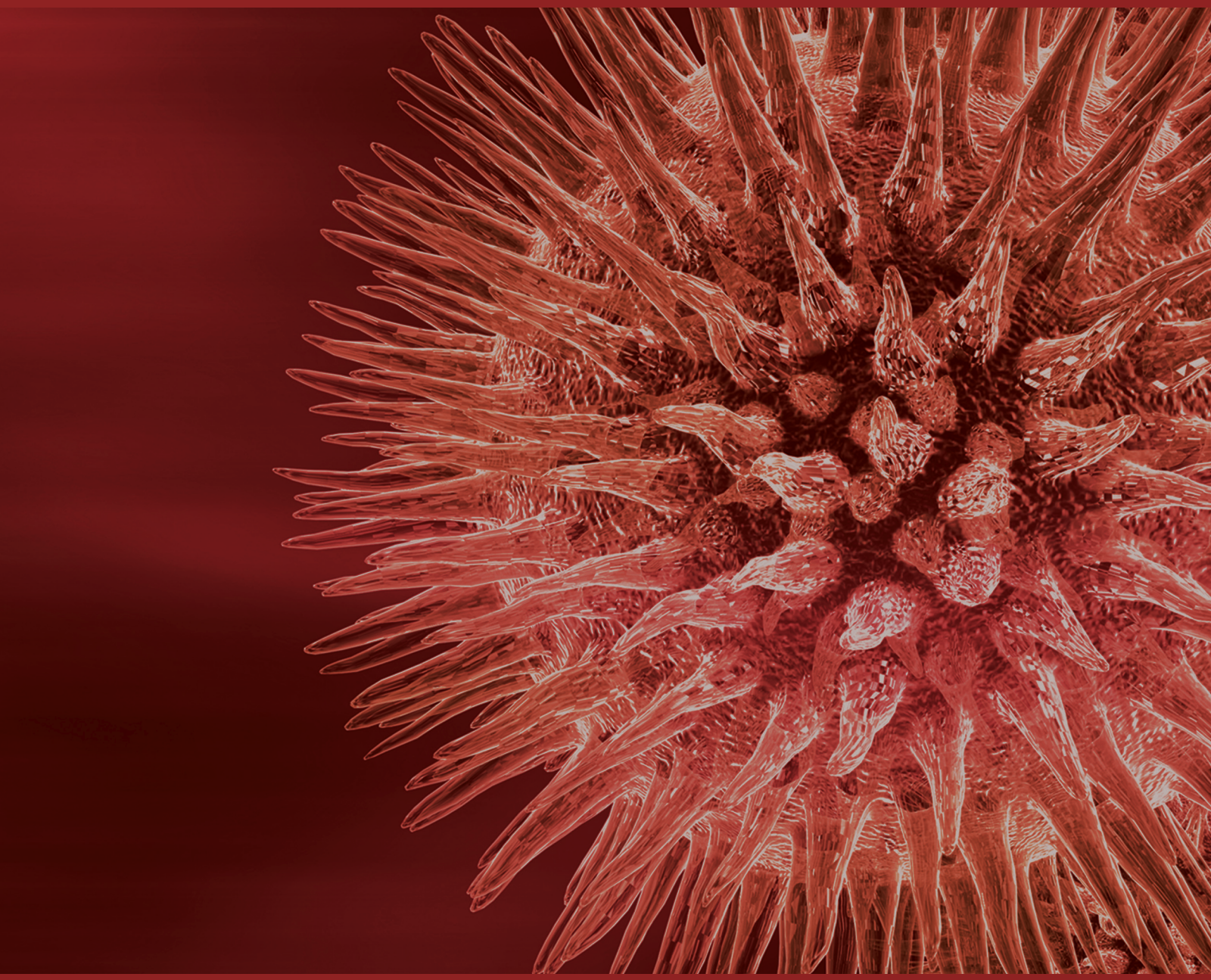
BioMed Research International

New Trends in Biotechnological Processes to Increase the Environmental Protection

Guest Editors: Ana Moldes, José Manuel Domínguez González, Ligia Raquel Marona Rodrigues, and Attilio Converti



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Editorial

New Trends in Biotechnological Processes to Increase the Environmental Protection

Ana Moldes,[1] José Manuel Domínguez González,[2] Ligia Raquel Marona Rodrigues,[3] and Attilio Converti[4]

[1] *Chemical Engineering Department, University of Vigo, School of Industrial Engineering (EEI), Campus As Lagoas Marcosende, Vigo, 36310 Pontevedra, Spain*
[2] *Chemical Engineering Department, University of Vigo, Faculty of Sciences, As Lagoas s/n, 32004 Ourense, Spain*
[3] *Institute for Biotechnology and Bioengineering (IBB), Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[4] *Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy*

Correspondence should be addressed to Ana Moldes; amoldes@uvigo.es

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A sustainable development can be achieved by deepening into more effective and eco-friendly products and technologies. From this point of view, the development of biotechnological processes to increase the environmental protection could be included in the best available techniques reference documents, the so-called BREFs, that cover, as far as practicable, the industrial activities to achieve an integrated pollution prevention and control. Members of the European Union are required to take these documents into account when determining the best available techniques, generally or in specific cases under the European Commission Directives.

In order to include biotechnological processes into the BREFs, for example, for obtaining food and pharmaceutical additives, these products have to be cost competitive with those synthesized by chemical ways. Biotechnological processes are advantageous compared to the chemical ones since various metabolites can be obtained simultaneously in the same process, and these metabolites are more eco-friendly than their chemical counterparts. Additionally, this feature also matches the increasing demand of consumers for natural products, which has intensified the biotechnological production of natural additives.

This special issue reports advances in the use of biotechnological processes for the treatment of contaminated soil or water as well as the revalorization of agroindustrial residues through the production of valuable metabolites such as biosurfactants or antioxidants, with applications in biomedicine, food industry, pharmaceutical industry, or environmental bioremediation.

Therefore, to avoid or at least to reduce the use of synthetic additives, it is necessary to identify natural alternative sources of food and pharmaceutical additives. For instance, in the recent years, there has been a growing interest in the use of natural antioxidants in the food industry not only for application as preservatives but also due to their benefits to human health. Moreover, the brewery industry generates waste that could be used to produce natural extracts containing bioactive phenolic compounds, which can be further used as natural antioxidants with potential applications in the food, cosmetic, and pharmaceutical industries.

On the other hand, water and soil pollution has become a significant environmental issue around the world. Toxic substances, such as endocrine disruptors, heavy metals, and excessive inflows of phosphorus, nitrogen, and other elements, all contribute to the water and soil pollution. Biotechnological processes, as well as metabolites extracted from plants, can be used to treat those contaminations enabling the use of mild operating conditions, as compared with other physical and chemical treatments, thus permitting a sustainable development. Accordingly, anammox bacteria can be used for the elimination of ammonium in wastewater, whereas phosphorus can be removed from water by using transgenic plants, which hyperaccumulate inorganic phosphate.

In addition, in this special issue it has been showed that epipsammic biofilms can play an important role in the environmental quality of river systems, increasing arsenic and phosphorous retention by the system, especially in environments where both arsenic and phosphorus occur simultaneously, whereas vegetables and vegetable residues can be used as sources of enzymes capable of removing colored compounds from water.

Regarding soil bioremediation, biosurfactants produced by *Lactobacillus pentosus* were found to improve the solubilization of hydrocarbons in the water phase of soil, leading to even better results than those reached with common chemical surfactants like sodium dodecyl sulfate.

Acknowledgments

We sincerely hope that the readers will find these articles helpful to their research pursuits. It has been our pleasure to put together this special issue. We also thank all the contributing authors for sharing their quality research through this special issue. Editors would like to thank the reviewers for their critical comments and suggestions, which helped to improve the quality of the Papers.

Ana Moldes
José Manuel Domínguez González
Ligia Raquel Marona Rodrigues
Attilio Converti

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Research Article

Arsenate Retention by Epipsammic Biofilms Developed on Streambed Sediments: Influence of Phosphate

D. M. Prieto,[1] R. Devesa-Rey,[2] D. A. Rubinos,[1] F. Díaz-Fierros,[1] and M. T. Barral[1]

[1] *Department of Soil Science and Agricultural Chemistry, Facultad de Farmacia, Campus Vida, 15782 Santiago de Compostela, Spain*
[2] *Defense University Center, Escuela Naval Militar, Plaza de España 2, 36920 Marín, Spain*

Correspondence should be addressed to D. M. Prieto; diego.martina@usc.es

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Natural geological conditions together with the impact of human activities could produce environmental problems due to high As concentrations. The aim of this study was to assess the role of epipsammic biofilm-sediment systems onto As (V) sorption and to evaluate the effect of the presence of equimolar P concentrations on As retention. A natural biofilm was grown on sediment samples in the laboratory, using river water as nutrient supplier. Sorption experiments with initial As concentrations 0, 5, 25, 50, 100, 250, and 500 $\mu g\,L^{-1}$ were performed. The average percentage of As sorbed was 78.9 ± 3.5 and 96.9 ± 6.6% for the sediment and biofilm-sediment systems, respectively. Phosphate decreased by 25% the As sorption capactity in the sediment devoid of biofilm, whereas no significant effect was observed in the systems with biofilm. Freundlich, Sips, and Toth models were the best to describe experimental data. The maximum As sorption capacity of the sediment and biofilm-sediment systems was, respectively, 6.6 and 6.8 $\mu g\,g^{-1}$ and 4.5 and 7.8 $\mu g\,g^{-1}$ in the presence of P. In conclusion, epipsammic biofilms play an important role in the environmental quality of river systems, increasing As retention by the system, especially in environments where both As and P occur simultaneously.

1. Introduction

Arsenic (As) is a ubiquitous contaminant which is widely distributed in the environment. Due to its toxicity, its presence in soils, sediments, and water, even at very low concentrations, may cause serious health hazards, increasing the incidence of cancer and dermatological, vascular, and cerebrovascular diseases. For this reason, it was one of the first chemicals recognized as carcinogens [1]. It is estimated that 40 million people worldwide are at risk from drinking As-contaminated water [2]. Several cases of people affected by As pollution have been reported; thus, for example, thousands of arsenic poisoned patients were identified in Bangladesh, suffering from skin lesions and gangrene in legs as well as various types of cancer [3]. Consequently, the World Health Organization (WHO) has set the level of arsenic allowed at 10 $\mu g\,L^{-1}$ in drinking water [4].

Environmental As problems are commonly the result of mobilization under natural conditions, such as weathering of arsenic-bearing minerals and geothermal sources, but human activities have contributed to an important additional impact by means of mining processes, fossil fuel combustion, and the use of arsenic in pesticides, herbicides, crop desiccants, and livestock feed [5].

Dissolved As can occur in aquatic systems in both organic and inorganic forms. Inorganic As species predominate in sediments and water, but, in contrast, organoarsenic compounds prevail in marine organisms [6].

The inorganic As can be present in natural aquatic systems in four oxidation states: +V (arsenate), +III (arsenite), 0 (elemental As), and −III (arsine). The oxidation state is determined by pH and Eh. As (V) and As (III) are the common valence states in natural waters. As (V) is the thermodynamically stable form that generally predominates in oxic surface waters, whereas As (III) is favoured in environments with low pH and low redox potential [7]. In natural waters and at normal pHs, arsenate and arsenite are present as oxyanions (such as $H_2AsO_4^-$ and $HAsO_4^{2-}$) and as neutral aqueous species (H_3AsO_3), respectively [8].

As previously mentioned, As may also occur in organic forms due to biological transformation of inorganic arsenic species. In the literature, this fact has been widely reported,

showing that microorganisms may methylate As species as monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), and trimethylarsine oxide (TMAO) [9, 10]. Additionally, arsenosugars could be produced by seaweed [11], whereas arsenobetaine and arsenocholine could be produced by marine animals [12–14].

Arsenic toxicity is dependent on the chemical form in which As is presented (inorganic or organic) and on its oxidation state. Traditionally, the inorganic forms of As have been considered more toxic than the organic forms [15]. Among inorganic forms, As (III) is in general considered more toxic, soluble and mobile than As (V) [16].

In rivers, sediments act as a significant sink of As, although changes in the river flow or in other environmental conditions (Eh, pH, and changes in water composition) may cause adsorption or desorption processes which should be controlled. In the last years, studies based on As adsorption onto sediments were reported by Rubinos et al., Bostick et al., Stollenwerk et al., Borgnino et al., and Mandal et al. [17–21]. Arsenic adsorption capacity has been related to the content of metal oxides, particularly of Al, Fe, and Mn [22, 23], and to the clay content of sediments [5].

A significant aspect to be taken into account when As (V) adsorption is studied is the potential competition between arsenate and phosphate for surface sorption sites. Phosphate concentration has been considered a critical factor in the adsorption or release of As from solid phases [24]. Arsenate and phosphate behave both as oxyanions and present striking similarities such as quasi-identical pK_a values and charged oxygen atoms [25]. Phosphate strongly competes with As (V) for surface sites, inhibiting As (V) adsorption by Fe and Al oxides [26]. In the literature, the mobilization of As by P from sediments has been widely reported by Kaplan and Knox, Bauer and Blodau, Stollenwerk et al., Rubinos et al., and Rubinos et al. [19, 27–30], amongst others.

The role of organisms that colonize the sediment water interface must also be taken into account. In recent years, several studies have treated the sorption and removal of arsenate by means of iron-oxidizing bacteria [31], the seaweed *Lessonia nigrescens* [32], and by sulphate-reducing bacteria [33]. Therefore, we hypothesize that As adsorption capacity may be affected by the presence of biofilms in the water-sediment interface. Costerton (2007) defines a biofilm as a universal community of microorganisms (bacteria, fungi, cyanobacteria, algae, and protozoa) linked to wet surfaces or interfaces and embedded by a polymeric matrix (EPS) which allows an efficient water, nutrients and gas exchange between constituent populations and the outside environment [34]. Biofilms play an important role in rivers systems as they constitute the interface between the overlying water and the sediments and are the first to interact with dissolved substances such as nutrients, organic matter, and toxicants [35].

The literature and investigations on the behaviour of epipsammic biofilms on the retention of heavy metals and metalloids are scarce, so as in natural river ecosystems such as at the microcosm and mesocosm scales. Published researches, as previously mentioned, are focused on As retention by sediments and by certain isolated organism but not on the whole river bed system with the presence of multispecies biofilms, which will be one of the objectives of this study.

In this work, the effect of epipsammic biofilms developed over riverbed sediments on As retention is evaluated as well as their environmental role in river systems with presence of problematic As (V) concentrations. The capacity of As (V) retention of biofilm-sediment systems will be compared to that of the sediment without biofilm, as well as the potential remobilization produced after the retention. The effect of the biofilm on As retention in the presence of equimolar P concentrations was also assessed.

