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Optimization of a food industry-waste-based medium for the production of the plant growth promoting microorganism *Pseudomonas oryzihabitans* PGP01 based on agro-food industries by-products

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ABSTRACT

In this study, three wastes based on potato peels and pulps, tomato seeds and wheat bran were used as basis for the preparation of a cheap medium to produce the bacterium *P. oryzihabitans* PGP01. In flasks experiments, *P. oryzihabitans* PGP01 growth at 25 °C in a medium based on frozen potato peels and pulp (FPP) with tryptone as a nitrogen source resulted in the maximum production compared to the commercial TSB medium. In the scale-up to 2 L bioreactors, FPP supplemented with tryptone, molasses, NaCl and K_2HPO_4 allowed to reach similar biomass production than in the TSB medium. A maximum growth of 4.4×10^9 CFU mL^{-1} after setting the agitation and the air flux conditions at 400 rpm and 0.75 vvm. Finally, *P. oryzihabitans* PGP01 growing in this optimized medium conserved its biological activity showing the expected effect in root development previously reported for this microorganism.

1. Introduction

From the beginning of the present century, the considerable increase in the amount of greenhouse gasses emitted into the atmosphere because of the application of synthetic fertilizers has led to abandon the existing agricultural practices, moving towards more sustainable ones. In this context, the use of plant growth promoting microorganisms (PGPMs) in agriculture is presented as a promising alternative since they are able to improve plant growth by several mechanisms including mineral solubilisation [1], nutrient uptake [2], changes in root morphology [3–5] or abiotic stress tolerance [6,7]. Due to their great variability of functions, the use of these microorganisms as biofertilizers can be potentially exploited [8].

Generally, PGPMs are divided in two categories, plant growth-promoting fungi (PGPF) and plant growth-promoting rhizobacteria (PGPR), being the second the greatest studied group so far [9]. Nowadays, a large amount of studies reported the effective use of rhizobacteria as biofertilizers, most of them involve bacteria belonging to *Pseudomonas* or *Bacillus* genus [10–12]. Within *Pseudomonas* genus, a growing interest in the species *Pseudomonas oryzihabitans* has emerged due to its potential role in plant growth promotion by the enhancement of root development. However, the plant growth-promoting activity of this bacterium species has remained relatively unexplored, and very little information is published in scientific literature. *P. oryzihabitans* is a soil-habiting rhizobacteria with the ability to enhance pea and mustard plant growth, even under adverse conditions, improving root elongation [13]. In the last years, an increase in the knowledge of this bacterial species has emerged, being those effects in roots demonstrated in other plant species such as potato plants [14] and in *Prunus and Pyrus* species cultured *in vitro* [15]. Other roles of this bacterial species including the biocontrol activity against root-knot nematodes or the degradation of toluene or organochlorine pesticides have also been reported [16–18].

The production of the microorganism is the first step to obtain a commercial microbial-based product and one of the main goals is to find a cheap cost medium that ensures the production of large amounts of microorganism cells without compromising their biological activity. In addition, the optimized medium must provide nutrients and energy that supplies microorganism's nutritional demands for cellular metabolism, growth and population stability [19]. For that purpose, the use of inexpensive commercial products and by-products has tended to be the

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most successful approach to obtain high quantities of microbial biomass [20]. This strategy allowed the successful implementation of an optimized biomass production protocol for several biological control agents including bacteria such as *Pantoea agglomerans* CPA-2 [21], *Bacillus subtilis* CPA-8 [22,23], or yeasts such as *Candida sake* CPA-1 [24,25] and *Rhodotorula minuta* [26]. In addition, production protocols of some PGPMs such as *Azospirillum brasilense* or *Pseudomonas trivialis* BIHB 745 as biofertilizers have been also developed [27,28].

Several efforts have been made to obtain cheap media to produce microorganisms in a commercial scale and in several cases, they were using by-products from food industries as a carbon or nitrogen sources [21,22]. More recently and under the framework of the European Project AGRIMAX (BBI-IA-DEMO-720,719), organic wastes from food and agriculture industries were used as by-products to enhance biological processes. It has been estimated that an approximate amount of 88 millions of tonnes of wastes are generated during food manufacturing within the European Union, representing economical losses valued at 143 billion of euros [29]. In this sense, organic wastes from food and agriculture industries are rich in bioactive compounds that could be used by living organisms to enhance biological processes [30]. These wastes have a significant relevance as they are obtained from products that are highly consumed within the European market, and they have been identified as raw materials for the extraction of a wide range of products such as biobased coatings and packaging, natural food additives and functional food products, fertilizers and biodegradable pots for agriculture [31]. This project was mainly focused on the sustainable utilization of by-products from potato, tomato, cereals and olive industries that may be useful in other industrial processes including low-cost microorganisms's production. Designing a waste-based medium for microorganism production will help in circular economy not only reducing the industry wastes but also in giving a second life and valorization of these products [32]. In previous studies conducted within the AGRIMAX project, several by-products from these food industries showing differences in manufacturing were characterised, and it was found that most of them displayed a high content (approximately 50%) of carbon source and a low proportion of nitrogen source (unpublished data). This fact has made possible the use of these by-products as the basis for the elaboration of a culture medium to grow the yeast *Saccharomyces cerevisiae*. In contrast, the nutritional requirements are quite different when growing bacteria, requiring compounds that provide a nitrogen supply since it is known that the growth of these microbes is favoured by the presence of nitrogen source [33].

The aim of this research was the development of a biomass production protocol for the PGPM *P. oryzihabitans* PGP01 using wastes from the agri-food industry as a more economic and sustainable alternative. This bacterial strain has previously shown to promote root growth and development in *Prunus* and *Pyrus in vitro* plantlets [15,34]. The objectives of this study were (1) to optimize the production of *P. oryzihabitans* PGP01 strain growth using a waste-based medium, (2) to scale-up and to optimize the bacterial production of 2 L bioreactor and (3) to use the bacterial population grown in the optimized media to promote explant rooting and improve plant quality *in vitro* conditions. The present research would set the bases to develop a product based on this microorganism for the fertilisation of fruit tree plants reducing the use of chemicals compounds, promoting a better environmental sustainability.

2. Material and methods

2.1. Microorganism

The bacterial isolate used in the study was *P. oryzihabitans* strain PGP01 belonging to the Postharvest Pathology Group of IRTA (Lleida, Catalonia, Spain). This bacterial strain was isolated from *Prunus* and *Pyrus in vitro* contaminated cultures which displayed a greater growth than those non-contaminated, and a root growth promotion ability was attributed in different *in vitro* plant materials [15]. Bacterial stocks were preserved at 4 °C after isolation and subcultured on nutrient dextrose agar (NYDA: 8 g L^{-1} nutrient broth; 5 g L^{-1} yeast extract; 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 25 °C for 48 h when needed. Bacterial cultures were stored for a long-term at -80 °C using criogenic beads.

2.2. Inoculum preparation

For all the experiments, *P. oryzihabitans* PGP01 inocula were obtained from 48-h-old cultures. Bacterial cells were transferred to phosphate buffer (70 mL KH_2PO_4 0.2 M; 30 K_2HPO_4 0.2 M and 300 mL deionized water). The fresh cell suspension was adjusted to OD 40 ± 5% measured at 420 nm with a spectrophotometer, which corresponded to, approximately 1×10^8 colony forming unit per mL (CFU mL^{-1}). This concentration was diluted to 1×10^5 or 1×10^6 CFU mL^{-1} using sterile distilled water for shake flasks or bioreactors inoculation, respectively. In both cases, the real applied concentrations were determined by dilution plate technique on NYDA medium.

2.3. Optimum growth temperature optimization in flasks

The effect of incubation temperature was tested to determine the optimal growth temperature of *P. oryzihabitans* PGP01. For that purpose, conical flasks containing *P. oryzihabitans* PGP01 growing in 50 mL of tryptone soy broth medium (TSB: 17 g tryptone; 3 g digested soy flour; 5 g sodium chloride; 2.5 g glucose, 2.5 g K_2HPO_4 in 1000 mL deionized water) were incubated at 20, 22, 25, 28, 30 and 32 °C under orbital agitation at 150 rpm for 48 h. Samples were taken at 24 and 48 h, and viable cell concentrations were determined by plate dilution technique on NYDA medium. For each temperature analysed, three independent replicates were used.

