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Designing a Waste-Based Culture Medium for the Production of Plant Growth Promoting Microorganisms Based on Cladodes Juice from *Opuntia ficus-indica* Pruning

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Abstract: The production of beneficial microorganisms is the first step to obtain a commercial-based product for application in agriculture. In this study, prickly pear (*Opuntia ficus-indica*) pruning waste was evaluated as a raw material for the production of large amounts of Plant Growth Promoting Microorganisms (PGPMs) reducing the number of generated wastes. Specifically, five PGPMs constituting a synthetic microbial consortium with complementing plant growth-promoting traits were grown on a laboratory scale and, subsequently, on a pilot scale using a 21-L bioreactor. Primarily, the physical-chemical characterization of the culture medium obtained from the juice of *Opuntia* cladodes was carried out, revealing the presence of sugars and organic acids with different molar ratios. Compared to conventional media, the waste medium did not show significant differences in bacterial growth efficiency. Instead, the survival rates of the bacteria grown in cladodes juice media, after air-drying on zeolite or freeze-drying, were significantly higher than those observed when they were grown in conventional media. The present work is the first conducted on a pilot-scale that maximizes the production of PGPMs in submerged fermentation using cladodes juice from *Opuntia*, reducing both economic and environmental impacts associated with the generation of wastes.

Keywords: *Opuntia* pruning wastes; cladodes juice; plant growth-promoting microorganisms; biofertilizers; microbial biomass

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1. Introduction

Increasing crop production without the use of environmentally harmful products as chemical fertilizers is a major challenge of the 21st century [1,2]. Among the strategies adopted to achieve this goal, the application of microbial biofertilizers represents a key driver for sustainable crop production, fostering the reduction of chemical pesticides and synthetic fertilizers. Microbial biofertilizers are formulations based on specific beneficial microorganisms such as single-strain inoculants or consortium products with plant growth-promoting (PGP) potential, which can improve soil fertility and crop productivity [3–5]. These microorganisms can exert their PGP functions through several mechanisms of action, such as fixing the atmospheric nitrogen, solubilizing soil phosphorus and potassium, stimulating plant growth through the synthesis of growth-promoting substances, and inhibiting the functioning of plant pathogens by stimulation of the plant's defense mechanisms or by their antagonistic suppression [6–8]. The development and production of a microbial biofertilizer include studies at different levels, from the screening and identification of the

beneficial microorganisms to the production processes of microbial biomass in bioreactors and stabilization and application techniques [9,10].

The global biofertilizer market is expected to grow and reach USD 2305.5 million by 2022. To respond to this market demand, the production of biofertilizers must be more economically and ecologically advantageous than chemical ones. In this context, even more cost-effective processes need to be developed. Generally, in the liquid fermentation process, the microbial biomass is cultured using synthetic media that represent the major cost in biofertilizer production, accounting for about 60–70% of the overall costs in fermentation processes [11,12]. Therefore, carbon substrates derived from waste are now being explored to make this process both cost-effective and environmentally friendly. This approach reduces the financial costs of the production and, at the same time, it is also an efficient step on the way towards the recycling process and sustainable use of resources from a circular economy perspective [13]. In fact, to achieve the Sustainable Development Goals (SDGs), the management and valorization of agricultural wastes also represent a fundamental step [14].

Particularly interesting are the juices and the extracts from suitable plant materials, as they contain all the necessary nutrients and growth factors for microorganisms, such as amino-acids, vitamins, and minerals. In fact, some growth substrates have been formulated using only vegetal materials to better reproduce the natural environmental conditions and to improve the in situ recovery, from the roots of plants and from soil, of microorganisms that cannot be cultured by traditional techniques, instead favoring in vitro growth and biomass production [15–18]. Furthermore, as reported by Mourad et al. [19], some *Rhizobacteria* can be cultured in plant-based liquid media with a growth rate, doubling time and cell biomass production compared to those observed using conventional synthetic media. In particular, they tested different waste plant materials including mowed turfgrass, berseem clover plants and cladodes of cactus prickly pears (*Opuntia ficus-indica*) [19].

O. ficus-indica already represents an attractive alternative feedstock for biofuels and other bioproduct production [20–24] due to its special adaptive mechanisms, which allow it to grow in particularly adverse conditions (such as high temperatures and poor-quality soils) with low water demand and high biomass productivity. *O. ficus-indica*, also known as prickly pear, is a succulent perennial shrub or tree ranging from 1.7–3 m in height with a lignified, well-defined, primary stem and with flattened green branches called cladodes, which are 30–60 cm long and 6–15 cm wide [25]. It is grown worldwide as a fruit crop and it is becoming more and more popular among consumers for the health benefits offered by its high content of polyphenols and antioxidant, anti-inflammatory, and anxiolytic compounds [22]. The chemical composition and nutritional contents of the *O. ficus-indica*, both cladodes and fruits, are already known and available in the literature [26]. Perucini-Avendaño et al. [27] reported that the main constituents of the cladodes of *O. ficus-indica* are carbohydrates and proteins which, in the percentage of dry weight, vary between 38.0–61.4% and 6.7–11.73%, respectively. Among the carbohydrates, the non-structural ones, such as soluble fibers (mucilage) and low-molecular-weight carbohydrates (monosaccharide), are reported to be very abundant. In particular, the mucilage fraction constitutes about 14% of the cladode dry weight [28]. Andreu et al. [29] observed that cladodes also present high contents of citric, malic, and succinic acids in a ratio that may vary according to the cladode age. Furthermore, if compared to other woody feedstocks, *Opuntia* cladodes show a lower lignin content. This characteristic allows the avoidance or reduction of some biomass pre-treatments, such as chemical and enzymatic hydrolysis processes [30].