2. Materials and Methods

2.1. Sediment Sample. The sediment sample was obtained in the Anllóns River, in a noncontaminated area upstream of the town of Carballo. A complex sample was collected with a small plastic shovel from the top 5 cm at various points at the same site and taken to the laboratory in hermetic plastic containers topped up to prevent oxidation. The Anllóns basin is located in the NW of Spain and was selected because gold mining activities were carried out in the area during the Roman Empire and between 1895 and 1910 [36]. Arsenopyrite associated to Au produced elevated As concentrations in the bed sediments downstream the mineralized areas [17, 29, 30, 36]. Nowadays, the exploitation of the mineralized area is under study, causing social concern and controversy among the locals.

2.2. Sediment and River Water Characterization. Grain size distribution of the sediment was determined as it was described by Guitián and Carballas, and the fractions were classified as coarse sand (2–0.2 mm), fine sand (0.2–0.05 mm), coarse silt (0.05–0.02 mm), fine silt (0.02–0.002 mm), and clay (<0.002 mm) [37]. Total P (P_T) was determined by acid digestion (HF, H_2SO_4, HCl, 10 : 1 : 10) followed by colorimetric determination with molybdenum blue, as described by Murphy and Riley [38].

Nitrogen was determined by wet digestion with H_2SO_4, by using the Kjeldahl method as described in Guitián and Carballas [37]. The concentration of total organic carbon (TOC) of the samples was determined according to the procedure proposed by Sauerlandt and modified by Guitián and Carballas [37], in an automatic titration system.

Sediment native As concentration was determined by X-ray fluorescence spectrometry (custom built, equipped with a Philips high-voltage generator and a Mo anade of 2.2 kW as X-ray source), following the method described by Devesa-Rey et al. [36]. The concentration of Al, Fe, and Mn was also determined.

River water was collected and filtered by 0.45 μm to be employed as biofilm growth medium in the laboratory in order to better reproduce the natural conditions for biofilm growth. pH and conductivity were determined, as well as soluble P by means of an acid digestion with H_2SO_4 followed by colorimetric determination with ammonium molybdate [39].

2.3. Native Biofilm Growth. A natural biofilm was grown in indoor systems during 15 days over 8 g of riverbed sediment, using 60 mL of natural river water as nutrient supplier, in small plastic containers of 100 mL. The samples were subjected to day-night cycles (12 h of light with 3,109 lux of intensity) to reproduce approximately the natural environmental conditions. The overlying river water was replaced each 5 days together with the addition of 0.5 mL of inoculum (fresh river biofilm) in order to stimulate the biofilm growth. Once the biofilm was developed, the overlying water was removed, and its total P was measured by acid digestion with H_2SO_4.

2.4. Arsenate Sorption Experiments. To evaluate the sorption capacity and desorption behaviour of the biofilm-sediment system, *batch* experiments were conducted with 8 g sediment and their corresponding formed biofilm. In parallel, samples without biofilm following the same treatment of the biofilm-sediment samples were used as controls.

60 mL of As (V) solutions with initial concentrations (C_0) of 0, 5, 25, 50, 100, 250, and 500 μg L^{-1}, prepared in 0.01 M $CaCl_2$ solutions as background electrolyte, were added to the systems. All the experiments were carried out in triplicate. Arsenate solutions were prepared from a stock standard solution of 1000 mg L^{-1} (Panreac, Barcelona, Spain). All the samples were prepared in triplicate. The batch experiments were carried out at room temperature (20 ± 2°C). Eh and pH measurements were carried out with a Thermo Scientific Orion Dual Star meter with a combined Redox/ORP electrode and with a AQUAPRO pH electrode (Beverly, USA), respectively. After 24 h, a pseudoequilibrium state was reached, and the overlying water was taken (pipetting without altering the system). Aliquots were filtered through a 0.45 μm Whatman filter, and As concentration (C_e) of the samples was determined by Inductively Coupled Plasma Spectrometry (ICP-MS, Varian 820 MS) with collision reaction interface (CRI) technology to reduce polyatomic interferences. The adsorbed As (V) concentrations (Q_{ads}) for the sediment or biofilm-sediment systems were obtained by the difference between C_0 and C_e, taking into account the water volume and sediment weight.

For the study of the desorption behaviour, 60 mL of 0.01 M $CaCl_2$ solutions were added to the previous loaded systems. After 24 h, aliquots of the overlying water were extracted by gently pipetting. Again, the samples were filtered and As concentration measured by ICP-MS. The weight of the samples was controlled in every moment to calculate the mass of As desorbed. All the experiments were carried out at pH 5.5 adjusted by addition of 0.1 M NaOH or HCl solutions.

2.5. Influence of Phosphorus Presence on Arsenate Sorption Process. To assess the influence of P presence on arsenate sorption, experiments with solutions of equimolar As(V): P concentrations were carried out, using the aforementioned procedure and concentrations used for the As (V) sorption experiments. P solutions were obtained by dissolution of KH_2PO_4 (Panreac, Barcelona, Spain). As and P concentrations in the supernatants were determined by ICP-MS.

2.6. Sorption Modelling. The adsorption experimental data were fitted using a linear equation, four two-parameters models (Freundlich, Langmuir, Dubinin-Radushkevich, and Temkin), and three three-parameters models (Redlich-Peterson, Sips, and Toth).

The linear equation was given by (1):

$$Q_e = AC_e - B, \tag{1}$$

where Q_e is the adsorbed or desorbed As concentration for the sediment or biofilm-sediment system, A is the slope, and B is the content of native arsenic.

The Freundlich equation (2) is used to describe heterogenous systems characterized by a heterogenous factor $1/n$:

$$Q_e = K_f C_e^{1/n}, \tag{2}$$

where K_f and n are empirical constants of the Freundlich model which are referred to as the capacity and intensity of adsorption, respectively [40].

The Langmuir equation (3) assumes monolayer coverage of adsorbate over a homogenous adsorbent surface:

$$Q_e = \frac{(Q_{\text{Max}} b C_e)}{(1 + bC_e)}, \tag{3}$$

where Q_{Max} is the maximum adsorption capacity of the system and b is a constant related to the energy bonds As-sediment and As-biofilm sediment interface [41].

The Dubinin-Radushkevich model isotherm is generally given by (4) [42]:

$$Q_e = q_D \exp\left(-B_D\left[RT\ln\left(1 + \frac{1}{C_e}\right)\right]^2\right), \tag{4}$$

where B_D is related to the mean free energy of sorption per gram of the sorbate as it is transferred to the surface of the solid from infinite distance in the solution [43].

The Temkin isotherm model contains a factor which takes into the account of adsorbent-adsorbate interactions and has been generally used in the form of (5) [44]:

$$Q_e = \frac{RT}{b_T}\ln\left(A_T C_e\right). \tag{5}$$

The Redlich-Peterson empirical equation (6) incorporates features of both Langmuir and Freundlich equations [45]. It can be applied to represent adsorption equilibrium over a wide concentration range:

$$Q_e = \frac{(K_R C_e)}{\left(1 + a_R C_e^{\beta}\right)}. \tag{6}$$

Sips model isotherm is also called Langmuir-Freundlich isotherm [46]. At low sorbate concentrations, it reduces to a Freundlich isotherm, and at high sorbate concentrations, a monolayer sorption capacity is predicted [47]:

$$Q_e = \frac{\left(K_S C_e^{1/b_S}\right)}{\left(1 + a_S C_e^{1/b_S}\right)}. \tag{7}$$

The Toth isotherm model is an empirical equation useful in describing heterogeneous adsorption systems [48]. Equation (8) exhibits the most general form of this model:

$$Q_e = \frac{(K_t C_e)}{\left[(a_t + C_e)^{1/t}\right]}. \quad (8)$$

The parameters of all studied models were estimated by nonlinear regression procedure employing Table Curve software (Jandel Scientific).

2.7. Statistical Analyses. Five error functions were tested in order to choose the best model to fit the experimental data. These error functions were the coefficient of determination (R^2), sum of absolute errors (EABS), hybrid fractional error function (HYBRYD), average relative error (ARE), and Marquardt's percent standard deviation (MPSD) and were calculated employing the equations described by Foo and Hameed [49].

The adsorbed concentrations of the different studied systems were evaluated by one-factor analysis of variance (ANOVA). Critical F values ($\alpha = 0.05$) were used to evaluate if the factor is significant. In the case of positive significance, post hoc analyses using the Duncan comparison test ($\alpha = 0.05$) were performed to establish statistical differences between the means (SPSS 19.0 statistical package).

2.8. Theoretical Aqueous Speciation. Visual MINTEQ V 3.0 was employed to theoretically calculate As species in the solutions and to determine their saturation degree, expressed as saturation index (SI) with respect to mineral phases, by means of thermodynamic calculations and (9):

$$\mathrm{SI} = \log\left(\frac{\mathrm{IAP}}{K_C}\right), \quad (9)$$

where IAP is the ionic activity product of the specific dissolution-precipitation reaction and K_C is equilibrium constant. Negative SI indicates a mineral which has potential to dissolve, whereas positive SI shows a mineral which has thermodynamic potential to precipitate [50].

To study the influence of the pH and organic matter in arsenic speciation, sweeps with values between 4 and 10 and 1 and 10 mg L^{-1}, respectively, were performed.

3. Results and Discussion

3.1. Sediment and River Water Characterization. The sediment sample collected for this study showed a predominance of the sandy fraction, with an average value of 86.3%, and only 6.7% of clayey fraction. Total organic matter content for the sediment was of 13.9 ± 0.6 g kg^{-1}. P and N concentrations presented average values of 471.9 ± 43.7 and 629.6 ± 98.3 mg kg^{-1}, respectively. The total As concentration of sediment determined by X-ray fluorescence spectrometry was 11.8 mg kg^{-1}, whereas the sediment content of Al, Fe, and Mn was 50.4, 51.9, and 1.2 g kg^{-1}, respectively.

The values of pH, electrical conductivity, and soluble P concentration in the river water were 6.87, 71.80 μS cm^{-1}, and 0.21 mg L^{-1}, respectively.

3.2. Arsenate Retention. Figure 1 shows the experimental data for As (V) sorption in function of the equilibrium As concentrations in the solution for the sediment and biofilm-sediment systems. Q_{ads} increased with increasing initial concentrations in all cases, thus indicating that the adsorption was not at its maximum. The values of Q_{ads} for sediment system without biofilm are in the range of the data reported by Stollenwerk et al. [19] and Borgnino et al. [20] but slightly lower because they used a different ratio (solution/sediment), lower native As concentrations in sediments, and higher added As concentrations.

It is noteworthy that Q_{ads} values for the samples with biofilm were higher than for the samples without biofilms. The average percentage of As adsorbed with respect to C_0 for the sediment and biofilm-sediment system was 78.9 ± 3.5 and 96.9 ± 6.6%, respectively. The difference between Q_{ads} for the biofilm-sediment system and for the sediment without biofilm increased in the range of studied concentrations from 6.81×10^{-3} up to 4.69×10^{-1} μg g^{-1} and was significant from As solutions of concentrations ≥50 μg L^{-1}. This may be explained by an increase in the specific surface area and the number of sorption sites and functional groups due to the presence of the biofilm, as well as arsenate biouptake by microorganisms which constitute the biofilm. Arsenate could enter cells through phosphate-transporting systems [51]. Arsenate bioaccumulation and biouptake in green algae was studied by Karadjova et al. [52] and by Wang et al. [53]. Karadjova et al. [52] reported that intracellular As increased linearly when As (V) concentrations increased up to 50 μM, followed by a single saturation plateau.

Studies about As retention onto episammic biofilm have not been reported. However, the removal of heavy metals by means of bacteria biofilm has been widely and successfully studied. For example, a biofilm of *Arthrobacter viscosus* was applied to remove Cr(VI), Cd(II), and Ni(II) [43, 54–56] whereas a biofilm of *Pseudomonas aeruginosa* was employed to the removal of Cr(III), Ni(II), and Co(II) [57].

These results highlight the important role that biofilms may play in river environments by increasing As (V) retention. Biofilms could promote As sequestration from the water column; therefore, they could be potentially employed as a bioremediation tool for contaminated waters due to the larger surface area of the biofilm, with more functional groups where As can be adsorbed.

Figure 2 presents Q_{ads} for As, in the presence of equimolar P for the sediment and biofilm-sediment systems. Again, Q_{ads} values for the biofilm-sediment system were higher than for sediment without biofilm. The difference between Q_{ads} for both systems increased in the range of studied concentrations from 0.00 up to 1.56 μg g^{-1} and was significant again from an initial concentration of 50 μg As L^{-1}. At the highest As concentration, this difference was three times higher than in the experiments without P. This behaviour could be attributed to the P presence which caused a significant reduction on

As (V) adsorbed by the sediment without biofilm (a 25% reduction at the highest added As concentrations), whereas no significant effect was detected in systems where biofilm took part.

The competition between phosphate and arsenate for sorption sites in sediments has been widely reported. Thus, for example, Stollenwerk et al. [19] reported that for concentrations of As (V) of 100 $\mu g\,L^{-1}$, the presence of 2 $mg\,L^{-1}$ of phosphate completely inhibited As (V) adsorption. Rubinos et al. [29] also showed that the addition of increasing concentrations of phosphate enhanced the As (V) release from sediments of the Anllóns River, and, in the same line, Rubinos et al. [30] confirmed that P increased the As mobilization from these sediments in a wide range of pH (3–10). Nevertheless, data have not been reported about the effect of P on As (V) sorption in the presence of biofilms. In this study, no significant effect of phosphate in As concentration of the overlying water was observed in systems with biofilm, which could be explained by the increase of sorption sites promoted by the biofilm and/or by the increase of intracellular arsenic uptake by microorganisms which constitute the biofilm.

Desorption processes were negligible at the lowest and middle concentrations in all the studied systems and represented less than 0.5% of the sorbed As concentrations at the highest initial As concentration.

3.3. Sorption Modelling. Figures 1 and 2 showed the sorption curves for all the studied systems with their corresponding fits. Sorption isotherms were of type I according to Brunauer's classification [58]. Table 1 shows the parameters for the different tested models. According to them, sorption data were satisfactorily adjusted by all the models. The values of the parameter A of the linear model were higher for the biofilm-sediment systems, especially in the presence of phosphate, while the lowest was obtained for sediment without biofilm in the presence of P.

The equilibrium arsenic concentration (EAC) is defined analogously to equilibrium phosphorous concentration (EPC) as the concentration of As that is supported by the sediment when in contact with an ambient solution such that no arsenate is either gained or lost by the sediment [17]. When As concentrations in water are higher than EAC, the sediment would act as a sink for As, whereas for water As concentrations lower than EAC, the sediment would act as a source of As. Calculated EAC values ranged from 2.62 to 18.28 $\mu g\,L^{-1}$; the lowest EAC corresponded to the biofilm-sediment system with P and the highest to the sediment system with P, thus pointing to a higher risk of As transfer towards the water column.

Among the analysed two-parameters models, the Freundlich model was considered the most suitable to fit experimental data in all cases; the highest R^2 values and the lowest values of other error functions are shown (Table 2). The better fits of Freundlich model are indicative of the heterogeneous surface of the solid phases studied. Langmuir model also successfully adjusted the experimental data, with R^2 values above 0.92 in all cases, whereas the Dubinin-Radushkevich and Temkin models were not completely satisfactory.

