRa (Japan). Plasmid DNA was extracted from the transformed *E. coli* cells by employing the Macherey-Nagel's "NucleoSpin Plasmid kit" (Germany). All sequencing reactions were carried out at Eurofins Genomics (Ebersberg, Germany). Amplicons were merged by the "CAP3 Sequence Assembly Program" of Huang and Madan [28] and assembled sequencing reads were deposited in GenBank (accession numbers MK332505-MK332511). The amplified 16S rRNA gene sequences were aligned with their related amplicons by using the "Clustal Omega" platform [29]. The phylogenetic tree was constructed by using the software MEGA 7 through the application of the Jukes-Cantor model and the inference of the phylogram topology by the "neighbor-joining" approach based on bootstrapping of 1000 trees [30–32].

2.4. Statistical Analysis of Enzyme Data

Analysis of variance (ANOVA) conducted by Duncan's multiple comparison test was employed to evaluate cellulolytic activities among the cellulose-degrading bacteria isolated, whereas Student's *t*-test was performed to assess significant differences between the bulk/surface and lysed orange juice processing waste.

3. Results and Discussion

A cultivation approach, which was based on the performance of an enrichment procedure, was employed in order to isolate cellulose-degrading microbiota from orange juice processing waste,

whereas a range of enzymatic assays were performed to assess their cellulolytic potential through estimation of their endo-1,4-β-D-glucanase, exo-1,4-β-glucanase, and β-1,4-D-glucosidase activities. The cellulose-degrading microorganisms were obtained from orange juice processing waste, which was characterized by low pH of 3.86 ± 0.02, extremely high organic matter (e.g., 99.05 ± 0.02% w/w), and high total soluble and anthrone-reactive carbohydrate content of 51.97 ± 2.00 and 4.12 ± 0.04% w/w, respectively (Table 1). The nitrogen content of the orange juice processing waste was equal to 0.68 ± 0.05% w/w, resulting in an extremely high C/N ratio of 84.5. The total phosphorus and phenolics concentrations were estimated to be 12.68 ± 0.14 and 12.48 ± 0.14 mg/g d.w., respectively (Table 1).

Table 1. Physicochemical composition of orange juice processing waste.

Parameter	Mean ± SE
pH *	3.86 ± 0.02
Electrical Conductivity (μS/cm) *	511 ± 0.33
Dry weight content (%)	14.89 ± 0.05
Organic matter (% w/w)	99.05 ± 0.02
Carbon (% w/w)	57.45 ± 0.01
Ash content (% w/w)	0.95 ± 0.03
Nitrogen (% w/w)	0.68 ± 0.05
Soluble carbohydrates (% w/w)	51.97 ± 2.00
Anthrone-reactive C (% w/w)	4.12 ± 0.04
Phosphorus (mg/g d.w.)	12.68 ± 0.14
Soluble phenolics (mg/g d.w.)	12.48 ± 0.14

* 1/10 w/v, waste-to-water ratio.

Similar endo-1,4-β-D-glucanase activities were detected in the bulk/surface (3.71 U/g d.w.) and in the lysed (4.00 U/g d.w.) orange juice processing waste, indicating that internal cleavage of cellulose occurred only extracellularly. On the other hand, exo-1,4-β-D-glucanase, which unchains cellobiose units from cellulose and cellulose oligomers ends, and β-1,4-D-glucosidase, which cleaves cellobiose and other β-1,4-linked oligomers to release glucose, exhibited significantly higher activities in the lysed biomass than in the non-sonicated bulk (for $p < 0.05$ and $p < 0.01$, respectively) (Table 2). This intimates that such enzymes acted both extracellularly and intracellularly, due to the fact that small molecules like cellulose oligomers and cellobiose can insert into the cytoplasm, inducing exo-1,4-β-glucanases and β-1,4-glucosidases.

Table 2. Cellulolytic activities of orange juice processing waste prior and after sonication.

	Endo-1,4-β-D-Glucanase Activity (U/g d.w.)	Exo-1,4-β-D-Glucanase Activity (U/g d.w.)	β-1,4-glucosidase Activity (U/g d.w.)
Sonicated waste	4.00 ± 0.11	2.60 ± 0.19	5.69 ± 0.23
Non-sonicated waste	3.71 ± 0.16	1.69 ± 0.16	3.93 ± 0.14
***p*-value**	0.200	0.022 *	0.003 **

Values are expressed as Mean ± SE; * and ** statistically significant for $p < 0.05$ and $p < 0.01$, respectively.