All these properties make *O. ficus-indica* a potential cost-effective raw material to formulate plant-based culture media for microbial biomass production at the industrial level. Notably, in specialized *O. ficus-indica* crops, to facilitate orchard practices and to improve fruit quality, it is necessary to periodically reduce the density of the vegetation through an important pruning operation [31,32]. Considering that the *Opuntia* wastes, including cladodes and immature fruits, range from 6 to 8 tons/hectares per year [33], their

utilization in biobased culture media production may be an interesting way to recycle them and reduce production costs.

Within the frame of the Horizon 2020 SIMBA project (Sustainable Innovation of Microbiome Applications in the Food Systems), we aimed to evaluate the potential use of *O. ficus-indica* juice from pruning wastes as a cheap medium for the production of microbial bio-fertilizers on a large scale. In previous works, new and multifunctional microbial consortia were developed for use as inoculants to improve plant growth in greenhouses and fields [6,34]. Here, five PGPMs constituting the multifunctional microbial consortium C, and belonging to the species *Azotobacter chroococcum*, *Bacillus amyloliquefaciens*, *Burkholderia ambifaria*, *Pseudomonas fluorescens,* and *Rahnella aquatilis,* were used. Our final goal is to optimize and scale up the fermentation processes of microbial consortia at an industrially relevant scale for product generation. To this end, during the first screening, all the strains were grown on a lab-scale; afterwards, in an optic of industrial production, we scaled up the process on a pilot scale using a 21-L bioreactor. For each strain, we monitored the microbial growth and evaluated the survival rate after freeze-drying and air-drying on zeolite, as a carrier for in-field application. Furthermore, the chemical composition of the most important sugars and organic acids present in cladode juice was determined.

2. Materials and Methods

2.1. Microorganisms

The bacteria used in the present study were *A. chroococcum* LS132, *B. amyloliquefaciens* LMG 9814, *B. ambifaria* MCI 7, *P. fluorescens* DR54 and *R. aquatilis* BB23/T4d. These strains were characterized by different specific functions (i.e., nitrogen fixation, biocontrol, amylolytic activity, auxin production) [6]. They were kindly provided by Agriges srl, LMG Bacteria Collection, University of Copenhagen (UCPH) andENEA microbial collection. Bacterial cultures were cryopreserved at −80° in 30% glycerol at ENEA Casaccia Research Center.

2.2. Plant Material and Juice Extraction

Cladodes of *Opuntia ficus-indica* (L.) Mill. were collected from wild orchards at the ENEA Research Centre Trisaia, in Basilicata, a region of southern Italy. Two- to three-year-old cladodes were collected in October from plants belonging to the cultivar *Muscaredda*. After collection, the cladodes were washed by dipping them in a tank filled with fresh water to eliminate impurities and then were cut transversely every 2–3 cm with a sharp knife. Subsequently, the shredded cladodes were homogenized for a few minutes in a professional cutter-mixer (Malavasi®, model P7002N) until a mush was obtained. This was then filter-pressed using a 50-liter traditional mesh basket press, and the juice obtained was further filtered by a nylon filter bag with pore sizes of 100 µm and immediately cryo-stored at −20 °C. Aliquots were used to determine physical-chemical parameters. The extraction was carried out in two stages, and approximately 40 kg of cladodes were used for each extraction.

2.3. Determination of Yield, pH, Total Soluble Solids, Density and Dry-Weight Matter on Juice

The juice obtained was gravimetrically determined and the yield, expressed as kg of juice for kg of cladodes, was calculated. The pH of the cladode juice was measured at 25 °C using a calibrated pH meter (Hanna Instruments, Halo® Wireless pH Meters). Brix measurement was performed by refractive index with a digital refractometer (Hanna Instruments, HI 96801) and juice density was estimated by a glass pycnometer method. Furthermore, 10 g aliquots of juice were placed in a Petri dish and dried by lyophilization. The dry matter was then determined, and the results were expressed as a percentage of the fresh juice weight. All measurements were replicated three times.

2.4. Determination of Sugars and Organic Acids in Juice

For organic acid and sugar quantification, an Agilent 1200 series high-performance liquid chromatography (HPLC) instrument (Agilent Technologies) consisting of an in-line degasser (G1379B), binary pump (G1312B), auto-sampler (G1367B), column temperature controller (G1316A), UV-Vis detector (G1314B), and Refractive Index Detector (RID), was used. Before analysis, a 1 mL juice sample was diluted with 3 mL of a 0.050 M phosphate buffer at pH 7.0 and centrifuged at 13,000× *g* for 5 min. Then, 1 mL of supernatant was filtered through a 0.22 µm Millipore filter into 2 mL vials. The analyses were performed with an Agilent Hi-Plex H analytical column (7.7 × 300 mm, 8 µm) using a 0.005 M sulphuric acid solution as a mobile phase under isocratic conditions, at a flow rate of 0.7 mL/min. For organic acids, the detection wavelength was set at 210 nm, while the refractive index detector (RID) was used to detect the sugars. The injection volume was 20 µL, and the column temperature was maintained at 60 °C. All data were collected and analyzed using the software OpenLAB CDS Chemstation Edition Rev. C.01.10(201).