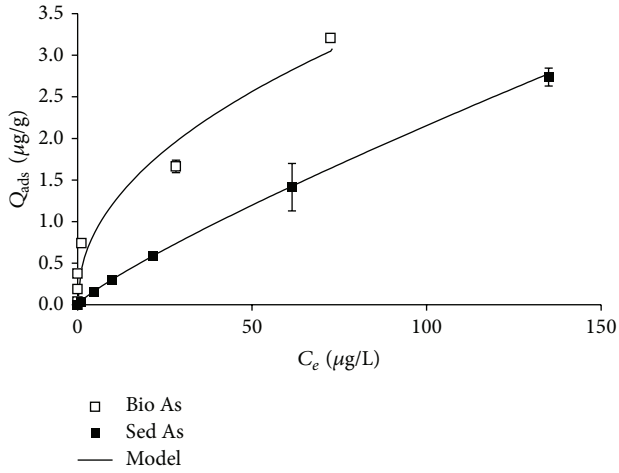

Figure 1: As (V) retention by biofilm-sediment and sediment systems in function of dissolved As equilibrium concentration.

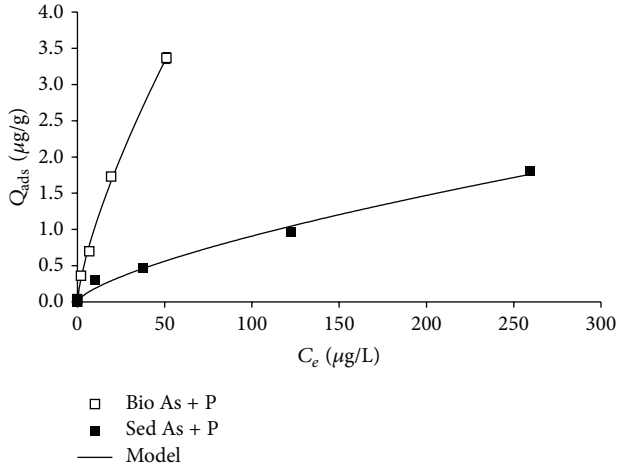

Figure 2: As (V) retention for biofilm-sediment and sediment systems in function of dissolved As equilibrium concentration in the presence of equimolar P concentrations.

The estimated maximum adsorption capacity of the sediment and biofilm-sediment systems, obtained from the Langmuir model, was 6.6 and 6.8 $\mu g\,g^{-1}$, respectively, and 4.5 and 7.8 $\mu g\,g^{-1}$, respectively, in the presence of P. These values fall within the range of, but slightly lower than, those reported by Stollenwerk et al. [19] when studying As adsorption oxidized aquifer sediments. Again, these results highlight the key effect that the presence of biofilm causes in the fate of As in the river system, mainly in the presence of P.

The essential characteristics of the Langmuir isotherm can be expressed in terms of an equilibrium parameter, R_L, which allows to determine if the adsorption process is favourable or unfavourable [59]. Equation (10) shows the relationship between R_L and C_0:

$$R_L = \frac{1}{(1 + bC_0)}. \tag{10}$$

Table 1: Parameters of theAs(V) sorption models.

	Sediment As(V)	Biofilm As(V) + P	Sediment As(V)	Biofilm As(V) + P
Linear				
A	2.02×10^{-2}	6.60×10^{-3}	4.15×10^{-2}	6.51×10^{-2}
B	7.19×10^{-2}	1.21×10^{-1}	2.83×10^{-1}	1.71×10^{-1}
Freundlich				
k_f	4.35×10^{-2}	3.71×10^{-2}	4.12×10^{-1}	1.91×10^{-1}
n	1.18	1.44	2.14	1.37
Langmuir				
Q_{Max}	6.60	4.52	6.79	7.78
b	4.94×10^{-3}	2.50×10^{-3}	1.22×10^{-2}	1.49×10^{-2}
Dubinin-Radushkevich				
q_D	3.23	1.54	3.614	3.72
B_D	5.07×10^{-4}	3.50×10^{-4}	1.04×10^{-4}	4.73×10^{-5}
Temkin				
A_T	2.48	1.28×10^{-1}	2.94	4.79×10^{-1}
b_T	4.93×10^{3}	5.68×10^{3}	4.84×10^{3}	2.66×10^{3}
Redlich-Peterson				
k_r	6.00×10^{-2}	3.87×10^{5}	1.25×10^{6}	7.80×10^{5}
a_r	5.59×10^{-1}	1.04×10^{7}	3.11×10^{6}	4.07×10^{6}
B	2.56×10^{-1}	3.05×10^{-1}	5.32×10^{-1}	2.70×10^{-1}
Toth				
k_t	4.69×10^{-2}	5.32×10^{-2}	2.59×10^{-1}	1.97×10^{-1}
a_t	3.91	6.02×10^{-3}	3.62×10^{-2}	3.60×10^{-1}
t	5.89	2.68	2.43	3.62
Sips				
k_s	4.09×10^{-2}	1.04×10^{-1}	1.07×10^{-2}	3.45×10^{-2}
a_s	1.15	4.28	330.52	66.13
b_s	7.18×10^{-4}	-2.15×10^{-1}	-9.84×10^{-1}	-9.32×10^{-1}

The values of R_L ranged between 0 and 1 for all the analyzed concentrations, which corresponds to a high affinity favorable adsorption process, being more favorable at the highest initial As concentrations. As it could be seen in Figure 3, the biofilm-sediment systems in the presence of phosphate present the highest affinity for As (the lowest R_L), whereas the sediments in the presence of phosphate show the lowest one (the highest R_L).

Gibbs free energy of adsorption process could be obtained from Langmuir and Temkin models by means of (11) and (12), respectively:

$$\ln\left(\frac{1}{b}\right) = \frac{\Delta G^0}{RT}, \tag{11}$$

$$\ln\left(\frac{1}{A_T}\right) = \frac{\Delta G^0}{RT}. \tag{12}$$

The values of Gibbs free energy yield negative values in all cases, which indicate that the adsorption process was always spontaneous. The biofilm-sediment system in the presence of P showed the most negative value by applying (11) ($-34.50\,\text{kJ mol}^{-1}$) and (12) ($-43.10\,\text{kJ mol}^{-1}$), whereas the sediment system with P presented the least negative value ($-30.08\,\text{kJ mol}^{-1}$ by (11) and $-39.83\,\text{kJ mol}^{-1}$ by (12)). The systems without P showed an intermediate behaviour, having more spontaneous As (V) retention in biofilm-sediment system. Therefore, the presence of biofilms jointly with P presence suggests that As retention process was more favoured.

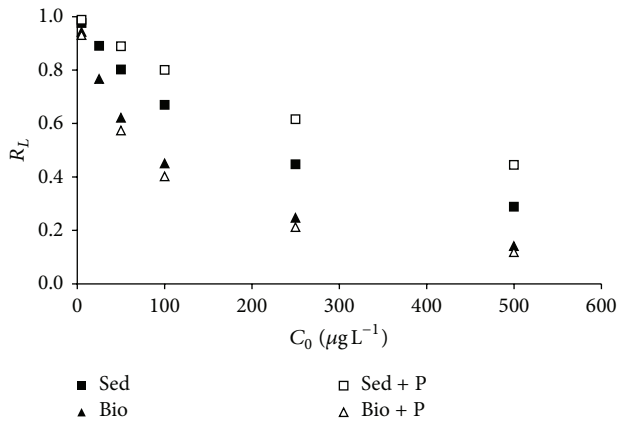

Figure 3: R_L values for all studied systems in function of initial As concentrations.

Table 2: Values of error functions for each model and for each analysed system.

	Sediment		Native biofilm	
	As(V)	As(V) + P	As(V)	As(V) + P
Freundlich				
R^2	0.999	0.990	0.953	0.998
EABS	7.83×10^{-2}	2.89×10^{-1}	1.36	2.26×10^{-1}
HYBRYD	8.31	3.77×10^{-1}	7.29×10^{1}	3.13×10^{1}
ARE	5.94	2.51×10^{1}	5.21×10^{1}	2.09×10^{1}
MPSD	1.40×10^{2}	5.37×10^{2}	8.00×10^{2}	5.06×10^{2}
Langmuir				
R^2	0.996	0.977	0.927	0.998
EABS	2.85×10^{-1}	4.31×10^{-1}	1.34	2.09×10^{-1}
HYBRYD	6.01	4.78×10^{1}	7.86×10^{1}	3.49×10^{1}
ARE	4.29	3.19×10^{1}	5.61×10^{1}	2.33×10^{1}
MPSD	6.18×10^{1}	5.98×10^{2}	8.68×10^{2}	5.30×10^{2}
Dubinin-Rabushkevich				
R^2	0.925	0.854	0.912	0.930
EABS	1.24×10^{1}	3.15	4.87	8.65
HYBRYD	1.73×10^{3}	1.79×10^{2}	9.36×10^{1}	3.68×10^{2}
ARE	1.24×10^{3}	1.19×10^{2}	6.69×10^{1}	2.46×10^{2}
MPSD	2.51×10^{4}	2.37×10^{3}	1.60×10^{3}	5.10×10^{3}
Temkin				
R^2	0.760	0.839	0.810	0.895
EABS	5.48	9.30×10^{-1}	1.19	1.51
HYBRYD	5.41×10^{2}	3.77×10^{1}	1.37×10^{1}	4.80×10^{1}
ARE	3.86×10^{2}	2.52×10^{1}	9.79	3.20×10^{1}
MPSD	6.74×10^{3}	4.20×10^{2}	1.91×10^{2}	5.98×10^{2}
Redlich-Peterson				
R^2	0.999	0.990	0.953	0.998
EABS	1.74×10^{-2}	2.44×10^{-1}	1.40	1.97×10^{-1}
HYBRYD	3.46	1.25×10^{1}	7.31×10^{1}	6.36
ARE	2.47	8.35	5.22×10^{1}	4.24
MPSD	6.70×10^{1}	1.96×10^{2}	8.02×10^{2}	7.87×10^{1}
Toth				
R^2	0.999	0.987	0.948	0.998
EABS	1.06×10^{-2}	3.60×10^{-1}	1.27	2.39×10^{-1}
HYBRYD	1.57	3.81×10^{1}	7.50×10^{1}	3.18×10^{1}
ARE	1.12	2.54×10^{1}	5.35×10^{1}	2.12×10^{1}
MPSD	2.83×10^{1}	5.22×10^{2}	8.26×10^{2}	5.07×10^{2}
Sips				
R^2	0.999	0.999	0.984	0.978
EABS	1.12×10^{-2}	3.34×10^{-1}	0.99	2.69×10^{-1}
HYBRYD	1.62	3.68×10^{1}	6.80×10^{1}	3.30×10^{1}
ARE	1.23	2.27×10^{1}	4.97×10^{1}	2.31×10^{1}
MPSD	2.89×10^{1}	5.01×10^{2}	7.98×10^{2}	5.29×10^{2}

Table 2 shows also that the three-parameters models, especially Sips and Toth models, also satisfactorily fit the experimental data based on the high R^2 values (>0.95) and low values of the error functions.

3.4. Theoretical Aqueous Speciation. The calculations performed by Visual MINTEQ indicated that in the studied systems, arsenic was present as inorganic As (V), with approximately 95% of total species present as $H_2AsO_4^-$. Negative values of SI were found in all studied cases, which indicated that the conditions are not favourable for the precipitation of As minerals. At pH 4, the monovalent $H_2AsO_4^-$ species prevails (approximately 98% of total aqueous As), whereas at pH 10, the bivalent $HAsO_4^{2-}$ species prevails (approximately 96% of the total aqueous As). In the studied conditions, the calculations did not predict significant complexation of As with the dissolved organic matter.

4. Conclusions

The biofilm increases the As (V) sorption capacity of the studied sediment. An input of P, at equimolar P: As concentrations, reduces the sorption of As (V) on the sediment, whereas no significant effect is exhibited by systems with biofilm. The Freundlich model is the best, amongst the two-parameters models, to fit the As retention in these

systems, which is indicative of their heterogeneous surface. The sorption of As is spontaneous and favourable in all cases, especially under the combined effect of biofilm and P, whereas the desorption this As retained is not significant. Overall, epipsammic biofilms play a key role in the fate and mobility of As in riverine environments and particularly in the transference of As from the water column to the sediment. They seem to enhance the sorption capacity and the affinity of the sediments for As, especially in environments where both As and P occur simultaneously.

Nomenclature

a_R: Redlich-Peterson model parameter (6) ($L\,\mu g^{-1}$)
a_S: Sips model parameter (7) ($L\,\mu g^{-1}$)
a_t: Toth model parameter (8) ($\mu g\,g^{-1}$)
A: Linear model parameter (1) ($L\,g^{-1}$)
A_T: Temkin isotherm equilibrium binding constant ($L\,\mu g^{-1}$) (5)
b: Langmuir model parameter (3) ($L\,\mu g^{-1}$)
b_S: Sips model parameter (7) (–)
b_T: Temkin model parameter (5)
B: Linear model parameter (1) ($\mu g\,g^{-1}$)
B_D: Dubinin-Radushkevich parameter (4)
C_e: Solution pseudoequilibrium concentration ($mg\,L^{-1}$)
C_0: Solution initial concentration ($\mu g\,L^{-1}$)
k_f: Freundlich model parameter (2) ($L\,g^{-1}$)
K_S: Sips model parameter (7) ($L\,\mu g^{-1}$)
K_R: Redlich-Peterson model parameter (6) ($L\,\mu g^{-1}$)
K_t: Toth model parameter (8) ($L\,\mu g^{-1}$)
n: Freundlich model parameter (2) (–)

q_D: Dubinin-Radushkevich model parameter (4) ($\mu g\,g^{-1}$)
Q_{ads}: Adsorbed concentration by sediment or biofilm-sediment interface ($\mu g\,g^{-1}$)
Q_{des}: Desorbed concentration by sediment or biofilm-sediment interface ($\mu g\,g^{-1}$)
Q_e: Solid-phase equilibrium concentration ($\mu g\,g^{-1}$)
Q_{Max}: Maximum adsorption capacity ($\mu g\,g^{-1}$) (3)
R_L: Langmuir equilibrium parameter (10) (–)
t: Toth model parameter (8) (–)
T: Absolute temperature (K) ((4), (5), (11), and (12))
R: Gas constant ($J\,mol^{-1}\,K^{-1}$) ((4), (5), (11), and (12)).

Greek Letters

β: Redlich-Peterson parameter (6) (–).