2.4. Agro-food industry by-products assays using shaking flasks

In this experiment, *P. oryzihabitans* PGP01 was tested for its ability to grow in media based on different by-products from several industry wastes (Frozen potato peels and pulps (FPP), tomato seeds after flotation (TS) and wheat bran (WB). Before media preparation, FPP was grinded to improve homogenization, and defrosted before mixing with distilled water for the elaboration of the medium. TS were sterilized to avoid microbial fermentation. Media were prepared in 250 mL conical flasks containing 50 mL of each medium (FPP, TS and WB at 300, 100 and 20 g L^{-1} in distilled water, respectively) and autoclaved at 121 °C for 15 min. For each by-product the selected concentration was the highest which could be dissolved for this amount of water without causing seals during growing. The laboratory medium TSB was used as a control. Initial pH of all media was measured before and after autoclaving, and as the different media showed different initial pHs values, different amounts of a sterile solution of K_2HPO_4 0.2 M were added to adjust the initial pH at 7. Upon inoculation with *P. oryzihabitans* PGP01 as described above, flasks were incubated at the optimum temperature and at 150 rpm for 48 h. Cultures were sampled at 24 and 48 h, and viable cell concentration (CFU mL^{-1}) was determined for both sampling periods by plate dilution technique on NYDA medium. Three independent replicates were used for each tested medium.

2.5. Optimization of nitrogen sources assays in shaking flasks

Media based on frozen potato peels wastes was supplemented with several nitrogen sources such as urea (Panreac, Barcelona, Spain), peptone (PEP, Peptic digest of meat USP, Biokar diagnostics, Allone, France), PROSTAR 510A (PS), yeast extract (YE, Biokar diagnostics, Allone, France), meat extract (ME, Biokar diagnostics, Allone, France), pea protein (PP, Naturalys F85M, Roquette Frères, France), maize protein (MP, Maizena®, Unilever) and tryptone (TRP, tryptone USP, Biokar Diagnostics, Allone, France), and were tested in order to fit the nitrogen requirements for the growth of *P. oryzihabitans* PGP01 in the waste-

based medium. PS is an isolated soy protein (Brentag Chemistry, Spain), and represents an adequate nitrogen source since it contains a 90% of protein content [23]. PEP and TRP are obtained by the controlled digestion of red muscles and casein, respectively, and they are rich in high molecular weight polypeptides (around 12.5% of total nitrogen). PP is a functional pea protein with a 84% of protein content. Both YE and ME are autolysates used for culture medium as a source of nitrogen (12% of total nitrogen). MP is a product used in gluten-free bakery, showing a 3% of the protein content. The different nitrogen sources were added at low and high concentration to select the best one. As previously, TSB medium was used as control. Samples were collected after 24 and 48 h of growth, and the *P. oryzihabitans* PGP01 population (CFU mL^{-1}) was determined at both sampling periods by plating serial dilutions on NYDA medium.

2.6. Optimization of the medium composition in 2 L bioreactors

Scaling-up of PGP01 production in FPP-based-media was performed at laboratory scale using 2 L BioFlo/CelliGen 115 modular bioreactors (Eppendorf AG, Barkhausenweg, Hamburg, Germany). In a first experiment, these bioreactors were filled with 1.8 L of the medium based on potato wastes (FPP) and the best nitrogen source at 10 and 20 g L^{-1} to optimise the concentration that provides the best bacterial growth. To improve bacterial production, the FPP-based medium supplemented with a nitrogen source was also complemented with 5 g L^{-1} of sodium chloride (NaCl), 5 g L^{-1} glucose + 5 g L^{-1} NaCl or 10 g L^{-1} of sugar cane molasses (MOL) + 5 g L^{-1} NaCl. All media were sterilized by autoclaving at 121 °C for 60 min. Bioreactors were inoculated with fresh *P. oryzihabitans* PGP01 inoculum at 1×10^6 CFU mL^{-1} as described above. In all cases, as the media showed different initial pHs values, different amounts of a sterile solution of K_2HPO_4 0.2 M were added to adjust the initial pH of the media to 7. Moreover, bioreactors containing the laboratory medium TSB were also inoculated and used in the experiment as standard control. The conditions of the process were set at 25 °C, 400 rpm and 0.5 vvm of air feeding, and pH, temperature and dissolved oxygen (pO_2) were constantly monitored. Antifoam (30% simethicone emulsion USP, Dow Corning®, USA) at 1 mL L^{-1} was added. Samples were taken at 0, 20, 22, 24, 26, 28 and 42 h of growth, and the viable cell concentration (CFU mL^{-1}) was determined at each sampling period by the dilution plate technique to obtain microorganism growth curve.

2.7. Optimization of growth conditions in the scale-up process to 2 L bioreactors

In order to maximize the production yield of *P. oryzihabitans* PGP01 in the potato wastes-based medium (300 g L^{-1} FPP supplemented with 10 g L^{-1} TRP, 5 g L^{-1} NaCl 10 g L^{-1} MOL and 2.5 g L^{-11} K_2HPO_4), different agitations (200, 400 and 600 rpm) and air fluxes (0.25, 0.5 and 0.75 vvm) at 25 °C were tested. After inoculation of bioreactors with fresh cell *P. oryzihabitans* PGP01 inoculum as described above, samples were taken at 0, 20, 22, 24, 26, 28 and 42 h for 200 and 400 rpm. In the case of 600 rpm, samples were taken at 0, 18, 20, 22, 24, 26 and 42 h due to the faster growth of *P. oryzihabitans* PGP01 in these conditions. Viable cell concentration (CFU mL^{-1}) was determined by dilution plate technique on NYDA medium.

2.8. P. oryzihabitans PGP01 growth under optimal conditions

Once defined the conditions of the whole production process, the growth curve of *P. oryzihabitans* PGP01 in the optimised medium was deeply studied with the aim of elucidating the performance of the microorganism in the culture medium. In this experiment, 2-L bioreactors containing the optimized medium were inoculated with *P. oryzihabitans* PGP01 at 1×10^6 CFU mL^{-1}. Growth conditions were set at 25 °C, 400 rpm and 0.75 vvm of air flux. Samples for the determination of the bacterial population size during the most active growing stage (from 0 to 30 h) were taken every 2 h. In addition, samples were also taken at the stationary phase of bacterial growth (40 and 48 h). Viable *P. oryzihabitans* PGP01 cell concentration was determined at every sampling time by dilution plate technique on NYDA medium and expressed as CFU mL^{-1}.

2.9. Determination of the root growth-promoting activity of P. oryzihabitans PGP01 in the optimized medium

The feasibility of the waste-based optimized medium for the growth and maintenance of *P. oryzihabitans* PGP01 effect on root development activity was tested in the *Prunus* rootstock Rootpac® 20 (RP-20) (Agromillora Group, Spain) *in vitro* rooted plantlets. For that purpose, *in vitro* micropropagated RP-20 explants were cultured in Murashige and Skoog medium [35] supplemented with 10 μM of the auxin indole-3-butyric acid (IBA) during 7 days in darkness to promote *in vitro* rooting. After exposition to IBA, RP-20 explants were transferred to the same medium without IBA during another 7 days, under light conditions, to promote root development. Shoots with visible roots were then cultured in root elongation medium with vermiculite to favour the *in vitro* coexistence of rooted plantlets with microorganisms. RP-20 *in vitro* plants were inoculated with 1 mL of fresh cell inoculum of *P. oryzihabitans* PGP01 set at 2×10^8 CFU mL^{-1} after 24 h of growth in synthetic TSB and optimized by-products based medium. Control plants were inoculated with 1 mL of sterile distilled water. Viable cell concentration was determined in both cases by dilution plate technique on NYDA medium.

2.10. Statistical data analysis

Data representing bacterial population (CFU mL^{-1}) were log transformed (Log CFU mL^{-1}) to improve homogeneity of variances. Data concerning the optimization of the temperature and the by-products as well as the biometrical data from root growth were analysed by one-way ANOVA. However, data concerning nitrogen source optimization as well as the scale-up to bioreactors were analysed by two-way ANOVA. Experiments concerning the optimization of the *P. oryzihabitans* PGP01 growth conditions in both flasks and 2 L bioreactors were repeated three times, using three independent replicates per treatment. For the validation of the biological activity of the microorganism *P. oryzihabitans* PGP01 growing in the wastes-based medium in RP-20 plantlets, ten *in vitro* seedlings per treatment (non-inoculated and *P. oryzihabitans* PGP01-inoculated seedlings growing in TSB and FPP media) were used. In all experiments, when the model was statistically significant, Tukey HSD test was used for the separation of the means. For all data analysis, JMP Pro-software was used (version 14.2.0, SAS Institute Inc., Cary, NC).