The HPLC sample peaks were identified by comparing their retention times with those of external standards. In particular, arabinose, xylose, glucose, fructose, sucrose, and maltose were used as sugar standards, while, as organic acids, oxalic, citric, malic, succinic, lactic, acetic, and tartaric acids were used. For each standard, the calibration curve was calculated over at least 3 concentrations (between 0.05 and 10 g L^{-1}), and each of them showed good linearity ($R^2 \geq 0.9999$). The analyses were carried out in three replicates and the results were expressed in grams per liter as mean ± standard deviations.

2.5. Shaken Flasks Cultivation of Five Microbial Strains on Different Dilution of Cladode Juice and Conventional Media

A loop of each bacterial strain (*A. chroococcum* LS132, *B. amyloliquefaciens* LMG 9814, *B. ambifaria* MCI 7, *R. aquatilis* BB23/T4d, and *P. fluorescens* DR54) was picked up from glycerol stocks and streaked onto nutrient agar (NA, Sigma-Aldrich, Italy) plates. Strains were grown at 26 °C for 24–48 h.

Bacterial strains were singly cultured on three dilutions with a 5 M phosphate buffer to achieve the following decreasing concentrations: 50%, 25%, and 15% (*v/v*). The pH value of these dilutions was adjusted at 7.0 by the addition of 4 M Sodium Hydroxide. Then, 20 mL were transferred in to 100 mL flasks and sterilized at 121 °C for 15 min. As a control, a standard liquid culture medium, nutrient broth (NB, Sigma-Aldrich, Italy) was used. The flasks were inoculated with each bacterial strain by adding 100 µL of overnight cultures and incubated at 26 °C and 130 rpm for 24 h in a thermostatic orbital shaker (Thermo Scientific Forma, model 420). Periodic samples from the resulting batch cultures were aseptically withdrawn from the flasks, and decimal dilutions with NaCl 0.85% were surface inoculated on agar plates to determine the Colony Forming Units (CFU) per milliliter. The experiment was carried out twice and three replicates were performed.

2.6. Production of Microbial Biomass in Bioreactor

To scale up the production process of microbial bio-fertilizers, each bacterial strain was grown in submerged cultures in a 21-L stirred-tank bioreactor (B. Braun Biotech International, Germany). In order to prepare the appropriate culture media, based on the bacterial growth observed for each strain during the culture in agitated flasks, cladode juice was diluted with a 0.05 M phosphate buffer (pH 7.0) to a concentration of 15% (*v/v*). In addition, the composition of the culture media was further optimized by adding 0.2% commercial sucrose as a low-cost supplement. The working volume used was 10 L and the medium was sterilized in the bioreactor at 121 °C for 15 min with gentle stirring. Each bacterial strain was also grown in a synthetic medium (NB) for the comparison of biomass yield in terms of survival cells.

Once the bioreactor was sterilized, 200 mL of starter culture, previously grown in shaken flasks, was inoculated, resulting in an initial bacterial density of 10^6–10^7 cells mL^{-1}. The pH, pO2, foam production, stirrer speed, temperature, and air-flow rate were controlled

by a bioprocess control unit (B. Braun Biotech International, Biostat® C) equipped with a gas mixing module during the process. For all five bacteria, the fermentation was performed at pH 7.0 and 26 °C. The pH was controlled by adding 2 M sodium hydroxide and 5% sulphuric acid solutions. The airflow was kept constant at 2.0 L min^{-1}. The foam level was controlled by adding the anti-foaming agent Anti-foam A (Sigma-Aldrich, Italy). Dissolved oxygen (DO) was set up at 20% of saturation by a cascade controller, first varying the rotation speed of the stirrer, between a minimum of 100 rpm and a maximum of 200 rpm, then, after a delay time of 5 min, if the rotation alone could not restore the set parameter, with the intervention of a second cascade controller that introduced O_2 pulses into the airflow. After 36 h, aliquots of the liquid culture were used to determine the viable cells and to quantify sugars and organic acids. Therefore, the liquid culture was split into two parts and the microbial biomass was dried using two alternating methods: air-drying on solid substrates (zeolite) and lyophilization.