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Research Article

Discoloration of Indigo Carmine Using Aqueous Extracts from Vegetables and Vegetable Residues as Enzyme Sources

A. Solís,[1] F. Perea,[1] M. Solís,[2] N. Manjarrez,[1] H. I. Pérez,[1] and J. Cassani[1]

[1] *Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana, Xochimilco, Calzada del Hueso 1100, Colonia Villa Quietud, 09460 Coyoacán, DF, Mexico*
[2] *Centro de Investigación en Biotecnología Aplicada, Instituto Politécnico Nacional, Ex-Hacienda San Juan Molino Carretera Estatal Tecuexcomac-Tepetitla Km 1.5, 90700 Tlax, Mexico*

Correspondence should be addressed to A. Solís; asolis@correo.xoc.uam.mx

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Several vegetables and vegetable residues were used as sources of enzymes capable to discolor indigo carmine (IC), completely or partially. Complete discoloration was achieved with aqueous extracts of green pea seeds and peels of green pea, cucumber, and kohlrabi, as well as spring onion leaves. The source of polyphenol oxidase (PPO), pH, time, and aeration is fundamental for the discoloration process catalyzed by PPO. The PPO present in the aqueous extract of green pea seeds was able to degrade 3,000 ppm of IC at a pH of 7.6 and magnetic stirring at 1,800 rpm in about 36 h. In addition, at 1,800 rpm and a pH of 7.6, this extract discolored 300 ppm of IC in 1:40 h; in the presence of 10% NaCl, the discoloration was complete in 5:50 h, whereas it was completed in 4:30 h with 5% NaCl and 2% laundry soap.

1. Introduction

The textile industry is one of the most polluting industries, due to the large amounts of water and the great quantity of unused dyes and other chemicals released in wastewaters. Previous studies have shown that textile dyes are toxic to flora and fauna and that their partial degradation products are mutagenic and carcinogenic [1]. Therefore, they present a potential health hazard to all forms of life [2]. Textile industries are the largest consumers of dyes, and it is estimated that 10–15% of the dyes are lost during the dying process and mainly released as sewage [3]. Effluents contaminated with colorants are usually treated by using physical and chemical methods [4]; however, these methods are expensive, lead to the formation of hazardous byproducts, and require high energy input [5]. The use of biological systems for the treatment of these wastewaters has become an attractive alternative [4, 6]. Phytoremediation is valuable to remove or destroy contaminants, because plants can be used to decontaminate soils, industrial sites, brownfields, sediments, and water containing metals and/or organic compounds. It is a low-cost, environmentally friendly technology for the extraction, degradation, or fixation of the contaminants [6, 7]. Oxidoreductases such as polyphenol oxidases (PPOs) and peroxidases have been shown to be effective in the degradation of recalcitrant pollutants from contaminated sites. Peroxidases are enzymes that require hydrogen peroxide or other redox mediators to oxidize a wide variety of inorganic and organic substrates, including dyes; examples of peroxidases used for the discoloration of dyes are those from *Trichosanthes dioica* [8], cauliflower [9], turnip [10, 11], bitter gourd [12], horseradish [13], and chayote [14]. PPOs are a low-cost alternative for the discoloration of dyes because they use free molecular oxygen as oxidant, for example, the PPO from potato and brinjal [15] and banana peel [16]. The purpose of the present study was to evaluate the potential of aqueous extracts of some vegetables and vegetable residues as PPO sources to discolor indigo carmine (IC). The advantages of the selected biological materials are that they are inexpensive and easily accessible;

in addition, some of the residues of the selected vegetables such as peels and leaves are considered waste.

2. Materials and Methods

2.1. Preparation of Aqueous Extracts of Vegetables and Vegetable Residues. The selected vegetables and vegetable residues were obtained from local markets, washed with soap, rinsed with distilled water, immersed in hypochlorite solution (1%) for 5 min, and rinsed again with distilled water. Then, 10 g of the biological material was blended with 20 mL of distilled water using a food processor. The mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was used as enzyme source.

2.2. Discoloration of IC Using Aqueous Extracts of Vegetables and Vegetable Residues. IC was added to 3 mL of the aqueous extracts of the vegetables (green pea seed, cucumber, horseradish, and leek) or vegetable residues (peels of green pea, cucumber, lemon, orange, chayote, kohlrabi, and cantaloupe; leaves of turnip, spring onion, and horseradish) to get a final dye concentration of 100 ppm. The mixture was then magnetically stirred (800 rpm) at room temperature until complete discoloration or for a maximum of 36 h. A sample was centrifuged (13,000 rpm for 5 min) to assay the % discoloration of the dye; that is, the absorbance of the supernatant was measured at 610 nm using a UV-Vis spectrophotometer Beckman DU 650 (Beckman Instruments, Inc., USA). The discoloration percentage was calculated as stated in (1):

$$\%\ \text{discoloration} = \frac{A_o - A_f}{A_o} \times 100, \quad (1)$$

where A_o = initial absorbance and A_f = final absorbance.

All discoloration experiments were performed in triplicate and average values were determined.

2.3. PPO Assay. PPO enzymatic activity of green pea seeds extract was measured using catechol as substrate according to Queiroz et al. [17] with some modifications. A mixture of 1.5 mL of the aqueous extract of green pea seeds (pH 6.32) and 0.5 mL of catechol (0.2 M) in phosphate buffer (pH 6.32, 0.01 M) was incubated at 25°C for 120 min. The blank did not contain enzymatic extract. Absorbance changes were recorded at 420 nm over 3 min, in 30 s intervals, using a spectrophotometer (Beckman DU 650). One unit of PPO activity was defined as the amount of enzyme that caused a change in the absorbance of 0.001 per min.

2.4. Protein Analysis. The protein concentration of the extract of green pea seeds was determined by using the dye-binding method of Bradford [18]; bovine serum albumin was used as standard.

2.5. PPO Substrate Specificity. The PPO activity of green pea seeds was measured with caffeic acid and pyrogallic acid at concentration of 0.2 mM in phosphate buffer (pH 6.32, 0.1 M). Changes in the absorbance were determined at 420 nm for caffeic acid and at 575 nm for pyrogallic acid. The relative PPO activity was described as the percentage of the activity compared with the activity measured using catechol as substrate.

2.6. Effects of pH, Dye Concentration, and Stirring on the Discoloration of IC Using Aqueous Extract of Green Pea Seeds Extract. Aqueous extracts of green pea seeds were made in phosphate buffer (0.1 mM) of various pHs (6.0, 7.0, 8.0, 8.7, and 9.3), prepared according to the procedure described in Section 2.1. Then, IC was added to get a final concentration of 100 ppm, and the mixtures were magnetically stirred at 800 rpm until complete discoloration. Increasing concentrations of IC (50, 100, 200, 500, and 1,000 ppm) were added to the aqueous extract of a pH of 8.7 and the mixtures were stirred at 800 rpm until complete discoloration or for 48 h. The effect of stirring was determined at a pH of 7.0 and 7.6, at 800 and 1,800 rpm, and at an IC concentration of 300 ppm. The reaction was followed until complete discoloration. The % discoloration was calculated using (1).

2.7. Effect of NaCl and Soap on the Discoloration of IC Using Aqueous Extract of Green Pea Seeds Extract. To a mixture of IC (300 ppm) and aqueous extract of green pea seeds (pH 7.6), NaCl was added at concentrations of 1, 2, 4, 7, and 10%. The mixtures were stirred at 1,800 rpm until complete discoloration; a sample without salt was used as control. To a mixture of IC (300 ppm) and aqueous extract of green pea seeds (pH 7.6), 5% of NaCl and 2% of commercial laundry soap were added; the mixture was then stirred at 1,800 rpm until complete discoloration.

2.8. Detection of the Biotransformation Product. Biotransformation of IC was detected using the aqueous extract of green pea seeds as enzyme source. A mixture of IC (1,000 ppm) and aqueous extract of green pea seeds (pH 7.6) was stirred (1,800 rpm) until complete discoloration. A sample was taken every 1:30 h, centrifuged, and analyzed by high performance liquid chromatography, using an Agilent 1100 Series chromatograph (Agilent Technologies, Inc., Japan) equipped with a diode array detector and a C18-Nucleosil column. Methanol water (50 : 50) was used as mobile phase at a flow rate of 0.5 mL/min. Absorbance was measured at 250 and 610 nm and compared against isatin-5-sulphonic acid (ISA5SA). Samples were analyzed until complete discoloration.

3. Results and Discussion

3.1. Discoloration of IC Using Aqueous Vegetable Extracts as Enzyme Sources. Several vegetables and vegetable residues were selected to achieve the discoloration of IC. The selected biological materials are easily available in local markets throughout the year. As shown in Figure 1, all tested materials discolored IC to some extent, some completely and others partially. However, green pea seeds are the most interesting material, because their extract was able to discolor 100 ppm of IC completely in about 7 h under stirring at 800 rpm. Almost complete discoloration was achieved with other materials but in longer times, between 24 and 36 h. In addition to edible vegetables such as green peas, cucumber, horseradish, and

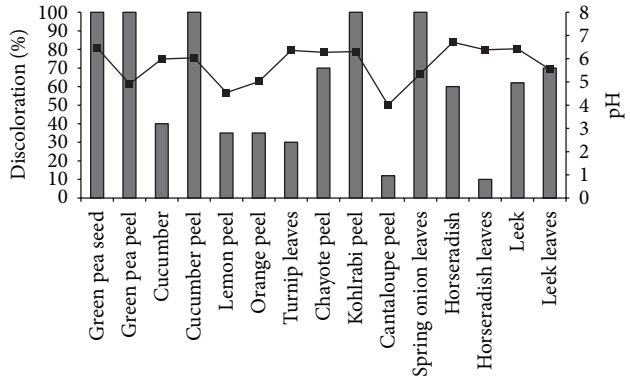

Figure 1: Discoloration of IC (100 ppm) using aqueous extracts of vegetables and vegetable residues as enzyme sources, at 800 rpm. pH –■–.

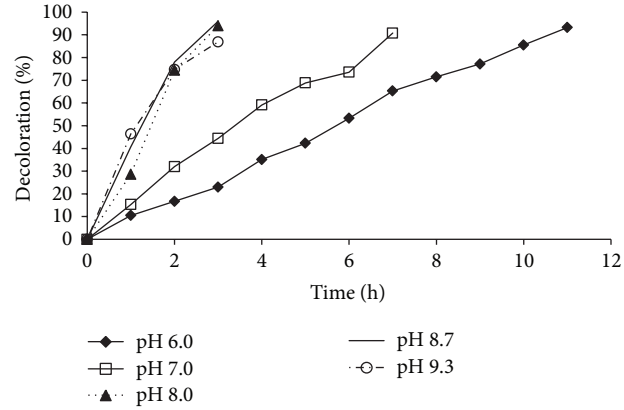

Figure 2: Effect of the pH on discoloration of IC (100 ppm) using green pea seed extract, under stirring at 800 rpm.

leek, we thought that it could be interesting to test the parts of plants that are considered waste such as peels and leaves. The vegetable residues that discolored IC completely were the peels from green peas, cucumber, and kohlrabi, as well as the leaves of spring onions; the other peels and horseradish leaves also discolored IC, but to a lesser extent. Some agricultural waste has been used for the discoloration of textile dyes because it involves an adsorption process [19–21]. In the case of the peels and leaves tested in this study, it is not possible that discoloration occurred due to an adsorption process, because we used aqueous extracts as enzyme sources, which means that discoloration was achieved via degradation of the dye. The pH of the reactions was not controlled. They were carried out at the physiological pH of the plants (Figure 1) as a screening to select the aqueous extract with the highest IC discoloration potential. Because the extract of green pea seeds had the highest activity toward the discoloration of IC compared to the other materials, the subsequent experiments were performed using this biological material only.

3.2. Enzymatic Analysis. Several monohydroxy-, dihydroxy-, and trihydroxyphenols have been used as substrates to determine PPO activity and specificity, but the most employed substrate for determining PPO activity in plants is catechol [15–17, 22]. Using catechol as substrate, the specific activity of the PPO from the green pea seed extract was determined as 1.288 U min^{-1} $protein^{-1}$. In addition to the fact that PPO oxidizes various phenolic substrates, the highest relative activity was observed in the presence of catechol (100% of the relative activity), followed by caffeic acid (48.23%) and pyrogallic acid (43.91%).

3.3. Effect of the pH, Dye Concentration, and Stirring on the Discoloration of IC Using an Aqueous Extract of Green Pea Seeds. Enzymes have a characteristic pH at which they show maximum activity. To determine the pH at which significant IC discoloration occurs, buffers of various pHs (6.0, 7.0, 8.0, 8.7, and 9.3) were used. The % discoloration of IC (100 ppm, at 800 rpm) at different pHs over time is shown in Figure 2. An acidic pH (6.0) was not favorable for the discoloration of the dye. A basic pH was more favorable for the enzymatic activity of the green pea seed extract. In the first 2 h, the degree of discoloration was similar at pH 8.0, 8.7, and 9.3, but within the next hour, 93% discoloration was achieved at pH 8 and 8.7, whereas only 84% was achieved at pH 9.3. Some authors have reported that other PPOs are more active at an acidic pH; for example, maximum discoloration with PPOs from potato (*Solanum tuberosum*) and brinjal (*Solanum melongena*) was observed at a pH of 3.0 [15], whereas the PPO from banana peel was more active at a pH of 7.0 [16].

Table 1 shows the % discoloration of IC at different dye concentrations, using the aqueous extracts of green pea seeds at a pH of 8.7 and magnetic stirring at 800 rpm. Under this condition, 200 ppm of IC was almost completely discolored in 4:30 h; however, in the presence of 1,000 ppm of IC, only 48.85% discoloration was achieved in 48 h. The next experiment was performed to find out if the enzymatic extract is stable at a pH of 8.7. The extract was stirred at 22°C and a pH of 8.7 for 5 h. Then, IC was added to the extract to get a final concentration of 100 ppm. Stirring was continued for an additional 24 h, but the % discoloration was negligible. It is possible that the maximum activity of the extract at a pH of 8.7 lasts only for a few hours, which could be explained by some kind of inactivation that is taking place at a higher pH.

In addition to the source of the enzyme, time, pH, and the concentration of the dye, we observed that aeration by stirring is also very important. The discoloration of IC (300 ppm) was studied at pHs of 7 and 7.6 and the reaction was carried out under stirring at 800 and 1,800 rpm until complete discoloration. The results are shown in Table 2. A high stirring rate and, consequently, high aeration are favorable for the reaction. The time required to discolor the dye was lowered considerably under stirring at 1,800 rpm compared with 800 rpm, and this could be because PPO requires free molecular oxygen as oxidant [22, 23]. The PPO activity of the green pea seeds was more efficient at a pH of 7.6. For example, at high-speed stirring (1,800 rpm), discoloration of 3,000 ppm of IC was achieved within 36 h with the aqueous extract of green pea seeds (pH 7.6).

Discoloration of 3,000 ppm of IC with green pea seed extract was achieved within 36 h at a pH of 7.6 and stirring at

Table 1: Effect of the concentration of IC on the discoloration using green pea seed extracts at a pH of 8.7 and stirring at 800 rpm.