3. Results

3.1. Temperature and by-product optimization in flasks experiments

Bacterial population data of *P. oryzihabitans* PGP01 when growing at different temperatures (ranged from 20 to 32 °C), and different by-product based media (potato, tomato and cereal) from industries wastes are shown in Fig. 1. After 24 h of growth in the commercial medium TSB, the highest growth of *P. oryzihabitans* PGP01 was obtained at 25 °C, reaching a value of 9.32 log CFU mL^{-1} (Fig. 1A). Conversely, a poor growth of *P. oryzihabitans* PGP01 was observed at 20 °C at this period. The highest growth achieved after 48 h of incubation was registered at both 22 and 25 °C of temperature (9.71 and 9.77 log CFU mL^{-1}, respectively) (Fig. 1A). According to the obtained results, 25 °C was the established temperature to be used in the following experiments since it was the temperature fixed in the experiments concerning the effect of this microorganism *in vitro* cultured plants.

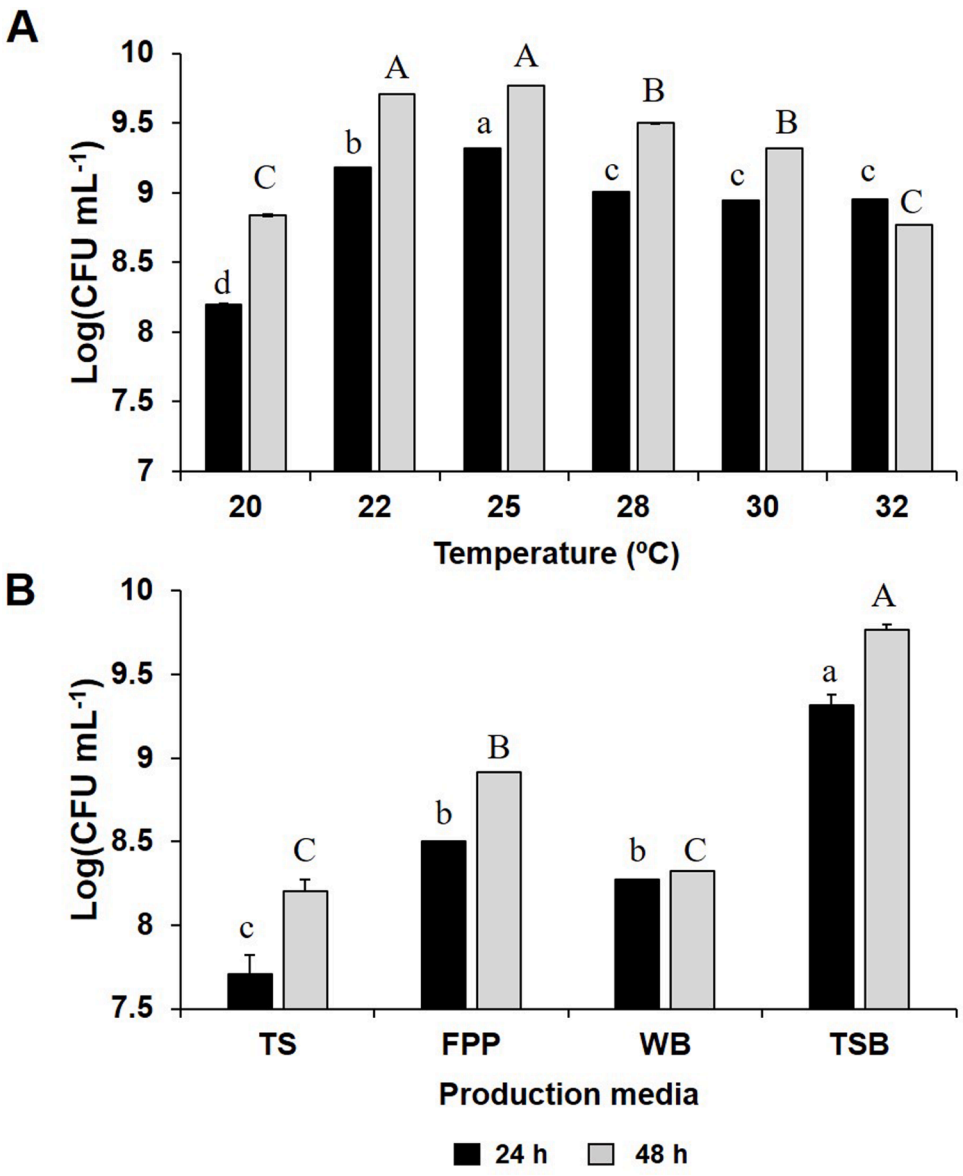

Fig. 1. - Growth in flasks of *P. oryzihabitans* PGP01 after 24 h (dark bars) and 48 h (grey bars) at different temperatures in the commercial medium tryptone soy broth (TSB) (A) and using media based on tomato seeds after flotation (TS), frozen potato peels and pulps (FPP) and wheat bran (WB) as agro-food wastes at 25°C (B). Media based on by-products were prepared by dissolving TS, FPP and WB at a concentration of 100, 300 and 20 g L^{-1} in 50 mL of distilled water using 250 mL conical flasks. Initial pH of all media based on agro-food industry wastes were adjusted to 7 by the addition of K_2HPO_4 0.2 M, and the growth of the microorganism *P. oryzihabitans* PGP01 in the different media was compared to the growth of the microorganism in the commercial medium TSB. Data represents mean ± Standard Error (SE) of three independent replicates. In both cases, different lower case letters represents significant differences amongst treatments at 24 h of culture, and different capital letters indicate significant differences amongst treatments at 48 h of culture according to Tukey HSD test ($P < 0.05$).

On the other hand, the growth of *P. oryzihabitans* PGP01 in the commercial medium TSB was 9.32 and 9.77 log CFU mL^{-1} at 24 h and 48 h, respectively. At the same sampling time, lower growth values were obtained when the bacterium was grown in media based on FPP and WB (8.50 and 8.27 log CFU mL^{-1}, respectively), and even lower in TS-based medium (Fig. 1B). Even though *P. oryzihabitans* PGP01 growth in the three by-product based media remained significantly lower than the observed for the TSB medium after 48 h (9.77 log CFU mL^{-1}), the medium with FPP provided the greatest values of bacterial production (Fig. 1B), being the candidate by-product for medium optimization in the later trials.

3.2. Selection of nitrogen source in flasks experiments

To improve the growth of *P. oryzihabitans* PGP01, FPP-based medium was supplemented with urea, PEP, PS, YE, ME, PP, MP or TRP as nitrogen sources to try to achieve similar amount of bacterial cells that the obtained in the commercial medium TSB. After 24 h, only the combination of FPP with the highest concentration (20 g L^{-1}) of peptone, PROSTAR 510A, yeast extract or meat extract led to a similar growth of *P. oryzihabitans* PGP01 than when the bacterium was grown in the commercial medium TSB (9.19, 8.29, 9.12 and 9.16 log CFU mL^{-1} for peptone, PROSTAR 510A, yeast and meat extract, respectively), and higher than the FPP-based media by itself (8.15 log CFU mL^{-1}) (Fig. 2A). FPP-based media supplemented with other nitrogen sources such as tryptone or pea protein at 20 g L^{-1} (8.72 and 8.66 log CFUvmL^{-1}, respectively) as well as meat extract at 10 g L^{-1} (8.59 log CFU mL^{-1}) were able to improve the growth observed when only the FPP by-product was used (Fig. 2A). However, at this period, the FPP-based medium supplemented with the lowest concentration (10 g L^{-1}) of yeast extract, pea protein, peptone, PROSTAR 510A or tryptone did not significantly affect *P. oryzihabitans* PGP01 growth compared to the observed in FPP-based media by itself (Fig. 2A). Urea and maize protein to the culture medium at both low and high concentration led to the poorest growth of *P. oryzihabitans* PGP01 recorded (<7.5 log CFU mL^{-1}) (Fig. 2A).