In comparison to previous studies, orange juice processing waste exerted among the highest cellulolytic activities reported in the literature for orange peel and pulp (Table 3). Dong et al. [33] estimated cellulase activity of 16.7 and 36.1 U/g f.w. in orange peels and pulp, respectively, which is approximately 38% and 34% lower and higher than the detected activity in the present research work (i.e., 26.9 U/g f.w.), respectively. In addition, the cellulase activities determined by Marín et al. [34] and Lei et al. [35] in orange peels and pulp respectively were 40-fold and 100-fold lower than the cellulase activity determined in the current study (Table 3).

Table 3. Cellulase activities exerted by orange peel and pulp.

Substrate	Cellulase Activity	Reference
Orange pulp Orange peel	6.5 mg/(g.min) or 36.1 U/g f.w. 3 mg/(g.min) or 16.7 U/g f.w.	[33]
Orange peel (mixed with compost and bulking agent)	7.8 mg/(g.h) or 0.1 U/g d.w.	[34]
Orange pulp	0.29 U/g f.w.	[35]
Orange juice processing waste (orange peel)	4.0 U/g d.w. or 26.9 U/g f.w.	Present study

U: μmoles glucose released/min.

None of the microbial strains growing in CMC liquid medium exhibited intracellularly any of the cellulolytic activities examined. The selected cellulolytic isolates showed a narrow range of extracellular endo-1,4-β-D-glucanase activities, fluctuating from 2.82 to 3.89 U/mg protein and from 1.52 to 3.03 U/L (Table 4). The highest endo-1,4-β-D-glucanase activity was expressed by the isolate CEL-11 (3.89 U/mg protein), whereas the respective lowest activities were exerted by the isolates CEL-3 and CEL-8 (2.87 and 2.82 U/mg protein, respectively), when the activity values were estimated as U/mg protein.

Table 4. Cellulolytic activities of cellulose-degrading microbial isolates obtained from orange juice processing waste.

	Endo-1,4-β-D-Glucanase Activity		Exo-1,4-β-D-Glucanase Activity		β-1,4-Glucosidase Activity	
Isolate	**U/mg protein**	**U/L broth**	**U/mg protein**	**U/L broth**	**U/mg protein**	**U/L broth**
CEL-1	3.52 ± 0.05 (b)	1.52 ± 0.02 (a)	4.42 ± 0.11 (d)	5.58 ± 0.14 (d)	0.13 ± 0.02 (a)	0.17 ± 0.02 (a)
CEL-3	2.87 ± 0.06 (a)	2.02 ± 0.04 (b)	5.55 ± 0.09 (e)	8.38 ± 0.14 (f)	6.30 ± 0.20 (c)	9.51 ± 0.30 (b)
CEL-6	3.41 ± 0.10 (b)	1.65 ± 0.05 (a)	3.61 ± 0.07 (c)	4.77 ± 0.09 (c)	0.07 ± 0.03 (a)	0.10 ± 0.04 (a)
CEL-7	3.64 ± 0.07 (b)	2.84 ± 0.05 (c)	0.98 ± 0.04 (a)	1.30 ± 0.05 (b)	9.52 ± 2.40 (d)	12.55 ± 3.16 (b)
CEL-8	2.82 ± 0.10 (a)	2.09 ± 0.07 (b)	1.96 ± 0.26 (b)	0.29 ± 0.04 (a)	3.29 ± 0.08 (b)	0.49 ± 0.01 (a)
CEL-9	3.40 ± 0.10 (b)	3.03 ± 0.09 (d)	10.67 ± 0.14 (f)	17.70 ± 0.23 (g)	7.26 ± 0.24 (c)	12.04 ± 0.40 (b)
CEL-11	3.89 ± 0.08 (c)	2.84 ± 0.06 (c)	5.59 ± 0.08 (e)	6.20 ± 0.09 (e)	10.69 ± 0.04 (d)	11.86 ± 0.04 (b)

Values are expressed as Mean ± SE; Lack of letter in common within the same column denotes statistically significant differences at $p < 0.05$.