IC (ppm)	Time (h)	% discoloration
50	2:30	97.74
100	3:00	95.99
200	4:30	98.41
500	24:00	84.05
1000	48:00	48.85

Table 2: Effect of stirring and pH on the time to achieve complete discoloration of IC, using green pea seed extract as enzyme source.

pH	IC (ppm)	Agitation (rpm)	Time (h) to complete IC discoloration
7.0	300	1800	3:20
7.0	300	800	7:00
7.6	300	1800	1:30
7.6	300	800	4.30
7.6	3000	1800	36:00

Table 3: Discoloration of IC (300 ppm) with an aqueous extract of green pea seeds in the presence of NaCl (pH 7.6 and 1,800 rpm).

%NaCl	1	2	4	7	10	Control
Time (h)	2:15	2:30	3:10	3:50	5:50	1:30

1,800 rpm. This is a very interesting improvement, compared with other studies on the discoloration of IC. For example, Neelamegam et al. [24] achieved 90% discoloration of IC (100 ppm) in 6 days using *Pleurotus ostreatus*. Ramya et al. [25] used a liquid culture of *Paenibacillus larvae* with solutions containing 100 ppm IC and achieved 100% of discoloration in 8 h. Podgornik et al. [26] reported the biodegradation of 30 ppm of IC in 2 h using isozymes of lignin peroxidase and manganese peroxidase obtained from *Phanerochaete chrysosporium*; an IC solution of 23.31 ppm was completely adsorbed on chitosan in 3.3 h [27].

3.4. Effect of NaCl and Soap on the Discoloration of IC Using an Aqueous Extract of Green Pea Seeds. In the textile finishing process, it is very common to use NaCl and soap, in addition to dyes. Thus, it was important to determine if the enzymes present in the green pea seed extract are able to degrade IC under such conditions, and if they are stable in the presence of NaCl and soap. The experiment was done with a solution of IC (300 ppm) and increasing quantities of NaCl, at a pH of 7.6 and stirring at 1,800 rpm. The control sample did not contain NaCl. From the results shown in Table 3, it can be perceived that the enzymes present in the aqueous extract of green pea seeds are able to discolor the dye completely, even in the presence of 10% NaCl. The biocatalytic activity of green pea seeds can be compared to that of halotolerant and halophilic microorganisms that are capable to discolor azo dyes in the presence of 4 to 15% of NaCl [28]. There are some reports about the reduction of the PPO activity in the presence of NaCl [29].

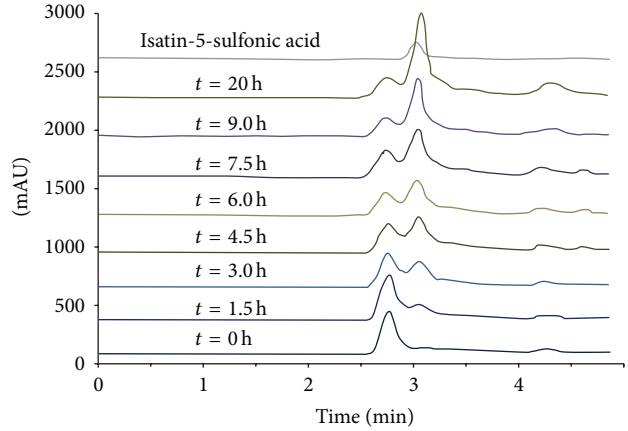

Figure 3: Chromatograms for the discoloration of IC using aqueous extracts of green pea seeds as PPO source, $\lambda = 250$ nm.

Complete discoloration of IC (300 ppm) was achieved in 4:30 h using an aqueous extract of green pea seeds in the presence of 5% NaCl and 2% commercial laundry soap (pH 7.6, 1,800 rpm). Thus, the enzymes present in the extract are active under the tested reaction conditions. This is a very important finding, because, in addition to colorants, high quantities of NaCl and soap are used in the textile industry. Therefore, the enzymes present in the extract may be useful in the wastewater treatment.

3.5. Biotransformation of IC. The discoloration of IC (1,000 ppm) with the aqueous extract of green pea seeds was measured by high performance liquid chromatography for 20 h and compared with ISA5SA, the previously reported IC degradation product [30, 31]. The results are shown in Figure 3. The peak corresponding to IC was detected at 610 nm with a retention time of 2.717 min; the peak of ISA5SA was detected at 250 nm and had a retention time of 2.992 min. At the 0 h time point, peaks corresponding to IC and some compounds present in the extract could be detected in the reaction mixture containing IC and the aqueous extract of green pea seeds. The principal peak corresponding to a compound from green pea seeds was detected at 2.645 min. Every 1.5 h, a sample was analyzed by high performance liquid chromatography. We observed that the peak at 2.992 min, corresponding to ISA5SA, was growing constantly, and the IC concentration diminished with time (data not shown). Thus, we can assume that IC is being degraded to ISA5SA by the action of a PPO in the extract of green pea seeds.

4. Conclusions

PPOs from vegetables and vegetable residues tested in this study are able to discolor IC without requiring any redox mediator. Both materials are inexpensive and easily accessible. The potential use of waste such as vegetable peels is very attractive because it can help to reduce the pollution due to food waste. In addition, the waste is a source of enzymes that are useful in bioremediation treatments. The PPO from green

pea seeds discolored IC even in the presence of NaCl and laundry soap, which is advantageous because those products are commonly employed in the dyeing processes in the textile industry. The results presented in this work are interesting because with this simple system that uses crude enzymatic extracts from inexpensive materials, that is, vegetables, we obtained similar or even better results than those described in other reports in which different or even more sophisticated and expensive processes were used.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Recovery of Stored Aerobic Granular Sludge and Its Contaminants Removal Efficiency under Different Operation Conditions

Zhiwei Zhao,[1] Shuo Wang,[1,2] Wenxin Shi,[1] and Ji Li[2]

[1] *State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China*
[2] *School of Environment and Civil Engineering, Jiangnan University, Wuxi 214122, China*

Correspondence should be addressed to Wenxin Shi; swx@hit.edu.cn

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The quick recovery process of contaminants removal of aerobic granular sludge (AGS) is complex, and the influencing factors are still not clear. The effects of dissolved oxygen (DO, air intensive aeration rate), organic loading rate (OLR), and C/N on contaminants removal characteristics of AGS and subsequently long-term operation of AGS bioreactor were investigated in this study. DO had a major impact on the recovery of AGS. The granules reactivated at air intensive aeration rate of 100 L/h achieved better settling property and contaminants removal efficiency. Moreover, protein content in extracellular polymeric substance (EPS) was almost unchanged, which demonstrated that an aeration rate of 100 L/h was more suitable for maintaining the biomass and the structure of AGS. Higher OLR caused polysaccharides content increase in EPS, and unstable C/N resulted in the overgrowth of filamentous bacteria, which presented worse NH_4^+-N and PO_4^{3-}-P removal. Correspondingly, quick recovery of contaminants removal was accomplished in 12 days at the optimized operation conditions of aeration rate 100 L/h, OLR 4 g/L·d, and C/N 100 : 10, with COD, NH_4^+-N, and PO_4^{3-}-P removal efficiencies of 87.2%, 86.9%, and 86.5%, respectively. The renovation of AGS could be successfully utilized as the seed sludge for the rapid start-up of AGS bioreactor.

1. Introduction

Aerobic granular sludge (AGS) was considered to be a special kind of biofilm structure composing of self-immobilized cells [1, 2]. With regular shape, smooth surface, and compact and strong microbial structure, AGS has the advantages of better settling property, lower consuming, higher biomass retention, and treatment efficiency than normal activated sludge [3, 4]. Therefore, AGS has been proposed as a promising technology which could be widely applied in the treatment of high organic wastewater [5] and wastewater with toxic components [6] as well as wastewater with toxicity and heavy metal [7, 8]. AGS technology possesses the ability to contribute to and improve the biological treatment of wastewater. Compared to normal wastewater treatment plants, similar efficiencies at lower costs could be achieved with the compact AGS technology [9].

Microbial forces by extracellular polymeric substances (EPS) were regarded as the significant factor in the formation process of AGS [10]. Protein content in EPS, rather than polysaccharides, was enriched in AGS [11, 12] which was known as the key component of EPS. It is believed that high protein content and relatively high PN/PS (the ratio of protein content and polysaccharides content in EPS) ratio would be a good situation to keep stable internal microstructure and high biomass retention. Enrichment of EPS assisted granulation, enhanced granules stability, which is important during the operation of AGS bioreactor, and reduced the loss of granules in storage [1].

However, AGS would lose its microbial activity under extended idle conditions or after long-term storage, which is one of the main problems hindering the practical application of AGS technology [13]. In addition, the cultivation of AGS, quick start-up, and stable operation of AGS bioreactor restricted the development of AGS technology, from lab scale to pilot scale as well [12]. Consequently, the long-term storage and quick recovery of contaminants removal of AGS are remarkably important for its full-scale application.

Table 1: Physical parameters of AGS before and after storage.

	Physical parameters					
	SVI (mL/g)	MLSS (mg/L)	MLVSS (mg/L)	MLVSS/MLSS (%)	ρ (g/cm^3)	Diameter (mm)
Before storage	49.3	8.45	7.73	91.5	1.040	2.8
After storage	64.1	8.45	7.25	85.8	1.032	2.6

The storage of AGS, including the bioactivity preservation and physical characteristics maintenance, was influenced by DO, OLR, and C/N [1, 14]. Moreover, the rapid recovery of contaminants removal and subsequent quick start-up of AGS bioreactor were also controlled by such factors.

In order to improve the flexibility and enhance the practicability of AGS technology, this study investigated the effects of DO, OLR, and C/N on the settling property, EPS, and microbial activity of AGS, and further to explore optimal operation conditions on contaminants removal.

2. Materials and Methods

2.1. Aerobic Granular Sludge Cultivation. The experiment was carried out in a sequencing batch airlift reactor (SBAR). The working volume of the reactor is 5.4 L, with a height of 100 cm and an internal diameter of 10 cm. The internal riser pipeline was 70 cm in height, 6 cm in internal diameter, and 2 cm leaving the bottom. Compressed air was supplied via a diffuser at the bottom of the reactor with a flux of 120 L/h. Effluent was discharged from the middle port of the reactor with a volumetric exchange ratio of 50%. The operating cycle time was 6 h, including 30 min for idle, 30 min for static feeding, 5 min for settling, 5 min for effluent discharge, and the rest of the time for aeration. Accordingly, the temperature of the mixed liquid was kept at ambient temperature, and influent pH and solids retention time (SRT) were adjusted to 7.0–7.2 (by 1 mol/L HCl and 1 mol/L NaOH) and 30 days, respectively.

2.2. Synthetic Wastewater and Seed Sludge. The components and concentrations in synthetic wastewater were listed as (mg/L) NaAc 830.0, $CaCl_2$ 60.0, $MgSO_4$ 42.0, NH_4Cl 240.0, EDTA 42.0, $NaHCO_3$ 250.0, and K_2HPO_4 58.0, KH_2PO_4 24.0 [14], in element solution 1 mL, which gave a total COD concentration of 1200 mg/L; the concentration of NH_4^+-N was 60 mg/L, and the concentration of PO_4^{3-}-P with 16 mg/L. Trace element solution contained the following components (g/L): $FeCl_3 \cdot 6H_2O$ 1.5, H_3BO_3 0.15, $CuSO_4 \cdot 5H_2O$ 0.03, KI 0.03, $MnCl_2 \cdot 4H_2O$ 0.12, $Na_2MoO_4 \cdot 2H_2O$ 0.06, $ZnSO_4 \cdot 7H_2O$ 0.12, and $CoCl_2 \cdot 6H_2O$ 0.15 [15]. The seed activated sludge was taken from the aerobic tank of Wenchang wastewater treatment plant (WWTP in Harbin, China) with an anoxic/oxic process (A/O process).

2.3. Storage and Recovery of Aerobic Granular Sludge. The inoculated AGS had been stored at a 4°C refrigerator for more than 6 months and then regained in the previously mentioned SBAR after washing by distilled water for 3 times, with the same operation conditions and synthetic wastewater components in different reactors. The temperature of the mixed liquid was kept at ambient temperature, and influent pH was adjusted to 7.0–7.2. DO (Do concentration was represented by air intensive aeration rate, 50, 100, 150, and 200 L/h), OLR (2, 4, 8 and 16 g/L·d), and C/N (100 : 5, 100 : 10, and 100 : 20) were regulated and controlled to investigate the recovery on contaminants removal characteristics.

2.4. Analysis Methods. COD, NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P, mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) were analyzed according to the Standard Method [16]. Granules size and wet density were determined according to the methods by Laguna et al. and Schwarzenbeck et al. [17, 18]. The microstructure and morphology of the AGS were observed by scanning electron microscope (SEM, S-4800N, Japan). Sludge volume index (SVI) was determined according to the settled bed volume after 30 min settling and the dry biomass weight [14]. The extraction of EPS was performed by the usage of ultrasound-formamide-NaOH method [1]. Total polysaccharides (PS) and total protein (PN) contents in EPS were quantified by Dubois et al. and Lowry et al. [19, 20].

3. Results and Discussion

3.1. Storage of Aerobic Granular Sludge. Mature AGS cultivated in the SBAR was kept at a 4°C refrigerator, and pH was adjusted to 7.0–7.2; especially, the feed liquid was replaced every two weeks. The morphology and integrity of AGS were both in good condition after 6 months of storage. The physical parameters were listed in Table 1. Granules size and wet density were basically unchanged after storage; however, AGS had apparent variation in its biomass and settling property. Biomass retention decreased from 7.73 to 7.25 mg/L, probably due to the release of soluble organic material and cell hydrolysis as reported by Tay et al. [21]. After 6 months of storage, its settling property deteriorated as shown from the values of SVI. SVI obviously increased from 49.3 to 64.1 mL/g, indicating that granules cannot maintain better settling property after long-term storage.

3.2. Recovery of Contaminants Removal Efficiency. AGS after the 6-month storage was utilized as the seed sludge for the quick start-up of the SBAR. Before the seeding, the granules taken from the 4°C refrigerator should be washed for three times to remove the fermentation products and the residual nutrient substances.

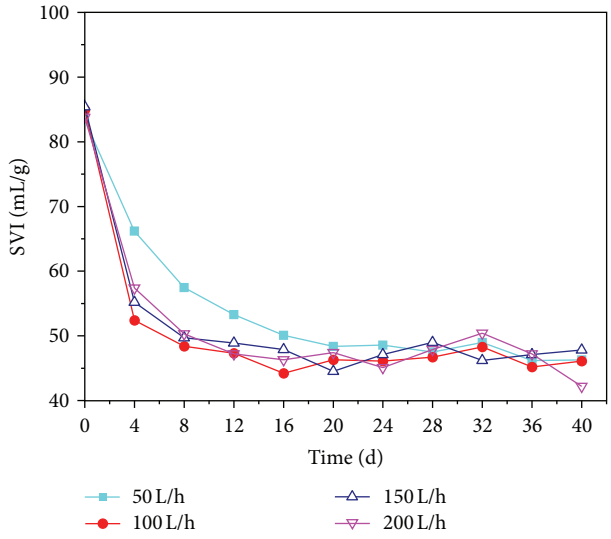

Figure 1: Variations of SVI at different air intensive aeration rates.