At 48 h of growth at 25 °C, the FPP-based media supplemented with PEP at 20 g L^{-1} or TRP at both 10 and 20 g L^{-1} provided a *P. oryzihabitans* PGP01 growth of 9.42, 9.54 and 9.56 log CFU mL^{-1}, respectively (Fig. 2B), being those values similar to the observed for the commercial medium TSB (9.62 log CFU mL^{-1}). The application of the YE at 20 g L^{-1} or PP at both concentrations only allowed to obtain

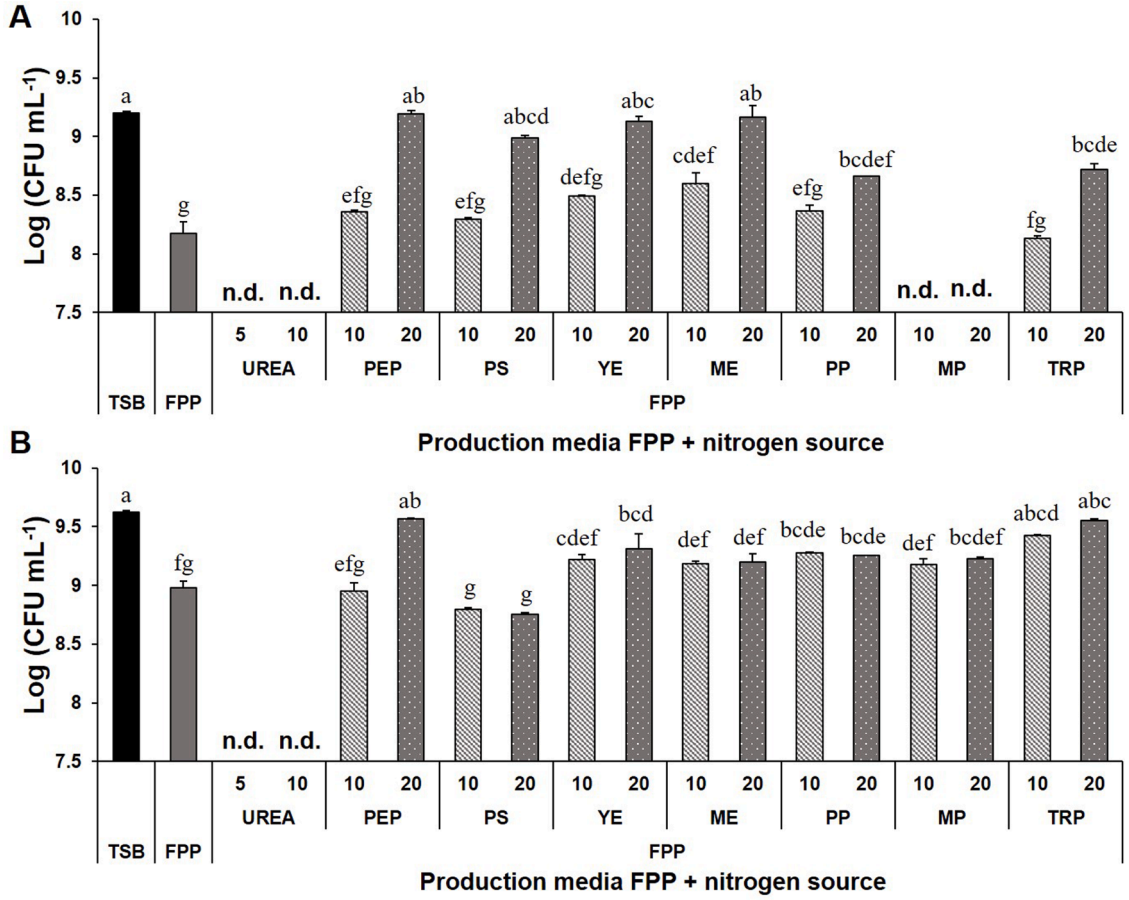

Fig. 2. - Growth in flasks of *P. oryzihabitans* PGP01 after 24 (A) and 48 h (B) using potato wastes (FPP = Frozen potato peels and pulps) supplemented with several nitrogen sources at low (▨) and high (■) concentration. *P. oryzihabitans* PGP01 cells were cultured at 25°C and 150 rpm. PEP = Peptone, PS = PROSTAR 510A, YE = Yeast extract, ME = Meat extract, PP = Pea protein, MP = Maize protein, TRP = Tryptone. Initial pH of all media were adjusted to 7 by the addition of K_2HPO_4 0.2 M. Data represent mean ± SE of three independent replicates, and different letters indicate significant differences between TSB and different media according to Tukey HSD test ($P < 0.05$).

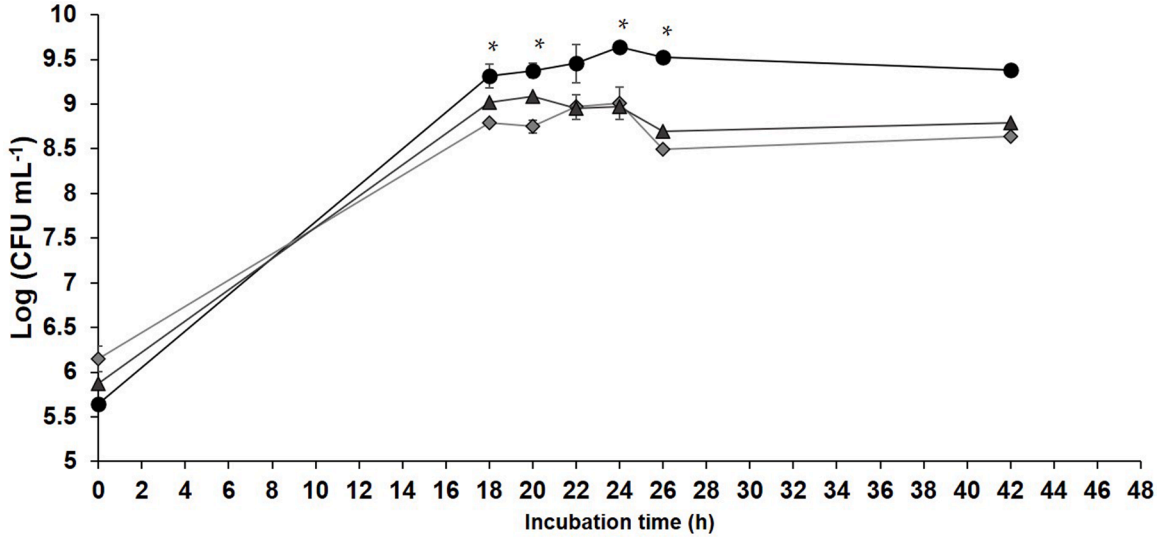

Fig. 3. - *P. oryzihabitans* PGP01 growing in the commercial medium tryptone soy broth (TSB) (—●—) and in media based on potato wastes (FPP) supplemented with 10 g L^{-1} (—◆—) or 20 g L^{-1} of TRP (—▲—) in a 2 L bioreactor at 25°C and 400 rpm. Data represent means ± SE of three independent replicates, and asterisks symbols (*) denote significant differences between commercial and by-products optimized media according to Tukey HSD test ($P < 0.05$).

significantly higher growth values than when the FPP was used alone for the elaboration of the medium (9.31, 9.27 and 9.25 log CFU mL^{-1}, respectively, against 8.98 log CFU mL^{-1}) (Fig. 2B). However, these growth values were significantly lower than those registered when the microorganism *P. oryzihabitans* PGP01 was growing in the commercial medium TSB (Fig. 2B). Again, urea provided the lowest values of viability since *P. oryzihabitans* PGP01 growth also failed to be detected under these conditions (Fig. 2B).

In the light of these results, and considering the lower price of the tryptone compared to the peptone, it was decided to select the first one as the best compound to provide the nitrogen supply on the basis medium in combination with the potato waste FPP. In the next step, the definition of the composition of this basis medium (FPP+TRP) for *P. oryzihabitans* PGP01 growth was carried out in the scaling-up of the production to 2 L bioreactors.

3.3. Optimization of the composition of the culture medium in the scale-up process to 2 L bioreactors

As a first step, the proportion of the chosen nitrogen source (TRP) needed to be optimised. For that reason, a preliminary experiment in bioreactors with *P. oryzihabitans* PGP01 growing at 25 °C in the presence of the FPP-based medium supplemented with TRP at 10 and 20 g L^{-1} was conducted, and the results are shown in Fig. 3. As it was observed in flask experiments, in bioreactors we also did not observe differences in *P. oryzihabitans* PGP01 growth when the potato waste FPP was combined with TRP at 10 or 20 g L^{-1} at the different sampling times (Fig. 3). Consequently, TRP 10 g L^{-1} was the definitive concentration used in the potato by-products-based medium (FPP). However, it was observed that *P. oryzihabitans* PGP01 growth on the commercial medium TSB still provided the best production values (Fig. 3), and further medium composition still required some improvement.