2.7. Drying Process of Microbial Biomass and Evaluation of Viable Cells

For each bacterial strain, grown on both cladode juice and NB, the microbial biomass was dehydrated by air-drying on zeolite and by freeze-drying, and the viability was determined. For desiccation on a solid support, micronized zeolite was used (Vebi s.r.l.), previously sterilized at 121 °C for 20 min. In particular, in 50 mL sterile tubes, 1 mL of liquid culture was added to 5 g of zeolite and manually shaken until a homogenized powder was obtained. After 24 h, to verify the viability of the dried microbial biomass, the powder was suspended in sterile flasks at 1 g 20 mL^{-1} ratio using a phosphate buffer saline (PBS–pH 7; 0.05 M; 0.85% NaCl) and left in agitation for 3 h in the orbital shaker before preparing the decimal dilutions and plating them on agar dishes. For each bacterial strain, the percentage of viability was calculated using the following equation:

$$\text{viability (\%)} = (\text{Ng}/\text{Ne}) \times 100 \quad (1)$$

where:

(Ng) was the number of viable cells determined in 1 g of product after 24 h from the drying process;
(Ne) was the number of theoretical viable cells in the product, obtained by dividing the number of CFU present in 1 mL of liquid culture by the final weight of the product (in this case, 5 g zeolite + 1 mL liquid culture).

For the freeze-drying process, the liquid culture was centrifuged at 15,000× *g* for 10 min, and the recovered microbial biomass was suspended in a pasteurized solution consisting of skimmed milk and sodium glutamate (10% and 1%, respectively). Then, 25 mL of the microbial suspension was dispensed in sterile bottles and immediately frozen and freeze-dried using a pilot lyophilizer (Christ, Loc-1M). Further, 1 mL aliquots of bacterial cell suspension were used to check the number of CFU mL^{-1} before and after the freeze-drying process. For each bacterial strain, the percentage of viability was calculated using the following equation:

$$\text{viability (\%)} = (\text{N}/\text{N0}) \times 100 \quad (2)$$

where:

(N0) and (N) were the number of viable cells from the vials before and after lyophilization, respectively.

2.8. Statistical Analysis

All CFU measurements, the shake flask cultures, and the drying process of microbial biomass were made at least in triplicate. For each bacterial strain, data were verified for homogeneity of variance and normal distribution. The means were separated by Tukey's HSD test when the analysis of variance showed Statistical significance ($\alpha = 0.05$).

3. Results and Discussion

3.1. Determination of Yield, pH, Total Soluble Solids, Density and Dry-Weight Matter on Juice

In Table 1, the yield and the physical-chemical proprieties of the juice extracted from the cladodes are reported. In particular, the yield was close to 20.2%, in accordance with data obtained by Boutakiout et al. [35], who carried out the extraction of the juice from cladodes by centrifugation, obtaining a yield between 20.83% and 51.96% on the basis of the harvest season. Specifically, cladodes harvested in August had the lowest yield, while those harvested in March had the highest. The observed difference was attributed to the water content of cladodes, which is strongly influenced by seasonality. In this work, a pH value of 4.3 ± 0.1 and a density of 1.03 g cm^{-3} in juice samples were determined. These values are similar to those reported by Procacci et al. [33] and are respectively due to the high percentage of organic acids, such as malic and citric acids, and the high water content, which characterize the species [29]. In addition, the content of total soluble solids (TSS), determined as °Brix, was 5.9 ± 0.2 and the dry mass after the lyophilization process of cladode juice was 6.83%. These results are consistent with the data reported by other authors in their independent studies [35,36].

Table 1. Yield and physical-chemical properties of cladodes juice.

	Mean Value ± SD
Yield (%)	20.2 ± 1.20
pH	4.3 ± 0.1
Density (g·cm^{-3})	1.03 ± 0.01
TSS as °Brix	5.9 ± 0.2
Dry mass (% fw)	6.83 ± 0.3

3.2. Sugars and Organic Acids Analyses of Cladodes Juice

The HPLC analysis of cladode juice, as shown in Table 2, revealed the presence of different simple sugars and organic acids. Among the sugars, glucose was the most abundant, detected with an amount of 8.82 g L^{-1}, followed in decreasing order by fructose (5.79 g L^{-1}), sucrose (2.38 g L^{-1}), maltose (1.08 g L^{-1}), and arabinose, with the latter observed only in traces. Among the organic acids, the predominant ones were citric acid (8.9 g L^{-1}), malic acid (5.8 g L^{-1}), and succinic acid (1.26 g L^{-1}). Although in lower amounts (0.13 g L^{-1}), the oxalic acid was also systematically detected, while Acetic acid was determined in traces only in a few samples. Furthermore, as can be observed from the chromatogram (Figure 1), there were other unidentified peaks that could be associated with undefined organic acids. These sugar and organic acid profiles, although with different molar ratios, were supported by data from other authors [28,30]. In particular, Yang et al. [30] found that the amounts of malic acid, citric acid, glucose, and fructose in samples from the juice of cladodes of *O. ficus-indica* were 10.9, 3.9, 1.0, and 1.6 g L^{-1}, respectively. Indeed, it is known that the chemical composition of *O. ficus-indica* can be affected by several factors, such as the seasonality and age of the plants.

Furthermore, especially in the cladodes, the organic acid content can change as a result of crassulacean acid metabolism (CAM metabolism), which characterizes the *Opuntia* species [29]. The sugar and acid content of *O. ficus-indica* suggest a potential for on-site composting of biomass to be utilized as an organic fertilizer or soil amendment [37], thereby reducing the use of chemical fertilizers.