3.2.1. DO (Air Intensive Aeration Rate)

Effects of DO on the Settling Property. As shown in Figure 1, AGS had good settling property in the reactivation process. SVI of the granules decreased obviously in the first 8 days, and the granules had faster decline rate at the air intensive aeration rates of 100, 150 and 200 L/h than 50 L/h. The SVI of AGS decreased to 57.5, 48.4, 49.7, and 50.3 mL/g at the 8th day; then, it was maintained at a low level indicating their excellent settling ability. The granules revived under different air intensive aeration rates (100, 150, and 200 L/h) obtained quick recovery on its settle capacity. The SVI of granules reactivated at air intensive aeration rate of 100 L/h varied from 84.9 to 46.1 mL/g, which demonstrated that he granules had better settling property. In addition, relatively low air intensive aeration rate could be more economic and conductive for large-scale production and practical application. Therefore, 100 L/h aeration rate is good in the recovery of settling property.

Effects of DO on Granules Structure. Bacterium can secrete sticky materials called EPS constituting proteins (PN), polysaccharides (PS), humic acids, and lipids, which could assist cell adhesion; thereby, it should be helpful to initiate the aerobic granulation process [12] (Schmidt et al., 2004). Protein and polysaccharides contents in EPS of AGS before the 6 months of storage were visually the same as those after the storage, with PN content of 80.3 mg/gMLSS and PS content of 27.0 mg/gMLSS. The ratio of PN/PS was 3.0. As illustrated in Figure 2(a), protein content was basically unchanged, implying the stable internal structure of AGS. PN content in EPS of the granules was 78.4, 82.5, 80.3, and 82.4 mg/gMLSS under different air intensive aeration rates (50, 100, 150, and 200 L/h). However, PS content under different air intensive aeration rates changed a lot (Figure 2(b)). PS content in EPS declined from 27.0 to 14.3 mg/gMLSS at air intensive aeration rate of 50 L/h, while it declined to 37.9 and 43.1 mg/gMLSS at air intensive aeration rate of 150 and 200 L/h. The variation of PS content caused unsteady PN/PS ratio and resulted in the fast disintegration of granules [13]. High PN content could be the cross-linked network by attraction of organic and inorganic materials [22] and the bridge of microbial cells once aerobic granules formed, which was consistent with the results of Adav et al. and Wang et al. [23, 24]. The present findings indicate that the induction of coaggregation and intracellular interaction by EPS played a significant role in the formation and maintenance of AGS.

Effects of DO on Contaminants Removal. DO was an important factor influencing nitrification and denitrification, which also expressed the key effect on the phosphorus release in anaerobic phase and the phosphorus uptake in aerobic phase [14]. Therefore, NH_4^+-N, and PO_4^{3-}-P removal efficiency would be greatly impacted by DO. Before the storage, AGS possessed good COD, NH_4^+-N and PO_4^{3-}-P removal performance, with COD, NH_4^+-N, and PO_4^{3-}-P removal efficiency of 90.2%, 93.5%, and 94.2%, respectively. However, the granules bioactivity to remove contaminants decreased after the storage. Microbial activity of aerobic granules began to revive, and NH_4^+-N removal ability was enhanced with the recovery progress (Figure 3(a)). As can be seen in the figure, NH_4^+-N removal rates were kept increasing in the first 8 days, especially for the air intensive aeration rates of 100 and 150 L/h. While for the air intensive aeration rate of 200 L/h, the story was somehow different, NH_4^+-N removal rate increased in the first 4 days and then decreased after 20 days. After 20 days reactivation, NH_4^+-N removal accomplished high removal efficiency of 77.4%, 85.7%, and 89.7% under aeration rates of 50, 100, and 150 L/h, which indicated that the granules gained better NH_4^+-N removal recovery performance. However, NH_4^+-N removal rate declined to 55.4% at the aeration rate of 200 L/h, probably because the sufficient oxygen supply led to the low bioactivity and slow specific growth rate of autotrophic bacteria including ammonia oxidized bacteria (AOB) and nitrite oxidized bacteria (NOB) [25, 26].

As displayed in Figure 3(b), the variations of PO_4^{3-}-P removal were basically the same and presented a good correlation with NH_4^+-N removal. PO_4^{3-}-P removal efficiency of the granules that recovered at aeration rate of 100 and 150 L/h increased up to 80% after activation for 16 days, which is higher than other recovery conditions. The granules had the characteristic of phosphorus accumulating potential with concomitant uptake of soluble organic carbon and release of phosphorus in the anaerobic stage, followed by rapid phosphorus uptake in the aerobic stage [27]. However, PO_4^{3-}-P removal of the granules that revived at an aeration rate of 200 L/h reached its maximum and then obviously declined to 47.2%. The result indicated that the sufficient oxygen supply might decrease the anaerobic zone inside AGS, which hindered the microbial activity of phosphorus accumulating organisms (PAO) and inhibited the phosphorus release and uptake process.

It is believed that the microstructure of the granules could be sustained and microbial activity retained good

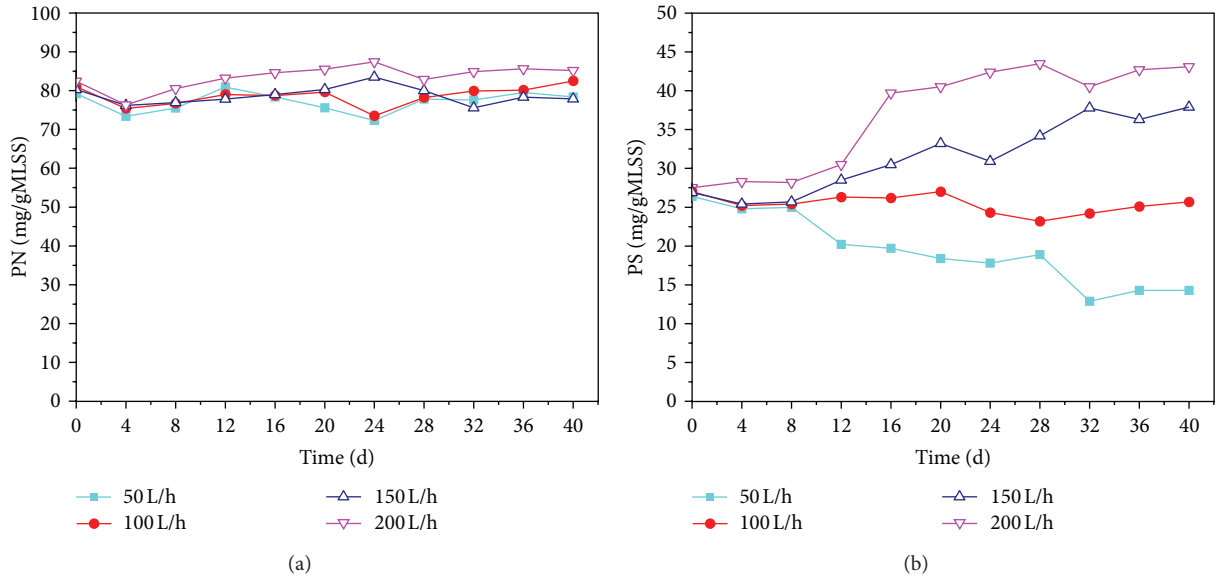

Figure 2: Variations of PN (a) and PS (b) contents in EPS at different air intensive aeration rates.

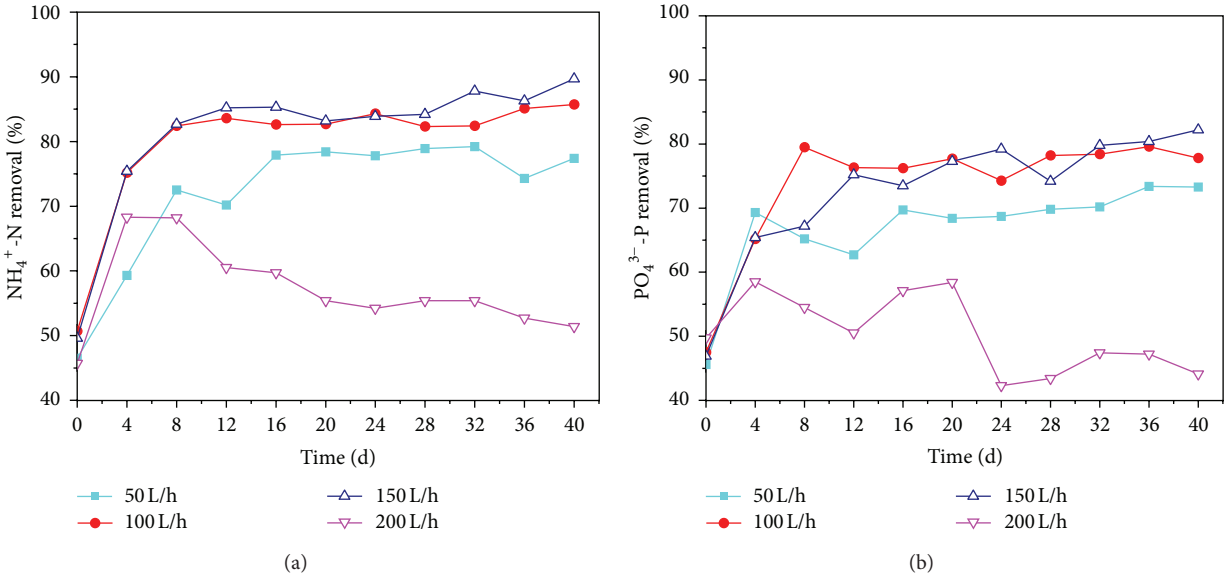

Figure 3: Variations of NH_4^+-N (a) and PO_4^{3-}-P (b) removal at different air intensive aeration rates.

performance. The results showed that DO had a major impact on the settling property and contaminants removal efficiency of AGS. In addition, protein content in EPS was almost unchanged, which demonstrated that air intensive aeration rate of 100 L/h was more suitable for maintaining biomass and the structure of AGS.

3.2.2. OLR

Effects of OLR on Granules Structure. It is believed that high protein content and relatively high PN/PS ratio would be good in keeping stable internal microstructure and high biomass retention [11, 12]. The variations of PN/PS ratio under different OLRs were illustrated in Figure 4. In the recovery process of AGS, PN and PS contents in EPS reactivated at 2 and 4 g/L·d were almost unchanged which possessed the stable structure of the granules. However, high OLR had a great impact on the PN/PS ratio. The variations of PN/PS revived at 8 and 16 g/L·d of OLR showed similar trend. PN content in EPS of AGS was stable, but PS content increased obviously, which were as high as 43.7 and 50.6 mg/gMLSS, resulting in the PN/PS ratio remaining at the low level of 1.5. Hence, relatively high PS content in EPS could reduce the integration and stability of the granules; however, Costerton

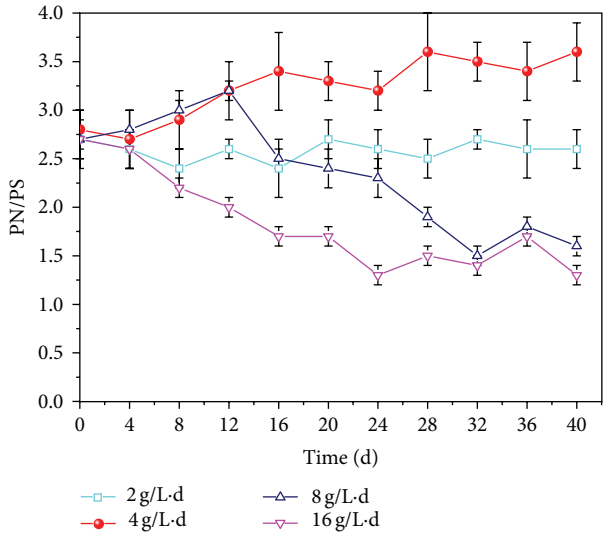

Figure 4: Variations of PN/PS ratio at different organic loading rates.

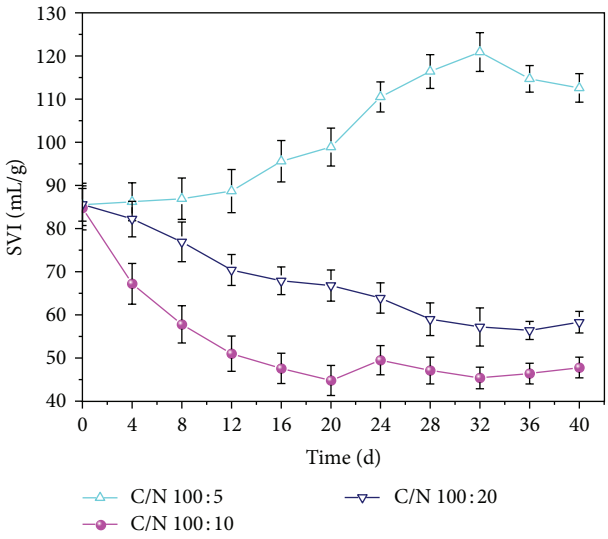

Figure 5: Variations of SVI at different C/N ratio.

et al. and Tay et al. discovered that high PS content was noted to facilitate cell-to-cell adhesion and strengthen the structure of granules through a polymeric matrix [28, 29].

Effects of OLR on Contaminants Removal. AGS did not lose all the contaminants removal abilities after the 6-month storage. The concentrations of contaminants in the effluent of the SBAR and contaminants removal efficiency after 31 days reactivation were listed in Table 2. COD removal efficiency reached about 80% under different organic loading rates in the first 8 days; then, COD removal was obtained at high efficiency of 89.2%, 89.4%, 90.5%, and 94.4%, respectively, which demonstrated good COD removal efficiency. Nonetheless, NH_4^+-N and PO_4^{3-}-P removal was quite different; relatively high OLR conditions resulted in lower NH_4^+-N and PO_4^{3-}-P removal as a result of the disintegration and deterioration of AGS [13, 30]. The granules revived under OLR of 4 g/L·d gained good NH_4^+-N and PO_4^{3-}-P removal efficiency of 90.5% and 80.7%.

The granules reactivated at OLR of 4 g/L·d could maintain good structural integrity and high contaminants removal efficiency. Meanwhile along the recovery process, PS content in EPS was progressively increased which led to the disintegration of AGS and worse NH_4^+-N and PO_4^{3-}-P removal efficiency under higher OLR conditions. Therefore, AGS revived at OLR of 4 g/L·d was more suitable for the long-term stable operation of AGS.

3.2.3. C/N

Effects of C/N Ratio on the Settling Property of AGS. It is apparent in Figure 5 that low C/N caused good settling property in the recovery process. SVI values were 47.8 and 57.2 mL/g at C/N of 100 : 10 and 100 : 20, respectively. The SVI of the granules that revived at C/N of 100 : 10 decreased significantly faster than that of the granules revived at C/N of 100 : 20. However, the SVI of AGS reactivated at C/N of 100 : 5 obviously increased up to 110.5 mL/g and then was retained at a high level that presented worse settling ability. The finding revealed that the compact AGS grew in size but gradually lost the stability corresponding with the outgrowth of filamentous bacteria. Therefore, unstable C/N resulted in the overgrowth of filamentous bacteria, which presented worse contaminants removal efficiency [14, 31].