The seven selected microbial isolates exerted extracellular exo-1,4-β-D-glucanase activities, which ranged from 0.98 to 10.67 U/mg protein and from 0.29 to 17.70 U/L (Table 4). The highest exo-1,4-β-D-glucanase activity was exhibited by the isolate CEL-9 (10.67 U/mg protein whereas the lowest exo-1,4-β-D-glucanase activities were exhibited by the isolates CEL-7 and CEL-8 (0.98 and 1.96 U/mg protein, respectively).

The β-1,4-D-glucosidase activities of the selected microbial isolates ranged from 0.07 to 10.69 U/mg protein and from 0.10 to 12.55 U/L (Table 4). Among them, microbial strains CEL-7 and CEL-11 exhibited the highest β-1,4-D-glucosidase activities (9.52 and 10.69 U/mg protein, respectively), whereas CEL-1 and CEL-6 expressed the lowest activities (0.13 and 0.07 U/mg protein, respectively).

The selected microbial isolates exhibiting cellulolytic activity were phylogenetically characterized through partial small subunit ribosomal RNA gene sequencing. DNA from these isolates was amplified with primers targeting the 16S rRNA gene, indicating that the selected strains belong to bacteria. All selected isolates were placed within the spectrum of the genus *Paenibacillus*, although they were divided into two distinct operational taxonomic units (OTUs) (Figure 1). The most abundant out consisted of the isolates CEL-1, CEL-6, CEL-7, CEL-9, and CEL-11 (OTU#1), whereas OTU#2 was comprised of the isolates CEL-3 and CEL-8. Members of OTU#1 were identical to 16S rRNA gene (100% similarity) with the species *Paenibacillus xylanexedens*, *P. tundrae*, and *P. pabuli*, whereas OTU#2 isolates were associated with the species *Paenibacillus wynnii*, *P. odorifer*, and *P. donghaensis*, showing equal phylogenetic similarities of 99.8% (Figure 1).

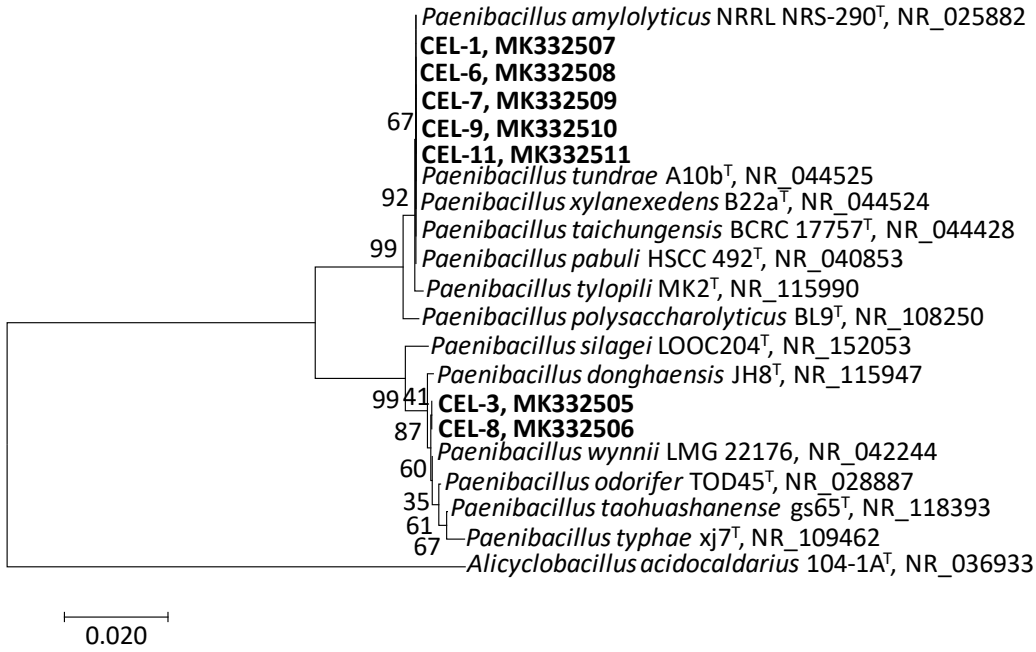

Figure 1. Phylogenetic position of cellulose-degrading bacteria isolated from orange juice processing waste.