Table 2. Sugars and organic acids determined by HPLC analyses in cladode juice of *O. ficus-indica.*

	RT (min)	Detector	Concentration (g L^{-1}) Mean ± SD
Sugars			
Maltose	7.405	RID	1.08 ± 0.28
Sucrose	7.495	RID	2.38 ± 0.58
Glucose	8.555	RID	8.82 ± 0.60
Fructose	9.285	RID	5.79 ± 1.28
Arabinose	9.785	RID	traces
Organic Acids			
Oxalic acid	6.362	UV 210 nm	0.13 ± 0.2
Citric acid	7.763	UV 210 nm	8.90 ± 0.93
Tartaric acid	7.951	UV 210 nm	n.d
Malic acid	8.990	UV 210 nm	5.80 ± 0.59
Succinic acid	10.988	UV 210 nm	1.26 ± 0.16
Acetic acid	13.79	UV 210 nm	traces

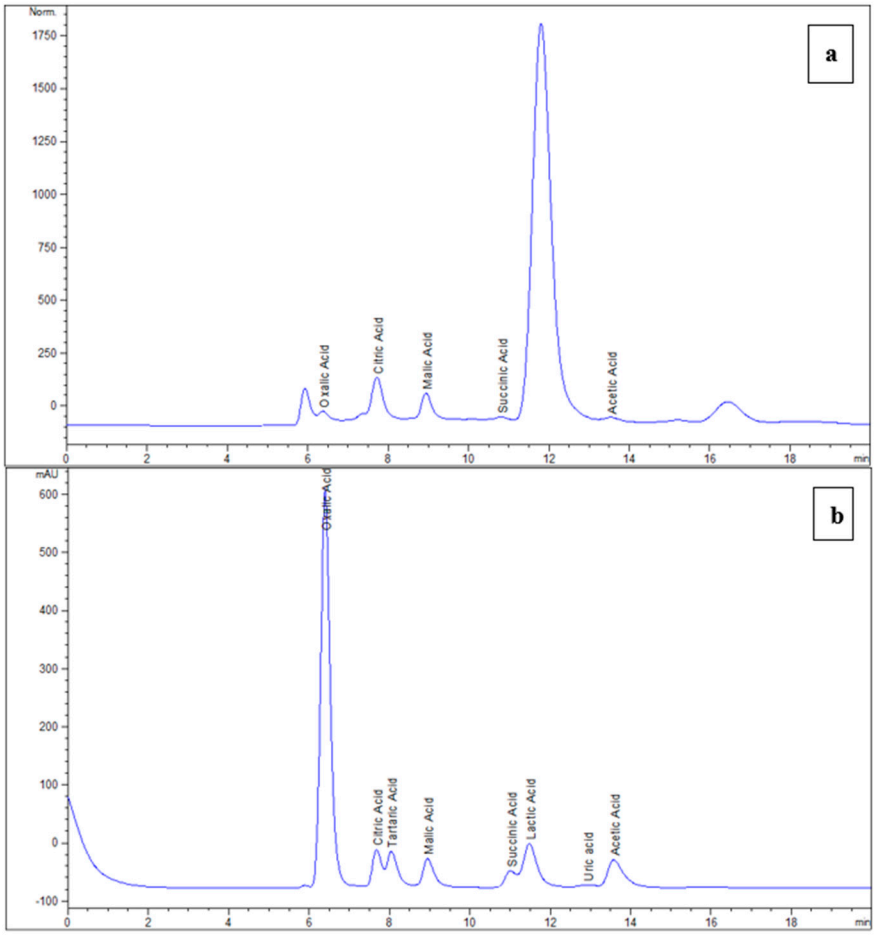

Figure 1. (**a**) HPLC chromatogram for organic acids of cladode juice (15% *v/v*) at 210 nm; (**b**) HPLC chromatogram of organic acid standards mix.

3.3. Shaken Flasks Cultivation of Five Microbial Strains on Different Dilution of Cladodes Juice and Conventional Media

When grown in liquid shaken flasks, all bacteria exhibited excellent growth in culture media prepared with different dilutions of *O. ficus-indica* juice and, at 16–18 h of incubation, a cell number in the range of 7.85–9.45 log units was achieved (Table 3).

Table 3. Determination of viable cells (Log CFU mL^{-1}**)** after 16–18 h of bacterial culture on nutrient broth and three different cladodes juice concentrations in shaken flasks.

Bacterial Strains	Nutrient Broth	Cladodes Juice Concentration		
		15 (%)	25 (%)	50 (%)
A. chrococcum LS 132	9.26 ± 0.08 a *	8.86 ± 0.05 b	9.24 ± 0.02 a	9.32 ± 0.04 a
B. ambifaria MCI 7	9.45 ± 0.15 a	9.04 ± 0.03 b	9.28 ± 0.04 a	9.4 ± 0.05 a
B. amyloliquefaciens LMG 9814	8.26 ± 0.04 a	7.85 ± 0.11 b	8.2± 0.01 a	8.3 ± 0.01 a
P. fluorescens DR54	9.4 ± 0.06 a	8.69 ± 0.01 b	8.86 ± 0.07 b	9.2 ± 0.02 a
R. aquatilis BB23/T4d	9.18 ± 0.06 a	8.61 ± 0.08 b	9.23 ± 0.05 a	9.43 ± 0.02 a

* Data are shown as means ± standard deviation. Within each row, mean values followed by the same letter are not significantly different according to Tukey's test (α = 0.05).