Effects of C/N on Contaminants Removal Efficiency. Effluent contaminants and contaminants removal efficiency after 31 days reactivation were listed in Table 2. Along with restoration, COD removal efficiency under different C/N conditions gained higher removal efficiency of 87.9%, 88.2%, and 89.6%, respectively, indicating excellent COD removal efficiency. Nevertheless, NH_4^+-N and PO_4^{3-}-P removal was quite distinct. NH_4^+-N removal efficiency at C/N of 100 : 5 was 62.4% because heterotrophic bacteria, whose growth rate was faster than those in a lower C/N, would be in a competitive advantage in inhibiting the activity of nitrifying bacteria and autotrophic bacteria [25]. Furthermore, PO_4^{3-}-P removal efficiency at C/N of 100 : 20 was 64.6% because of the competition of soluble organic carbon between PAOs and denitrifiers that hindered the microbial activity of PAOs [32]. The granules reactivated under C/N of 100 : 10 achieved both good NH_4^+-N and PO_4^{3-}-P removal efficiencies of 88.2% and 89.1%.

In this study, different C/N conditions had significant impacts on the settling property of AGS during the reactivation process. High C/N rate resulted in the overgrowth of filamentous bacteria, which presented worse NH_4^+-N and PO_4^{3-}-P removal efficiency. Hence, the granules that recovered at C/N of 100 : 10 were most stable with little variation on SVI and good NH_4^+-N as well as PO_4^{3-}-P removal efficiency after 12 days of reactivation.

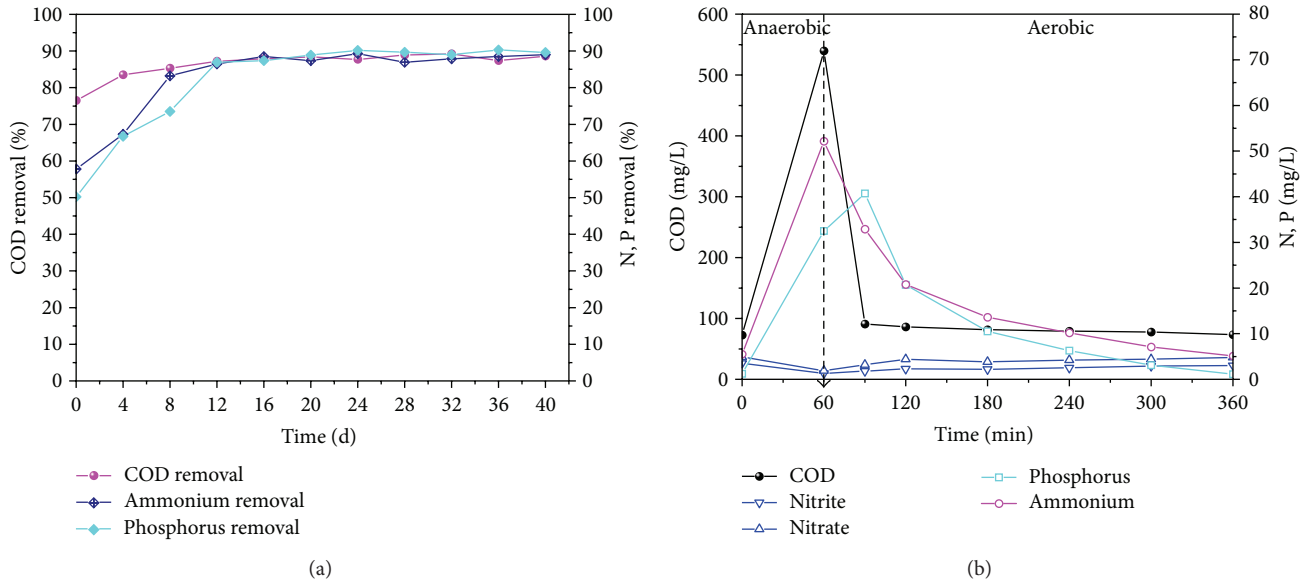

Figure 6: Contaminants removal under optimized operation conditions ((a) process; (b) cycle).

Table 2: Effluent characteristics and contaminants removal under different operation conditions.

		Effluent							
		COD (mg/L)	NH_4^+-N (mg/L)	NO_2^--N (mg/L)	NO_3^--N (mg/L)	PO_4^{3-}-P (mg/L)	COD[a] (%)	NH_4^+-N[b] (%)	PO_4^{3-}-P[c] (%)
OLR (g/L·d)	2	56.0 ± 5.2	4.4 ± 0.3	13.3 ± 0.2	—	8.0 ± 0.4	89.2	89.3	52.5
	4	73.8 ± 6.9	4.4 ± 0.3	3.1 ± 0.2	—	3.3 ± 0.3	89.4	90.5	80.7
	8	107.8 ± 8.9	7.6 ± 0.4	3.5 ± 0.1	0.5 ± 0.3	4.1 ± 0.4	90.5	81.3	75.9
	16	120.0 ± 10.3	9.2 ± 0.4	4.2 ± 0.2	—	5.2 ± 0.3	94.4	77.0	68.9
C/N	100 : 5	63.0 ± 6.4	10.1 ± 0.3	1.8 ± 0.1	3.3 ± 0.1	4.7 ± 0.2	87.9	62.4	71.3
	100 : 10	58.0 ± 6.1	3.7 ± 0.2	1.9 ± 0.1	3.1 ± 0.1	4.5 ± 0.2	88.2	89.1	72.7
	100 : 20	57.5 ± 6.5	7.7 ± 0.3	2.9 ± 0.1	7.5 ± 0.5	5.8 ± 0.3	89.6	77.4	64.6

a: COD removal; b: NH_4^+-N removal; c: PO_4^{3-}-P removal; —: not detected.

3.3. Quick Recovery of Contaminants Removal. As displayed in Figure 6(a), the bioactivity of AGS progressively revived along with the recovery process under optimized operation conditions. According to the defined conditions previously mentioned, the optimal conditions were as follows: air intensive aeration rate 100 L/h, OLR 4 g/L·d, and C/N 100 : 10. Initially COD, NH_4^+-N, and PO_4^{3-}-P removal was low; however, with the reactivation progress, COD, NH_4^+-N, and PO_4^{3-}-P removal kept increasing. After 12 days of reactivation, AGS achieved the best recovery performance on microbial activity; COD, NH_4^+-N, and PO_4^{3-}-P removal efficiency could be quickly recovered to 87.2%, 86.9%, and 86.5%, respectively.

It is noticeable in Figure 6(b) that COD, NH_4^+-N, and PO_4^{3-}-P in effluent were 73.5, 5.1, and 1.1 mg/L in the stable cycle during the reactivation process, and the respective COD, NH_4^+-N, and PO_4^{3-}-P efficiencies were 86.4%, 90.2%, and 93.1%. Moreover, the nitrification and denitrification coefficiency was 75.2%, and the simultaneous nitrification and denitrification rate was 0.41 mmol/L·h. The results demonstrated that the granules revived under optimal operation conditions gained overall recovery performance. Furthermore, AGS technology could deal with a large number of conversion processes including COD-oxidation, ammonium oxidation, and biological phosphorus removal. The renovation of AGS in store could be successfully utilized as the seed sludge for the rapid start-up of AGS bioreactor.

4. Conclusions

The morphology and integrity of AGS were both in good condition after 6 months of storage. Dissolved oxygen had significant impacts on the recovery of the granules, and OLR and C/N had comparatively slight influence.

The microstructure of the granules could be sustained; the microbial activity retained good performance; and protein content in EPS was almost unchanged, which demonstrated that air intensive aeration rate of 100 L/h was more suitable

for maintaining its biomass and the structure of AGS. The granules reactivated at OLR of 4 g/L·d could maintain good structural integrity and high contaminants removal efficiency. PS content in EPS was progressively increased which led to the disintegration and worse NH_4^+-N and PO_4^{3-}-P removal efficiency under higher OLR conditions. Different C/N conditions had significant impacts on the settling property of AGS during the reactivation process. High C/N resulted in the overgrowth of filamentous bacteria, which presented worse NH_4^+-N and PO_4^{3-}-P removal efficiency.

Correspondingly, quick recovery of contaminants removal was accomplished in 12 days at the optimal operation conditions of air intensive aeration rate 100 L/h, OLR 4 g/L·d, and C/N 100 : 10, in which COD, NH_4^+-N, and PO_4^{3-}-P removal efficiencies were 87.2%, 86.9% and 86.5%, respectively. The renovation of AGS in store could be successfully utilized as the seed sludge for simplifying the start-up and enhancing the long-term stable operation of AGS bioreactor.

Acknowledgments

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Research Article

Enhancement of Phosphate Absorption by Garden Plants by Genetic Engineering: A New Tool for Phytoremediation

Keisuke Matsui,[1] Junichi Togami,[2] John G. Mason,[3] Stephen F. Chandler,[4] and Yoshikazu Tanaka[1]

[1] *Research Institute, Suntory Global Innovation Center Limited, 1-1-1 Wakayama-dai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan*
[2] *Safety Science Institute, Quality Assurance Division, Suntory Business Expert Limited, 57 Imaikami-cho, Nakahara-ku, Kanagawa Kawasaki 211-0067, Japan*
[3] *Biosciences Research Division, Department of Environment & Primary Industries, AgriBio, Centre for AgriBioscience, 5 Ring Road, La Trobe University, Bundoora, VIC 3083, Australia*
[4] *School of Applied Sciences, RMIT University, P.O. Box 71, Bundoora, VIC 3083, Australia*

Correspondence should be addressed to Keisuke Matsui; keisuke_matsui@suntory.co.jp

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Although phosphorus is an essential factor for proper plant growth in natural environments, an excess of phosphate in water sources causes serious pollution. In this paper we describe transgenic plants which hyperaccumulate inorganic phosphate (Pi) and which may be used to reduce environmental water pollution by phytoremediation. At*PHR1*, a transcription factor for a key regulator of the Pi starvation response in *Arabidopsis thaliana*, was overexpressed in the ornamental garden plants *Torenia, Petunia, and Verbena*. The transgenic plants showed hyperaccumulation of Pi in leaves and accelerated Pi absorption rates from hydroponic solutions. Large-scale hydroponic experiments indicated that the enhanced ability to absorb Pi in transgenic torenia (At*PHR1*) was comparable to water hyacinth a plant that though is used for phytoremediation causes overgrowth problems.

1. Introduction

Water pollution has become a serious problem around the world. Contamination by toxic substances such as endocrine disruptors and heavy metals and excessive inflows of phosphorus, nitrogen and other elements all contribute to water pollution. Eutrophication is one of the major problems associated with water pollution and is caused by inflow of excess amounts of nutrients (especially phosphorus and nitrogen) [1]. The sources of excessive amounts of phosphorus and nitrogen are agricultural run-off, sewage, industrial effluents, and natural erosion from soil and rocks. Eutrophication is due to rapid growth of phytoplankton causing algal blooms or "red tides," the result of which are serious environmental problems such as bad odor and fish death as a result of oxygen depletion and accumulation of toxic cyanotoxins [2].

Phosphorus can be removed by physical, chemical, and biological methods [3–6]. Physical and chemical methods (e.g., electrolytic, crystallization, filtration, and aggregation/separation methods) are superior in terms of removal efficiency and throughput capacity. However, these methods require complicated equipment and large quantities of chemicals, resulting in high cost and environmental burdens. A biological method, the anaerobic-anoxic-oxic method (A2O), is one of the advanced activated sludge methods and has been widely examined in sewage plants. However this method is also very expensive [7], and presently, there are no practically useable technologies to remove inorganic ions such as phosphorus and nitrogen during sewage treatment using activated sludge methods. Thus, though various types of water purification systems have been developed for water and sewage plants [8], these technologies are often difficult to apply directly to aquatic environments due to cost and the need for special equipment. Eutrophication therefore remains a problem.

Concurrently with improving sewage treatment technology, a low-cost and highly efficient method is still needed

for sustainable water purification in aquatic environments. A treatment for environmental pollution using plants (phytoremediation) is a possible solution [9, 10]. Since phosphorus is an essential and often limiting nutritive substance for plants, plants actively absorb it from environments through the roots. Phytoremediation of aquatic systems has been attempted using water plants such as water hyacinth and *Phragmites*, as these plants absorb phosphorus relatively efficiently in comparison to terrestrial plants, and they also grow rapidly [11]. However, the high cost of collection and disposal of water plants (especially water hyacinth) presents difficulties in habitat management, and the impact of the plants on preexisting ecosystems hamper their wide application. In addition, the ability of these water plants to eliminate phosphorus in aquatic ecosystems is still inadequate as an even higher efficiency is needed for effective phytoremediation.

Inorganic phosphate (Pi) transporter is a key component in Pi absorption by plant roots. In *Arabidopsis thaliana*, 9 high-affinity transporters are known [12]. One of these, At*PHT1,* encodes a cell membrane-located Pi transporter with high affinity for Pi. It has been reported that overexpression of At*PHT1* in cultured cells of *Nicotiana* leads to an acceleration of Pi absorption and an increase in cell growth rate [13]. In contrast, when the same Pi transporter was overexpressed in *Hordeum vulgare*, an increase in absorption of Pi was not observed [14]. These two contradicting reports suggest that merely increasing the number of Pi transporters does not necessarily lead to enhanced Pi absorption.

Several Pi starvation-related genes have been identified in *A. thaliana* mutants [15]. One of the known control factors which function when plants enter a state of Pi starvation is the At*PHR1* gene. At*PHR1* gene encodes a transcription factor which activates the transcription of genes in response to states of Pi starvation [16]. Recently, it is reported that overexpression of At*PHR1* in *A. thaliana* increases the Pi concentration in aerial plant parts [17].

In this study, we introduced the At*PHR1* gene into the garden plants *Torenia*, *Petunia,* and *Verbena*, in order to enhance Pi absorption. Small and large-scale hydroponic trials with transgenic torenia plants expressing the At*PHR1* gene were performed. We demonstrate for the first time that over expression of the At*PHR1* gene results in enhanced Pi absorption rate in different plant species. The At*PHR1* transgenic plants can possibly facilitate effective phytoremediation in polluted aquatic environments.

2. Materials and Methods

2.1. Plant Materials. Plants of *Torenia hybrida* cv. Summer Wave blue, *Petunia hybrida* cv. Surfinia purple mini, and *Verbena hybrida* cv. Temari scarlet (Suntory Flowers, Ltd.) were grown in soil and supplied with full nutrients every week in a green house or a growth chamber in controlled conditions (22–25°C, 12 hours light).

2.2. Constructs for Expression in Plants and Plant Transformation. Molecular biology techniques were employed according to the methods described by Sambrook et al. [18], unless otherwise specified.