For that reason, in the following steps of optimization, the effect of NaCl as well as glucose and sugar cane molasses as mineral and carbon sources were studied in order to improve *P. oryzihabitans* PGP01 growth in the FPP-based medium FPP+TRP10. *P. oryzihabitans* PGP01 growing in TSB medium peaked its maximum (9.64 log CFU mL^{-1}) after 24 h of culture in 2 L bioreactors (Fig. 4). It is important to highlight that the addition of NaCl at 5 g L^{-1} together with molasses at 10 g L^{-1} allowed to reach a 9.68 log CFU mL^{-1} of *P. oryzihabitans* PGP01 production at the same point, very similar to that obtained when growing the bacterium in TSB medium (Fig. 4). When the FPP + TRP10 medium was supplemented only with NaCl 5 g L^{-1}, a production of 9.20 log CFU mL^{-1} was obtained after 24 h of incubation, being this value significantly lower than the observed in the synthetic medium TSB (Fig. 4). On the other hand, the combined addition of 5 g L^{-1} glucose and 5 g L^{-1} NaCl provided the poorest growth since a maximum growth of 7.20 log CFU mL^{-1} was achieved after 18 h of culture. After that, bacterial growth decreased, and it was not detected from 22 h to the end of the production process (Fig. 4). Constant pH monitoring revealed a decrease of this factor after the combined addition of 5 g L^{-1} of glucose and NaCl, registering values of 4.5 from 20 h of culture that remained invariable until the end of the fermentation process (data not shown). Thus, the composition of the FPP-based medium which provided similar growth than the observed in the laboratory medium TSB was 300 g L^{-1} FPP, 10 g L^{-1} TRP, 10 g L^{-1} MOL 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4.

3.4. Effect of agitation and oxygen flux

Once the final composition of a competitive medium based on potato wastes for *P. oryzihabitans* PGP01 growth was established, the agitation and aeration conditions were optimized to obtain the highest bacterial production. In these experiments, the agitation speed had a significant impact on *P. oryzihabitans* PGP01 production at the different sampling times ($P < 0.0001$), while the air flux only showed a significant effect at 20, 22 and 24 h of growth (Table 1). The interaction between the two factors only was significant at 22 and 24 h, coinciding with the maximum growth period (Table 1). Comparing the aeration depending on the agitation speed, it was shown that the lowest values of *P. oryzihabitans* PGP01 production were obtained at 0.25 vvm for both 200 and 400 rpm of agitation at 20 and 22 h of growth (Table 1). In contrast, 0.5 and 0.75 vvm of air flux produced similar *P. oryzihabitans*

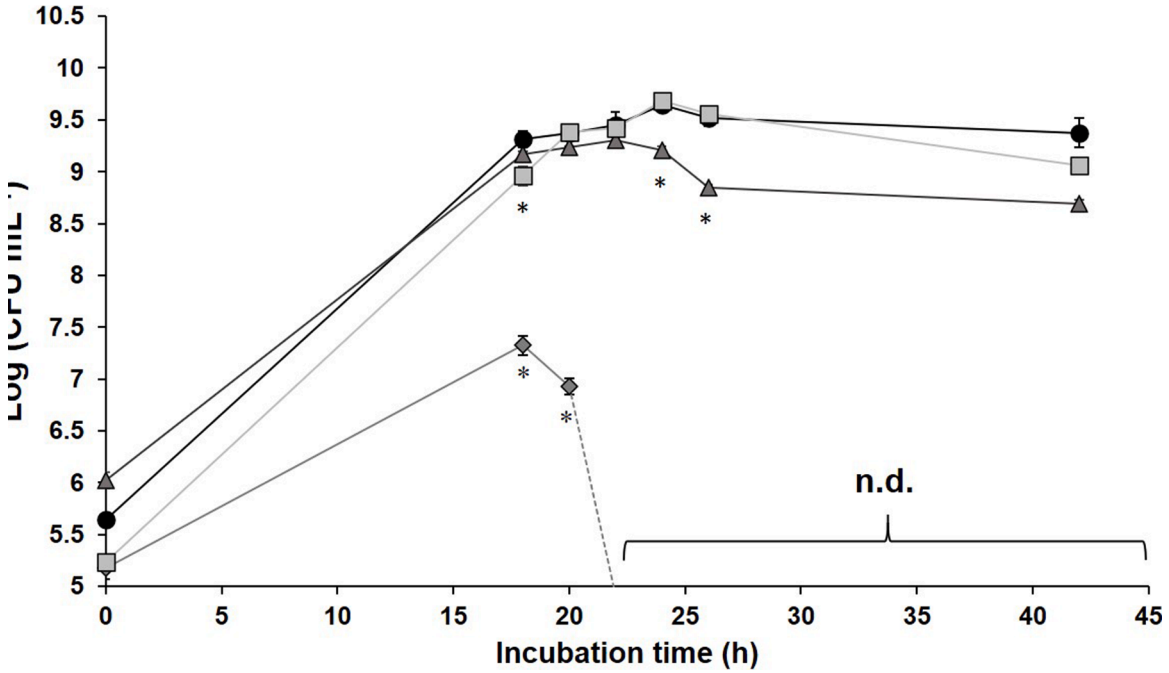

Fig. 4. - ***P. oryzihabitans*** **PGP01 growing in the commercial medium tryptone soy broth (TSB) (□) and in media based on frozen potato peels and pulps and 10 g L^{-1} of TRP (FPP + TRP10) supplemented with 5 g L^{-1} of NaCl (◆), 5 g L^{-1} of NaCl, 5 g L^{-1} of GLU (◇) and 5 g L^{-1} of NaCl, 10 g L^{-1} of MOL (□) at 25°C and 400 rpm. TSB** Data represents the mean ± SE of three independent replicates. Asterisks (*) symbols within each sampling period denote significant differences between TSB and optimized media according to Tukey HSD test ($P < 0.05$).

Table 1
- Effect of different stirring speeds (200, 400 and 600 rpm) and different air feedings (0.25, 0.5 and 0.75 vvm) during the production process at different periods of time on *P. oryzihabitans* PGP01 cells growth in 2 L bioreactors using an agro-food by-product medium based on 300 g L^{-1} of FPP supplemented with 10 g L^{-1} TRP, 10 g L^{-1} MOL, 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4. Data represent means ± SE of at least three independent replicates. For each air flux, lowercase letters indicate significant differences amongst agitation speeds according to Tukey HSD test ($P < 0.05$). For each agitation speed, air fluxes with different uppercase letters are significantly different according to Tukey HSD Test ($P < 0.05$).

	18 h	20 h	22 h	24 h	26 h	28 h
			200 rpm			
Air flux (vvm)						
0.25		8.82 ± 0.001 b C	8.89 ± 0.033 b B	8.70 ± 0.067 a B	8.71 ± 0.084 a B	8.51 ± 0.145 a
0.5		8.88 ± 0.056 ab B	8.96 ± 0.029 ab B	8.83 ± 0.035 a B	8.63 ± 0.071 a B	8.63 ± 0.096 a
0.75		9.02 ± 0.025 a B	9.06 ± 0.007 a C	8.94 ± 0.014 a B	8.93 ± 0.066 a B	8.78 ± 0.053 a
			400 rpm			
Air flux (vvm)						
0.25		9.20 ± 0.022 b B	9.35 ± 0.023 b A	9.36 ± 0.013 b A	9.33 ± 0.105 a A	9.26 ± 0.019 a
0.5		9.33 ± 0.052 ab A	9.49 ± 0.080 a A	9.57 ± 0.051 a A	9.40 ± 0.087 a A	9.10 ± 0.088 a
0.75		9.40 ± 0.035 a A	9.59 ± 0.017 ab A	9.52 ± 0.04 ab A	9.32 ± 0.100 a A	9.05 ± 0.070 a
			600 rpm			
Air flux (vvm)						
0.25	9.20 ± 0.012 a	9.36 ± 0.047 a A	9.50 ± 0.055 a A	9.50 ± 0.040 a A	9.46 ± 0.004 a A	
0.5	9.24 ± 0.033 a	9.39 ± 0.023 a A	9.41 ± 0.011 a A	9.52 ± 0.023 a A	9.41 ± 0.060 a A	
0.75	9.23 ± 0.039 a	9.37 ± 0.013 a A	9.44 ± 0.036 a B	9.44 ± 0.014 a A	9.23 ± 0.033 a AB	
			***P* - values**			
Agitation (A)		< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***
Air flux (B)	0.697	0.0006 ***	0.0102 *	0.001***	0.966	0.854
(A) × (B)		0.077	0.007**	0.012**	0.053	0.051