In particular, at the same culturing time, significant differences in growth among the juice concentrations of 50% and 25%, and the synthetic standard media (NB) for all bacteria strains were not observed. The only exception was found for *P. fluorescens* DR54 at a concentration of 25%, which showed the lowest value of CFU log (8.86). Our results confirmed the data previously observed by Youssef et al. [15] who, in liquid batch cultures, for the growth of different *Rhizobacteria*, used a slurry homogenate of *O. ficus-indica* (diluted 1:20 with distillate water). Indeed, the authors found results of cell growth and biomass production similarly to those reported using the synthetic culture media.

Instead, at a concentration of 15%, - less bacterial growth was achieved, probably due to the high dilution grade. In fact, as observed by the HPLC analysis (data not shown), at 16 h of culture, glucose, fructose, and other simple sugars in liquid media were completely assimilated only at a concentration of 15%. However, a high number of cells was achieved—specifically, 7.85, 8.69, 9.04, 8.86, 8.61 log CFU mL^{-1} for *B. amyloliquefaciens* LMG 9814, *P. fluorescens* DR54, *B. ambifaria* MCI7, *A. chrococcum* LS 132, and *R. aquatilis* BB23/T4d, respectively.

Our results support the findings of Mourad et al. [19], who stated that plant-only-based culture media are sufficient and efficient for biomass production of rhizobacteria. In particular, the authors investigated the cell growth of three *Rhizobacteria* (*Klebsiella oxytoca*, *Enterobacter agglomerans,* and *Azospirillum brasilense*) in liquid batch cultures prepared from slurries and/or powders of clover, cactus, and turfgrass.

3.4. Production of Microbial Biomass in Bioreactor

For each bacteria strain, the scale-up process was carried out in a 21 L stirred-tank bioreactor using cladode juice in order to optimize the medium composition, which was recently defined by Vassileva et al. [38] as one of the crucial steps in achieving high biomass production of plant beneficial microorganisms. As shown in Table 4, the growth in the conventional medium is the same as that observed in flask cultivation, while for cladode juice, it was clearly highlighted as the fermentation process performed in the bioreactor, and the addition of 0.2% sucrose improved the growth of all bacterial strains. After 36 h of fermentation, differences between the log of viable cells determined in cladode juice and conventional medium (NB) were not observed. In fact, the cell number determined was in the range of 7.91–9.08 and 8.08–9.35 log unit mL^{-1} in the cladode juice and NB, respectively. This result demonstrates that cladode juice is a viable alternative to the conventional medium. Nowadays, several efforts are being made with the aim of developing suitable

economic growing media for the production of biofertilizers. In some cases, the use of agro-industrial wastes has been investigated both in the laboratory and on a large scale [39–41]. These residues include multiple plant-based materials whose utilization has become a central issue in recent years, e.g., for energy generation [42], for the green synthesis of silver nanoparticles [43], as a substrate for the production of microbial pigments [44] as well as for new materials, chemicals, and valuable products [45].

Table 4. Number of viable cells determined at the stationary phase of batch culture performed in 15 L bioreactor.

Bacterial Strains	Nutrient Broth (Log CFU mL^{-1})	Cladodes Juice 15% + Sucrose 0.2% (Log CFU mL^{-1})
A. chroococcum LS132	9.12 ± 0.01 a *	9.03 ± 0.01 a
B. ambifaria MCI7	9.08 ± 0.03 a	8.98 ± 0.04 a
B. amyloliquefaciens LMG 9814	8.08 ± 0.05 a	7.91 ± 0.06 a
P. fluorescens DR54	9.00 ± 0.02 a	8.96 ± 0.03 a
R. aquatilis BB23/T4d	9.35 ± 0.01 a	9.08 ± 0.02 a

* Data are shown as means ± standard deviation. Within each row, mean values followed by the same letter are not significantly different according to Tukey's test (α = 0.05).

To the best of our knowledge, in the scientific literature the data concerning the optimization of low-cost media for the PGPMs are limited and suitable for only a few genera. In a very recent study, Cantabella et al. [46] used different industry wastes to develop a low-cost medium for the cultivation of *Pseudomonas oryzihabitans* PGP01. These authors reported that in a 2 L bioreactor with a medium based on frozen potato peels and pulps, supplemented with 10 g L^{-1} of tryptone, a concentration of 9.56 log CFU mL^{-1} was achieved. Pastor-Bueis et al. [47] reported that anaerobic digestate (AD) at 50% dilution, supplemented with 2.3% sugar beet molasses, was the optimum growth medium to produce the *Bacillus siamensis* at a bacterial concentration of 9 log CFU mL^{-1}. Furthermore, Damir et al. [48] and Mukhtar et al. [49] investigated, in two independent studies, the growth of *Azotobacter chroccocum* and *Azotobacter* spp., respectively, using the basal medium with the supplement of different carbon sources. In both of these studies, the authors concluded that to obtain appropriate culturing media for the growth of *Azotobacter* strains a supplement of 2–2.5% of sugar (sucrose and mannitol) is needed.