The phylogenetic distinctness among the two OTUs is also reflected in the expression of endo-1,4-β-D-glucanase, where the respective activities of the isolates of OTU#2 were statistically lower than those of the members of OTU#1 (Table 4). However, intra-strain variation in the expression of endo-1,4-β-D-glucanase and β-1,4-D-glucosidase activities appeared to occur among members of each OTU (Table 4).

The cellulase activities determined in the current study were greater than those measured in allochthonous fungi, when expressed as U/mg. In particular, the endo-1,4-β-D-glucanase activities of the selected *Paenibacillus* species ranged within 2.82 to 3.89 U/mg, whereas the respective activities of *Emericella variecolor*, *Aspergillus niger*, *A. fumigatus*, *A. awamori*, *Fusarium moniliforme*, and *Rhizopus oryzae* strains fluctuated within 7×10^{-4} to 39.2×10^{-3} U/mg (Table 5). As expected, endo-1,4-β-D-glucanase activities were greater in allochthonous fungi when expressed as U/L (Table 5), since the *Paenibacillus* species of the present study were grown in CMC as the sole carbon and energy source in contrast to the fungal strains reported in Table 5, which were grown on the various carbonaceous substrates (apart from cellulose) of the orange peels and pulp.

Even though orange waste is considered to be a preferable substrate for solid state fermentation applications, attempts at isolating indigenous cellulolytic microbiota have been limited, probably due to the important fraction of sugar monomers and easily hydrolyzed oligomers (e.g., anthrone-reactive carbohydrates, Table 1), which are available to microorganisms, favoring the growth of non-cellulolytic microbial communities and restricting the isolation of the slow cellulase-producing microbiota. The implementation of the successive enrichment steps overcame this limitation, permitting the isolation of the most effective cellulose-degraders.

Thus, we can conclude that the high cellulase activities of *Paenibacillus* isolates selected in the current study demonstrate their biotechnological potential to serve as biocatalysts, whose application is not limited to juice clarification, but also in their valorization in solid state fermentation and energy recovery from cellulosic residues.

Table 5. Cellulase activities of microorganisms growing on orange peels, pulp and residues.

Cellulase-Producing Microorganism	Fermentative Substrate	Cellulase Activity	Isolation Source	Reference
Xanthomonas axonopodis pv. *citri* Xac 306	citrus pulp	0.12 U/mL	host: sweet orange	[9]
Emericella variecolor NS3	orange peel	31 U/g	rotten wood	[36]
Clonostachys byssicola RCFS6	orange peel	0.27 U/mL	soil	[37]
Aspergillus terreus MTCC 7600	orange peel	15.8 U/mL	soil	[38]
Aspergillus japonicus PJ01	orange peel	1.6 U/mL	soil	[39]
Aspergillus niger strain-*Trichoderma viridae* strain	orange peel	0.30 U/mL-0.62 U/mL	soil	[40]
Pycnoporus sanguineus CS2	orange peel	0.16 U/mL	oak forest	[41]
Pleurotus ostreatus GK10-*Fomes* sp. KA20-*Ganoderma* sp. GM 04	orange peel	22.1 U/mL-17.3 U/mL-5.9 U/mL	soil	[42]
Aspergillus niger P47C3-*Aspergilus fumigatus* P40M2	orange bagasse	39.2 U/g 39.0 U/g	soil	[43]
Aspergillus niger NS2-2	orange peel	1.0 U/g	agricultural residues	[44]
Aspergillus awamori 2B.361 U2/1	orange peel	2.8 U/g	not reported	[45]
Trichoderma longibrachiatum strain-*Aspergillus niger* strain-*Saccharomyces cerevisiae* strain	orange albedo, orange pulp & orange peel	1.8, 1.3 & 1.1 U/mL-1.9, 1.6 & 1.2 U/mL-1.6, 1.4 & 1.1 U/mL	rotten wood and palm wine	[46]
Fusarium moniliforme NCIM1276	wheat bran & orange pulp	27.5 U/g	soil	[47]
Rhizopus oryzae strain	orange peel	2.4 U/g	soil	[48]
Rhizopus oryzae NBRC 4707	orange peel	0.7 U/g	not reported	[49]
A. niger A-20-*A. oryzae* 1911-*Memnoniella* sp. 6-*P. chrysogenum* 3486-*P. oxalicum* 7	dried orange peels	1.3 U/mL-1.1 U/mL-3.4 U/mL-0.9 U/mL-2.4 U/mL	not reported	[50]

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

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