PGP01 growth values at the same sampling times (Table 1). After 24 h, no differences amongst the three air fluxes were found on bacterial production at 200 rpm. However, at 400 rpm of agitation, both of the analysed air fluxes (0.5 and 0.75) still produced significantly higher values of *P. oryzihabitans* PGP01 than 0.25 vvm (Table 1). At 600 rpm of agitation, the three air fluxes did not produce significant differences in *P. oryzihabitans* PGP01 production (Table 1). Comparing the agitation depending on the air flux, we found that an agitation of 200 rpm led to the poorest bacterial growth at the three air fluxes and at all times of incubation (Table 1). Overall, agitations of 400 or 600 rpm produced the highest *P. oryzihabitans* PGP01 growth values under 0.25, 0.5 and 0.75 vvm, being these values similar at all of the times of incubation except for 20 and 22 h of incubation (Table 1). Therefore, due to economic reasons, the lower agitation speed (400 rpm) was selected for the production protocol. In addition, this decision was reinforced by the fact that at the most active growth period (22 h), the highest production of *P. oryzihabitans* PGP01 was obtained at 400 rpm and 0.75 vvm of air flux. Regarding air feeding, the highest 0.75 vvm was chosen to provide an adequate oxygen supply since this factor does not show an important economic impact on the production process.

3.5. Growth curve under optimal conditions

At the initial stages of incubation in the optimized medium (FPP*: 300 g L^{-1} FPP, 10 g L^{-1} TRP, 5 g L^{-1} NaCl, 10 g L^{-1} MOL and 2.5 g L^{-1} K_2HPO_4) at 25 °C, 400 rpm of agitation and 0.75 vvm of aeration, *P. oryzihabitans* PGP01 rapidly reached a 7.19 log CFU mL^{-1} after 8 h of growth (Fig. 5). During this period, medium pH remained stable between 6.42–6.48. Then, a 12 h exponential growth phase of *P. oryzihabitans* PGP01 occurred coinciding with a rise on the medium pH until 8.02 after 20 h of growth in the optimized medium (Fig. 5). Maximum population of *P. oryzihabitans* PGP01 was reached at 22 h, and it remained stable until 26 h of culture. During this period, a maximum population of 9.64 log CFU mL^{-1}, corresponding to 4.4×10^9 CFU mL^{-1} of *P. oryzihabitans* PGP01 was registered at 24 h (Fig. 5). After 26 h, *P. oryzihabitans* PGP01 population decreased while the medium pH continued increasing during the whole fermentation process reaching a maximum of 8.85 after 48 h of culture (data not shown).

3.6. Maintenance of the root growth-promoting activity of *P. oryzihabitans PGP01* growing on the optimized medium

The final step of this biomass production protocol was to validate that the *P. oryzihabitans* PGP01 growing in the FPP optimized medium (300 g L^{-1} FPP, 10 g L^{-1} TRP, 10 g L^{-1} MOL and 5 g L^{-1} NaCl) at 25 °C, 400 rpm and 0.75 vvm maintained its biological activity. In this sense, a significant 64% increase in the number of roots was observed in RP-20 *in vitro* plantlets compared to those non-inoculated plantlets (Fig. 6A1 and B). However, although plants inoculated with *P. oryzihabitans* PGP01 grown in synthetic medium TSB also showed higher number of roots than non-inoculated plants, these differences were not statistically significant. The root-located effect of the bacterium *P. oryzihabitans* PGP01 growing in the optimized FPP-based medium in RP-20 *in vitro* plants was corroborated since both root/shoot as well as root/plant fresh weight (FW) ratio were increased in a 54 and 35% in relation to non-treated plantlets (Fig. 6A2 and A3). A similar trend was recorded in response to the inoculation with *P. oryzihabitans* PGP01 growing on the commercial medium TSB, showing significant 60 and 35% increase in these two parameters (Fig. 6A2 and A3). Moreover, the inoculation of RP-20 *in vitro* plants with *P. oryzihabitans* PGP01 also showed differences in the roots appearance, observing a more robust root system than those non-inoculated plants (Fig. 6B1, 2 and 3).

4. Discussion

Due to the beneficial effects on plant growth and development induced by PGPM, the development of products based on these microorganisms constitutes a necessary strategy for their large scale application. In this sense, the optimization of an economic medium that allows obtaining an equal microbe population at a lower price without affecting its biological activity must be firstly considered. In the present study, a protocol for the economic production of the PGPM *P. oryzihabitans* PGP01 was set up using a frozen potato peels and pulp-based medium, providing more evidence about the exploitation of agro-food industries wastes as nutrient sources for microorganism's production. Similarly, by-products generated from dairy sludge or the processing of sweet potato have been also successfully used to produce other bacterial PGPMs including *Rhizobium* spp. [36], *Bacillus thuringiensis* [37] and *Paenibacillus polymyxa* [38] in a more sustainable way. For all those reasons, this research represents an interesting approach to give a second use to wastes obtained from potato processing, which is one of the

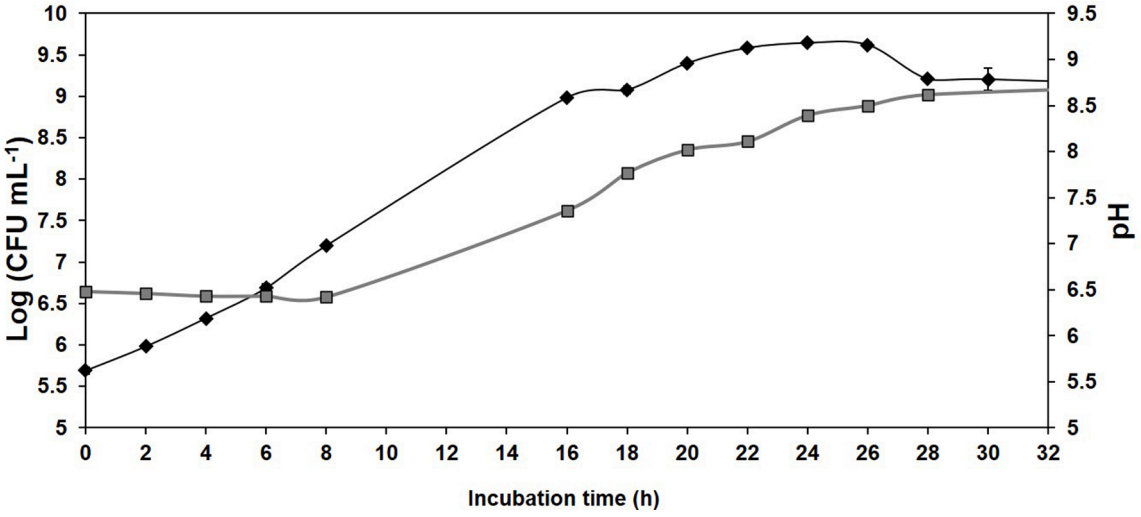

Fig. 5. – **Bacterial growth curve (log CFU mL^{-1}) (◆) and pH (▣) evolution of growth curve in a 2-L bioreactor of the bacterium *P. oryzihabitans* PGP01 in the optimized-wastes-medium based on frozen peels and pulps (FPP*: 300 g L^{-1} FPP, 10 g L^{-1} TRP, 10 g L^{-1} MOL and 5 g L^{-1} NaCl) at 25°C, 400 rpm and 0.75 vvm of oxygen flux.** Initial pH of the culture medium was adjusted to 7 by the addition of K_2HPO_4 0.2 M. Each value of the log (CFU mL^{-1}) of the curve represents the mean ± SE of at least three replicates.