Furthermore, for all bacterial strains, an apparent cell size difference between the cells obtained with the two different media by examination under an optical microscope at 100× magnification was observed. In our study, the bacterial cells grown in the cladode juice medium appeared smaller than those grown in NB; for *B. amyloliquefaciens* LMG 9814, particularly interesting was the presence of spores in the cladode juice medium, which were not checked at the same culturing time by examination under a phase-contrast microscope on the conventional substrate.

3.5. Drying Processes of Microbial Biomass and Evaluation of Viable Cells

The viability of the bacteria grown on two culture media (cladode juice and NB) after different drying processes was evaluated in order to investigate the potential production of powder formulations for subsequent application in the field. As shown in Figures 2 and 3, a different survival rate was observed among the different bacterial species. *B. amyloliquefaciens* LMG9814 reached the maximum survival rate, ranging from 14.13% to 98%. Conversely, *P. fluorescens* DR54 and *B. ambifaria* MCI7 showed the lowest survival rate and, when grown on nutrient broth, viability after air-drying on zeolite was less than 0.01%. The cell survival rate of *A. chroococcum* LS132 and *R. aquatilis* BB23/T4d ranged from 2.69% to 35.12%.

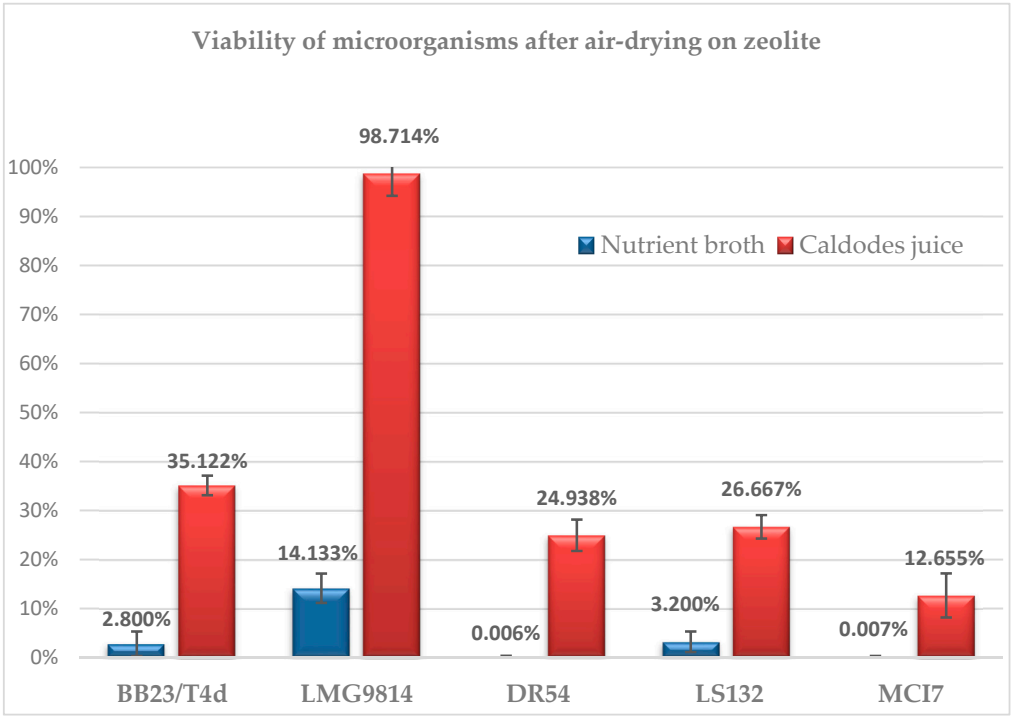

Figure 2. Percentage of survival of microorganisms grown in cladodes juice and NB after air-drying on zeolite. Strains: *R. aquatilis* BB23/T4d, *B. amyloliquefaciens* LMG 9814, *P. fluorescens* DR54, *A. chroococcum* LS132, and *B. ambifaria* MCI 7.