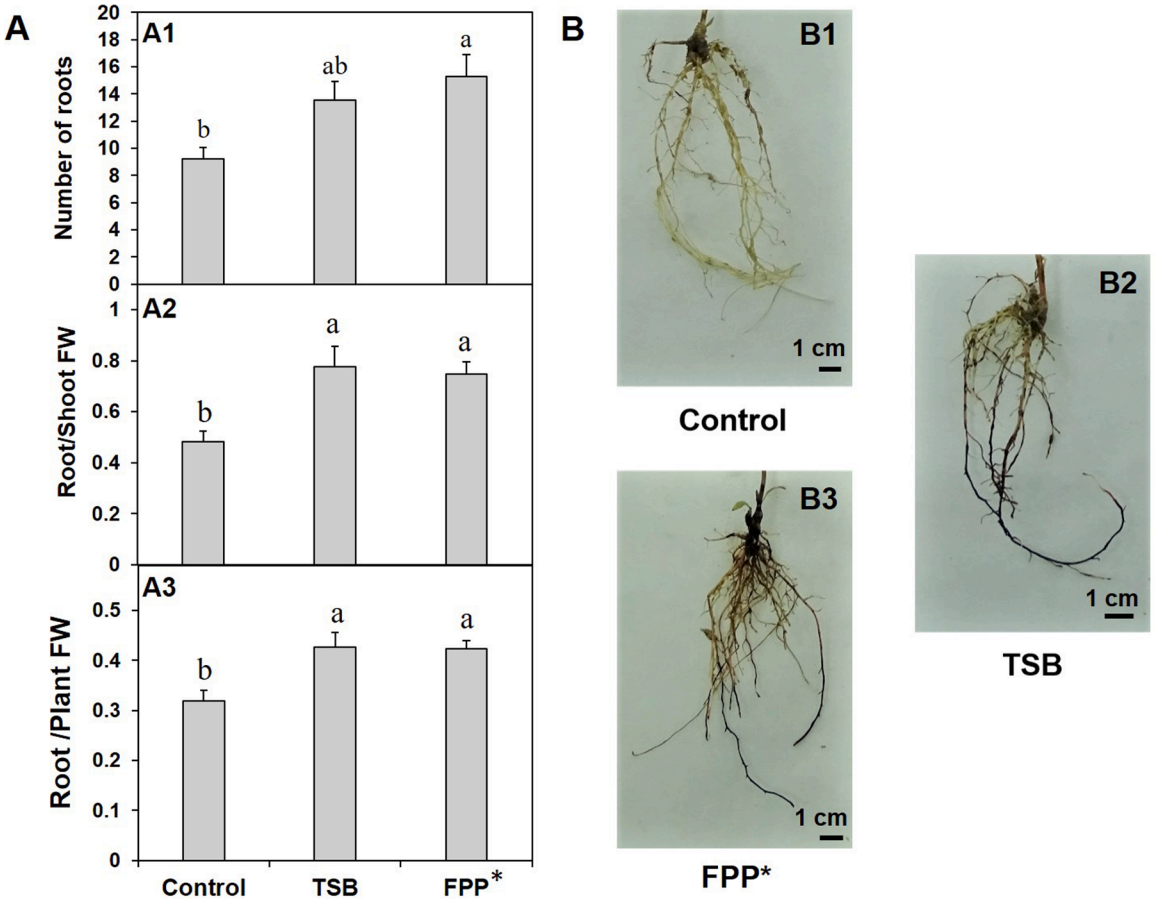

Fig. 6. - **Effect of the bacterium *P. oryzihabitans* PGP01 in the number of roots (A1), root/shoot fresh weight (FW) (A2) and root/plant FW ratio (A3) as well as in root morphology (B) of *in vitro* rooted Rootpac® 20 plantlets when growing in the absence of microorganism (control) (B1), or in the presence of *P. oryzihabitans* PGP01 growing in the commercial medium tryptone soy broth (TSB) (B2) or in the optimized potato wastes-based-medium FPP* (300 g L^{-1} FPP + 10 g L^{-1} TRP + 10 g L^{-1} MOL + 5 g L^{-1} NaCl + 2.5 g L^{-1} K_2HPO_4) (B3).** Data represents the mean ± SE of at least ten independent replicates. Different letters indicate significant differences amongst control, commercial and optimized medium according to Tukey HSD test ($P < 0.05$).

most widely consumed food products in the European market.

At the very early stages of optimization, the effect of the incubation temperature on *P. oryzihabitans* PGP01 growth was studied. Growth temperature should be one of the first factors to optimize in a biomass production protocol as it is determinant for microbial functionality and development [17,39,40]. In our study, the maximum bacterial population was obtained at both 24 and 48 h when *P. oryzihabitans* PGP01 was grown at 25 °C, being this the chosen temperature for the biomass production of *P. oryzihabitans* PGP01. The nature of the food industry wastes-based medium was the following step in the developing of the low cost medium. For these experiments, culture medium based on frozen potato, tomato and cereals wastes from AGRIMAX project, were selected as a potential alternative to the use of commercial products due to their wide diversity of nutrients such as carbohydrates, phenolic compounds, proteins or vitamins that might be used by microorganisms [31,41–43]. According to the obtained results, FPP seemed to be the most promising by-product as an almost 9 log CFU mL^{-1} after 48 h of culture was achieved. Nonetheless, similar production levels than the obtained in the TSB medium were not so far reached. Although the use of TS and WB may have contributed to a cheaper *P. oryzihabitans* PGP01 production protocol due to their lower amount required for the preparation of the media (100 and 20 g L^{-1}) and ease handling in comparison to FPP (WB is a ready-to-use waste), the lower bacterial growth obtained led to the discard these by-products for following experiments. Moreover, the characterization of the three wastes revealed that TS and WB had higher contents of carbon and nitrogen than FPP (AGRIMAX project, unpublished data). However, the carbon and nitrogen forms existing in TS and WB might not be accessible for *P. oryzihabitans* PGP01 metabolism, leading to a poorer bacterial production. It is known that, in some cases, the effective use of wastes by microorganisms depends on the ability to degrade their components [31]. For instance, the yeast *S. cerevisiae* is not able to degrade starch, and complementary strategies such as the addition of starch-degrading enzymes must be followed to favour its utilisation [44]. Accordingly to this study, other authors have reported the effective implementation of potato peels wastes for the reduction of the costs of secondary metabolites and single-cell protein processes by microorganisms [45,46]. Considering all those reasons, a wastes medium based on FPP was optimized in further experiments for *P. oryzihabitans* PGP01 production.

Another important aspect that must be considered, especially when growing bacteria, is the nitrogen source present in the culture medium [33]. In the present study, several commercial products were tested at a lower and a higher dose. In this case, the addition of peptone at 20 g L^{-1} or tryptone at both 10 and 20 g L^{-1} to FPP resulted in *P. oryzihabitans* PGP01 populations of 9.56, 9.42 and 9.55 log CFU mL^{-1} after 48 h of growth, respectively. These values were similar to those reached when *P. oryzihabitans* PGP01 was grown in the TSB medium. Peptides such as tryptone and peptone are efficiently used as a substrate in culture media due to their high content in amino acids [47,48]. In our research group, both nitrogen sources have been previously tested showing different results depending on the bacterial species. While these two commercial products were not the most effective nitrogen sources for *P. agglomerans* CPA-2 production, peptone resulted in one of the most suitable compounds for the *B. subtilis* CPA-8 mass production protocol [22,33]. In the view of the obtained results, we were able to reduce the concentration of tryptone without seriously affecting *P. oryzihabitans* PGP01 growth. In addition, this tryptone concentration was also lower than the required for the TSB medium composition (17 g L^{-1}). For this reason, tryptone was selected as the best nitrogen source to be added to the FPP-based medium and to produce *P. oryzihabitans* PGP01 in a low cost medium.

In the scale-up to 2 L bioreactors, glucose at 5 g L^{-1} and molasses at 10 g L^{-1} were selected to study the effect of the carbon source on *P. oryzihabitans* PGP01 production, and NaCl at 5 g L^{-1} was added to the optimised medium to maintain osmotic balance. In this regard, it has been reported that the mineral supplementation of the culture medium with trace elements provided a stimulatory effect in cell growth of some *Bacillus* species improving the production process effectiveness [49,50]. In our study, the addition of 5 g L^{-1} NaCl to the potato waste-based medium (FPP + TRP10) allowed to reach similar values to those obtained in TSB medium after 20 and 22 h of culture (9.23 and 9.42 log CFU mL^{-1}, respectively). However, after 24 h of growth, *P. oryzihabitans* PGP01 population significantly decreased. On the contrary, when the medium was supplemented with both 5 g L^{-1} NaCl and 10 g L^{-1} molasses, it was able to reach a 9.68 log CFU mL^{-1} of *P. oryzihabitans* PGP01 population, being these values similar to the obtained in the synthetic medium after 24 h of growth. Molasses represent a low cost waste from sugar beet, and its high relevance in microorganisms production relies on its high content of mono- and disaccharide such as glucose, fructose and sucrose (approximately 50% w/w) [21,51]. In previous studies conducted by Yánez-Mendizábal et al. (2012), the combination of molasses with other by-products resulted in the best option to produce the *B. subtilis* CPA-8 strain. A similar response was observed when molasses were used for the mass production of *Bacillus siamensis* using an anaerobic digestate [52]. The addition of 5 g L^{-1} glucose and 5 g L^{-1} of NaCl to the FPP + TRP10 medium did not produce a positive effect in *P. oryzihabitans* PGP01 production as considerably lower population values of this microbe were recorded after 18 and 20 h of culture, and undetectable values were registered from 22 h to 42 h. The concentration of this substrate added to the optimized medium was the same than the used in the elaboration of the TSB medium (5 g L^{-1}). It is widely known that glucose represents the main carbon sources used for many bacteria genus to ensure cell growth [53]. Nevertheless, the presence of glucose in the FPP + TRP10 medium produced a decrease on the pH of the culture medium, reaching values of 4.5 after 20 h of incubation. In this sense, it is widely known that acidic values seriously affect bacterial growth [54]. Therefore, the better results obtained in the *P. oryzihabitans* PGP01 production terms using molasses instead might suggest that other compounds existing in this by-product likely favoured bacterial cell growth. In fact, although the 50% of the composition of molasses are sugars, other compounds potentially used for microorganism's growth such as nitrogen compounds or amino acids are also present [55]. Considering the aforementioned, our findings provide a great value to the study, developing an economic protocol for the mass production of *P. oryzihabitans* PGP01 by the re-utilisation of wastes from the two highly consumed products potato and sugar beet.

Once the composition of the medium was established, (300 g L^{-1} FPP + 10 g L^{-1} TRP + 5 g L^{-1} NaCl + 10 g L^{-1} MOL + 2.5 g L^{-1} K_2HPO_4), we focussed on the optimization of the growing conditions in the bioreactors such as the agitation and air flux. Both parameters have been previously shown as critical factors for the successful scaling-up of the production process to bioreactors [56,57]. In our study, three different agitation speeds (200, 400 and 600 rpm), and three air feeding fluxes (0.25, 0.5 and 0.75 vvm) were analysed in order to maximize the *P. oryzihabitans* PGP01 production in bioreactor. Both parameters and its interaction had a significant impact on *P. oryzihabitans* PGP01 production during the most active period of growth (22 and 24 h). At those points, it was observed that higher bacterial production values were reached when the air flux of the process were set at both 0.5 and 0.75 vvm under 200 and 400 rpm of agitation. In contrast, the higher speed agitation of 600 rpm might provide an adequate oxygen supply, and an effect of aeration was not observed. Regarding agitation speeds, while low agitations (200 rpm) led to a maximum of 9 log CFU mL^{-1} at 22 h under the highest air flux (0.75 vvm) around a 9.5 log CFU mL^{-1} of optimum *P. oryzihabitans* PGP01 growth was reached when the agitation was set up at either 400 or 600 rpm. However, after 22 h of incubation in 2 L bioreactors, the maximum *P. oryzihabitans* PGP01 production was reached at 400 rpm under the highest air flux (0.75 vvm). In this context, a high oxygen supply in bioreactors increase bacterial production since *P. oryzihabitans* is a strictly aerobic bacterium [58]. Our results differ significantly from those obtained in the production of *A. brasilense* in laboratory-scale pneumatic bioreactors [59]. These authors demonstrated that the highest bacterial biomass was obtained at aerations of

0.1 vvm. Considering the results, 400 rpm and 0.75 vvm were the chosen agitation and air flux conditions for the production of *P. oryzihabitans* PGP01 in 2 L bioreactors. Therefore, in order to reduce the costs of the *P. oryzihabitans* PGP01 process, the selected agitation speed was 400 rpm. Regarding air flux conditions, no differences between 0.5 and 0.75 vvm were found in terms of *P. oryzihabitans* PGP01. In our study, considering that air feeding is not an expensive factor, the highest air feeding flux 0.75 vvm was fixed for the production protocol of *P. oryzihabitans* PGP01 production.

Observing the growth curve, when the bacterium *P. oryzihabitans* PGP01 was grown in the optimised medium, an exponential growth of this bacterium was recorded from the beginning during the first stage of growth (from 0 to 8 h), increasing the bacterial population from 5.68 to 7.19 log CFU mL^{-1}. This response is quite different to that obtained for the *B. subtilis* CPA-8 and *P. agglomerans* CPA-2 production protocols where a lag phase occurred during the first 4–6 h of bacterial growth [22,33]. Those results might suggest that an adaptation to the waste based medium by the bacterium *P. oryzihabitans* PGP01 is not required, obtaining high production values in a shorter time. In this study, the maximum cell growth was obtained at 22 h of culture and a pH of 8.4, reaching a *P. oryzihabitans* PGP01 population of 9.64 log CFU mL^{-1}. In this sense, our study could provide valuable knowledge about the optimum pH for *P. oryzihabitans* PGP01 growth as very little information in this regard is available in literature. Moreover, bacterial population decreased after 26 h of culture in the optimised medium likely due to the depletion of the nutrients in the culture medium or the accumulation of undesirable products that may inhibit bacterial growth.

Although obtaining a high cell biomass using inexpensive compounds constitutes a significant requirement for the development of a microbe-based product, the maintenance of the biological activity of the microorganisms must be ensured for commercial purposes [21–23]. For that reason, micropropagated explants of the rootstock Rootpac 20 (RP-20) belonging to *Prunus* genus were inoculated with *P. oryzihabitans* PGP01 grown in optimized medium to corroborate the same effects in roots observed in previous studies with this bacterium grown in laboratory medium [15]. In this case, our study demonstrated the effectiveness of the optimized medium based on by-products on the maintenance of the root development enhancing activity of *P. oryzihabitans* PGP01, observing an increase of the number of roots over non-inoculated plantlets in a greater extent than the laboratory medium. Similarly to previous studies [34], the root-located activity of this microorganism grown in the optimized medium was corroborated by the 54 and 35% increases on the root/shoot and root/plant FW ratio observed in RP-20 *in vitro* plantlets. These findings might constitute the first step on the development of a more sustainable biofertilizer with important applications in agriculture. Following a similar strategy, *Paenibacillus polymyxa* produced by the use of wastewater from sweet potato starch production produced an increase of tea yield production as well as polyphenol contents under field conditions [38]. However, to achieve that goal, it will be necessary to optimize the pilot-scale production and formulation of *P. oryzihabitans* PGP01.

5. Conclusions

In light of the obtained results, we can conclude that an inexpensive medium to maximize the production of the plant growth promoting rhizobacterium *P. oryzihabitans* PGP01 was developed using low cost industry waste's-based medium and commercial products. This medium was based on 300 g L^{-1} of FPP supplemented with 10 g L^{-1} TRP, 10 g L^{-1} MOL and 5 g L^{-1} NaCl, and the optimal culture conditions were 25 °C of temperature, 400 rpm of agitation and 0.75 vvm of aeration during 22 h. The microorganism growing in this cheap culture medium also preserved its biological activity, increasing several parameters involving root development. Using a food industry waste as a main component of the medium to grow *P. oryzihabitans* PGP01, the production process was carried out in a more sustainable manner. Although pilot-scale production experiments will be required to finally develop a product based on this bacterium, this study has provided evidence about the revalorisation of food by-products, reducing both economic and environmental impact associated to the generation of wastes in industry.

CRediT authorship contribution statement

Daniel Cantabella: Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Ramon Dolcet-Sanjuan:** Conceptualization, Writing – review & editing. **Cristina Solsona:** Data curation, Formal analysis. **Laura Vilanova:** Formal analysis, Writing – review & editing. **Rosario Torres:** Formal analysis, Data curation. **Neus Teixidó:** Conceptualization, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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