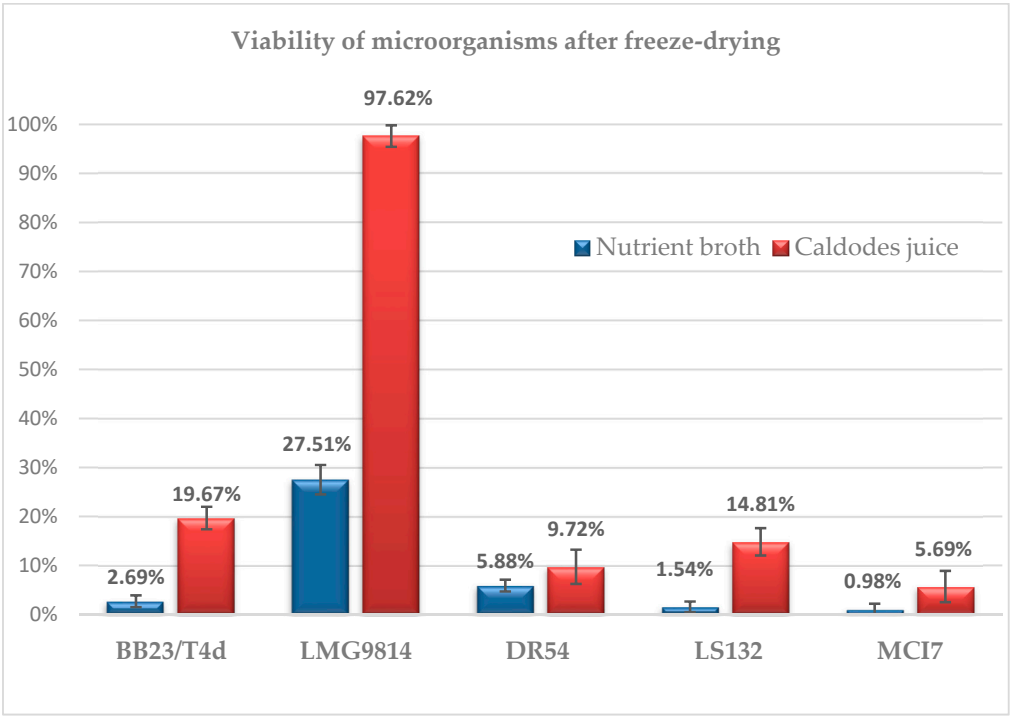

Figure 3. Percentage of survival of microorganisms grown on cladodes juice and Nutrient Broth after freeze-drying. Strains: *R. aquatilis BB23/T4d, B. amyloliquefaciens LMG 9814, P. fluorescens DR54, A. chroococcum* LS132, and *B. ambifaria* MCI 7.

In both processes, air-drying on zeolite and freeze-drying, the viability was systematically higher when grown in cladode juice rather than in NB for all bacterial strains. In particular, the growth of *B. amyloliquefaciens* LMG9814 in cladode juice was not affected

by drying processes, showing, in any case, a survival rate close to 100%, as a possible consequence of the abundant presence of the observed spores. Instead, the survival rate of NB was 14.13% and 27.51% after air-drying and freeze-drying, respectively. However, other bacterial strains, when grown in cladode juice, also showed viability rates of at least one order of magnitude higher than when grown in NB.

Overall, it is worth noting the relevant difference in the survival rates observed for *P. fluorescens* and *B. ambifaria* when they were air-dried with zeolite. In this case, when the two strains were grown on cladode juice, they showed a viability rate of four orders of magnitude higher than that found when grown in Nutrient Broth. Therefore, we can assume that performing the bacterial growth in the cladode juice medium had a positive effect on the survival of bacteria during both drying processes.

This result might be due to different physiological and morphological characteristics of the bacterial cells in the two different culture media (cladode juice and NB). Similarly, Zhang et al. [50], observing in *Pseudoalteromonas nigrifaciens* a greater survival rate in the freeze-drying during the stationary phase compared to that in the logarithmic phase, attributed the result to the different morphological and physiological state of cells during the two phases. It is possible that in cladode juice the bacteria were grown under biophysical and metabolic stresses due to the nutritional complexity of the carbon sources, so they developed a greater resilience compared to that observed when they grew in a rich substrate such as NB.

On the other hand, it has been reported that microorganisms adopt numerous strategies to respond to stress conditions, including changes in the cell envelope (plasma membranes and cell wall), production of extracellular polymeric substances (EPS), spore and cyst formation, and much more [51–53]. Additionally, Hallsworth [54] stated that vitality, vigor, and resilience tend to increase in moderately stressed cells.

4. Conclusions

In order to boost the use of microbial biofertilizers on a large-scale, their production needs to be more economically and ecologically advantageous. Therefore, identifying low-cost growth substrates, obtained from waste materials, in the context of a circular economy, is a priority objective for sustainable agriculture. The results obtained in this study respond adequately to these needs, proposing pruning waste from *O. ficus-indica* crops as a potential raw material for the production of microbial biofertilizers. Specifically, five PGPMs belonging to different genera were effectively grown in a low-cost substrate based on cladode juice, obtaining concentrations comparable to those obtained with conventional substrates. *O. ficus-indica* represents a versatile substrate that, properly optimized, could allow the growth of different types of bacteria. Another interesting result concerns the increased viability of microorganisms grown in cladode juice after drying processes which, in the case of *B. amyloliquefaciens* LMG9814, can be correlated with the abundant and early sporulation. Generally, as the drying procedures are the most critical steps that influence the viability of microbial cells [55], an appropriate culture medium that can increase the viability of both non-sporulating and sporulating bacteria will render formulation development more efficient. In the case of air drying on zeolite, we conclude that from 1 Kg of cladodes, 10^{11}–10^{12} CFU of bacterial strains (depending on the microorganism used) are obtained, which are the quantities normally applied per hectare of cereal crops [56]. Further investigations are needed to assess whether the increased viability observed in cladode juice also results in increased effectiveness in field trials. This work is the first pilot-scale study and represents the first step toward the large-scale use of *Opuntia* waste in the production of cheap and environmentally friendly microbial biofertilizers.

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