The At*PHR1* gene was amplified by PCR using primers PHRf (5′-ATGGAGGCTCGTCCAGTTCAT-3′) and PHRr (5′-TCAATTATCGATTTTGGGACGC-3′) and subcloned into the pCR2.1 vector using a TOPO-TA cloning kit (Life Technologies) according to the manufacturer's instructions. A fragment of the At*PHR1* gene was inserted into binary vector pBinPLUS [19] which contains an enhanced cauliflower mosaic virus 35S promoter [20] and a nopaline synthase (nos) terminator. This plasmid was named pSPB1898.

Transformation with transformation vector pSPB1898 was carried out as described previously for Torenia [21], Petunia [22], and Verbena [23] using *Agrobacterium tumefaciens* strain AGL0 [24].

RNAs were extracted from leaves of the obtained recombinant plants using the RNeasy Plant Mini Kit (Qiagen). Positive strains were selected by RT-PCR.

2.3. Method for Measuring Phosphorus Concentration. Phosphorus concentration was measured according to a modified method of Ames [25]. Leaves were weighed (approximately 100 mg per sample) and inserted into a 2 mL tube for crushing with zirconia beads (4 mm diameter), at −80°C. The frozen sample was taken to room temperature, and 500 μL of 1% (v/v) acetic acid was added to each tube. The mixture was then shaken and crushed for 6 minutes using a TissueLyser (Qiagen). After crushing, the mixture was centrifuged at 15,000 rpm for 5 minutes using a desktop centrifuge to obtain 500 μL of supernatant. This Pi extract was diluted with distilled water (from 10 to 100-fold dilution) to a final concentration of 800 μL. To this solution, 160 μL of measuring buffer (1.25 M sulfuric acid, 30 mM ascorbic acid, 0.405 mg/mL antimony potassium tartrate, and 24 mg/mL ammonium molybdate) was added, and the mixture was stirred well and left for 10 minutes. The absorbance was measured at 880 nm using a spectrophotometer BioSpec-mini (Shimadzu, Japan). The amount of phosphorus in 1 g of leaf was calculated from phosphorus concentration and weight of the sample. For calculations on a dry weight basis, samples were dried at 80°C for about 2 days.

An independent Student's t-test was used to compare differences between host and transgenic plants. All tests were two-sided, and $P < 0.05$ was considered statistically significant. Data are the mean ± SD from at least three different samples.

2.4. Hydroponic Experiment. Wild-type torenia or transgenic torenia was grown on a support made of polystyrene foam with holes to allow the root systems of the plants to grow into the hydroponic solution. Plants were floated on 5 liter of hydroponic solution (0.5 mM KNO_3, 0.2 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$, 0.161 mM KPO_4, 5 μM Fe-EDTA, 7 μM H_3BO_3, 1.4 μM $MnCl_2$, 0.05 μM $CuSO_4$, 0.1 mM $ZnSo_4$, 0.02 μM Na_2MoO_4, 1 μM NaCl, and 0.001 μM $CoCl_2$). The initial phosphorus concentration in the hydroponic solution was 5 mg/L. Four plants were used in each support. The Pi concentration in the hydroponic solution was measured each day. Since the fluid volume of the hydroponic solution decreased due to transpiration and evaporation, on every fourth day, deionised water was added to the solution. For

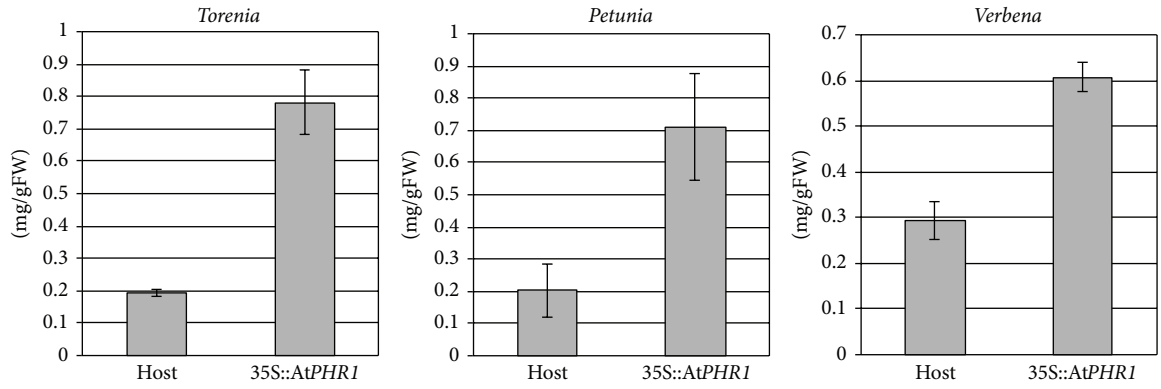


Figure 1: Phosphorus measurements of At*PHR1* transgenic plants. Phosphorus concentrations in the leaves of At*PHR1* transgenic plants of potted torenia, petunia, and verbena were measured. The longitudinal axis shows the phosphorus amounts per gram fresh weight (mg/gFW). Significant differences in means between host and transgenic plants were detected for all three species.

large container experiments, the same solution was used, but the volume of hydroponic solution was 400 liter, and 13 plants were used per container. The volume of each container was adjusted with deionised water on a weekly basis.

3. Results and Discussion

3.1. Overexpression of AtPHR1 Enhances Pi Accumulation and Absorption in Transgenic Plants. It has been shown in *A. thaliana* that over expression of At*PHR1* causes enhanced Pi accumulation in aerial parts [17]. To examine whether At*PHR1* is effective in otherplant species, we transformed torenia, petunia, and verbena with At*PHR1*. These plants were transformed with the plasmid pSPB1898, which contains the At*PHR1* gene under the control of the constitutive 35S promoter. We screened over 30 transgenic plants for each species for the presence of the transgene with RT-PCR and for leaf Pi concentration 4 weeks after potting up from tissue culture. Concentration of phosphorus per fresh leaf weight was then measured for selected lines. In each of the 3 plant species, phosphorus concentration in the leaves of the transgenic plants was 2 to 3-fold higher than that of control host plants (Figure 1).

We examined other Pi starvation-related genes (At*PHT1;1*, At*PHT1;2*, At*IPS1,* and At*PHO1*) from *A. thaliana* by constitutively overexpressing them in transgenic torenia and petunia (data not shown). None of these transgenic plants showed enhanced Pi accumulation. This result is consistent with the observation that over-expression of the Pi transporter did not cause any change to Pi accumulation in *H. vulgare* [14]. Thus, we focused on At*PHR1* in the following experiments.

To confirm that introduction of the At*PHR1* gene accelerates Pi absorption rates, we grew plants of a transgenic torenia line in a hydroponic system. Torenia was chosen as this plant grows luxuriantly and roots tolerate being submerged in water. The torenia plants were grown in 5 liters of hydroponic solution containing 5 mg/L phosphorus for 1 to 2 months in a green house or a growth chamber. The phosphorus concentration of the hydroponic solutions was measured daily. The superior transgenic line expressing At*PHR1* (35S::At*PHR1*) showed enhanced Pi absorption from the hydroponic solution (Figure 2(a)). Enhanced accumulation of Pi in the transgenic leaves was also confirmed by measurements of leaf phosphorus concentration (Figure 2(b)). The phosphorus concentration of the hydroponic solution in which 35S::At*PHR1* was grown decreased during the two weeks of the experiment. The Pi absorption rate observed for 35S::At*PHR1* was up to 0.091 mgP/day/plant in this experiment compared to 0.056 mgP/day/plant for the host (Figure 2(a)). This result suggests that the enhanced Pi accumulation observed in the potted At*PHR1* transgenic torenia plants is mainly due to enhanced Pi absorption rate.

To see if the decrease of Pi concentration in the hydroponic solution was also reflected in an increase in Pi accumulation in the plant, Pi accumulation in the aerial parts of the plants was measured. Three plants each of the transgenic and the host torenia were hydroponically cultivated in the solution containing 5 mg/L phosphorus for about 2 months. The aerial parts of those plants were collected and dried on the phosphorus concentration measured (Figure 2(b)). The Pi concentration in the transgenic plants was approximately 2.5-fold that of the host.

We weighed aerial and root parts of the tested plants after each hydroponic experiment. Even though slightly less weight was measured in the host, there was no statistically significant difference between the transgenic and host (Figure 2(c)). This suggests that excessively absorbed Pi is not used for plant growth but is accumulated and stored in the aerial part of the plants. As a result, overexpression of At*PHR1* does not retard plant growth. Since the transgenic plants did not show any morphological or reproductive abnormalities, overexpression of the At*PHR1* gene can enhance Pi accumulation with no negative effects on plant growth.

3.2. Limitation of Pi Capacity. Sections of dead tissues in the leaves were often observed in transgenic torenia during the 4 weeks of the hydroponic experiments (Figures 3(a)–3(c)). We collected the dead sections and compared them to the unaffected areas of the leaves from the same plants. The harvested leaves were dried and then measured for phosphorus concentration. The phosphorus concentration in

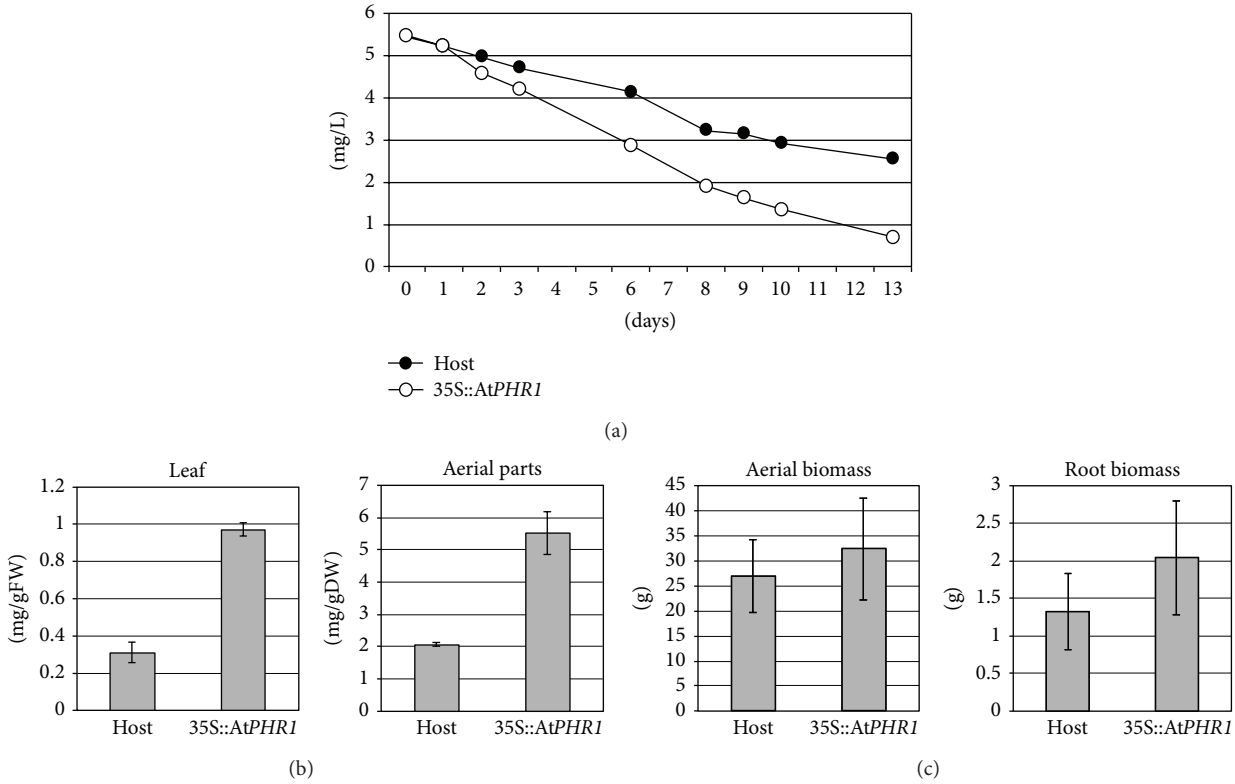


Figure 2: Pi accumulation and growth properties of At*PHR1* transgenic torenia. (a) Changes of Pi concentration in hydroponic solutions. The phosphorus concentration in a hydroponic solution in which host (filled circle) and At*PHR1* transgenic torenia (empty circle) were cultured was measured. The longitudinal axis shows the phosphorus concentration (mg/L), and the horizontal axis shows the number of days after exchange of the hydroponic solution. (b) Pi concentration in the leaves and aerial parts of hydroponically-cultivated torenia. The longitudinal axis shows the phosphorus concentration per gram fresh weight of samples (mg/gFW) (left) and the phosphorus concentration per gram dry weight of samples (mg/gDW) (right). There were significant differences in means between host and transgenic plants. (c) Comparison of growth rate. Weights of aerial parts and root parts of the torenia plants were measured at the end of hydroponic experiments. There was no statistically significant difference between transgenic and host.

the dead sections was slightly higher than that of unaffected portions of leaves (Figure 3(d)). Since excess Pi may cause cell toxicity [26], the death may have been the result of exceeding a critical limit of Pi concentration in the torenia leaf cells. It thus appears that the critical limit of Pi accumulation level in At*PHR1* transgenic torenia is approximately 20 mg/gDW. One possible way to overcome the death of leaf tissues due to high Pi accumulation is to convert Pi to a nontoxic form of phosphorus that is phytic acid. Genetic modification could be used to achieve this, resulting in transgenic plants accumulating even more Pi than reported here.

3.3. Large-Scale Hydroponic Experiment. To access the potential for phytoremediation using the transgenic torenia at a larger scale, we performed longer term hydroponic experiments. Thirteen torenia plants were put each into 400-liter tub and incubated for approximately 2 months (Figure 4). There was no significant difference in average biomass between transgenic and host plants after 65 days incubation (Figure 4 and Table 1). However, approximately 3-fold more Pi accumulation was seen in the transgenic plant when compared to the host. This confirmed that transgenic torenia shows the accelerated absorption as well as accumulation of Pi in the leaves when grown on a larger scale. From the daily calculation of Pi accumulation of the transgenic torenia plant, Pi accumulation rates were able to be compared to water hyacinth (Table 1). The At*PHR1* transgenic torenia showed an equivalent efficiency of Pi accumulation to that of water hyacinth [27, 28].

Table 1: Comparison of phosphate absorption performances. Phosphorus content, total biomass, and absorption rate after 65 days of the hydroponic experiment are indicated. Data are the mean ± SD from 13 plants. Values of water hyacinth were calculated from values listed in [27, 28].

	Phosphorus in leaf (mg/gFW)	Total biomass (g/plant)	Absorption rate (mg/plant/day)
Host	0.18 ± 0.11	396.34 ± 146.06	1.08
35S::At*PHR1*	0.69 ± 0.20	382.95 ± 178.85	4.15
Water hyacinth	0.38		1.79

Overexpression of At*PHR1* gene might drive a Pi starvation response in the transgenic plants. As a result, excessive

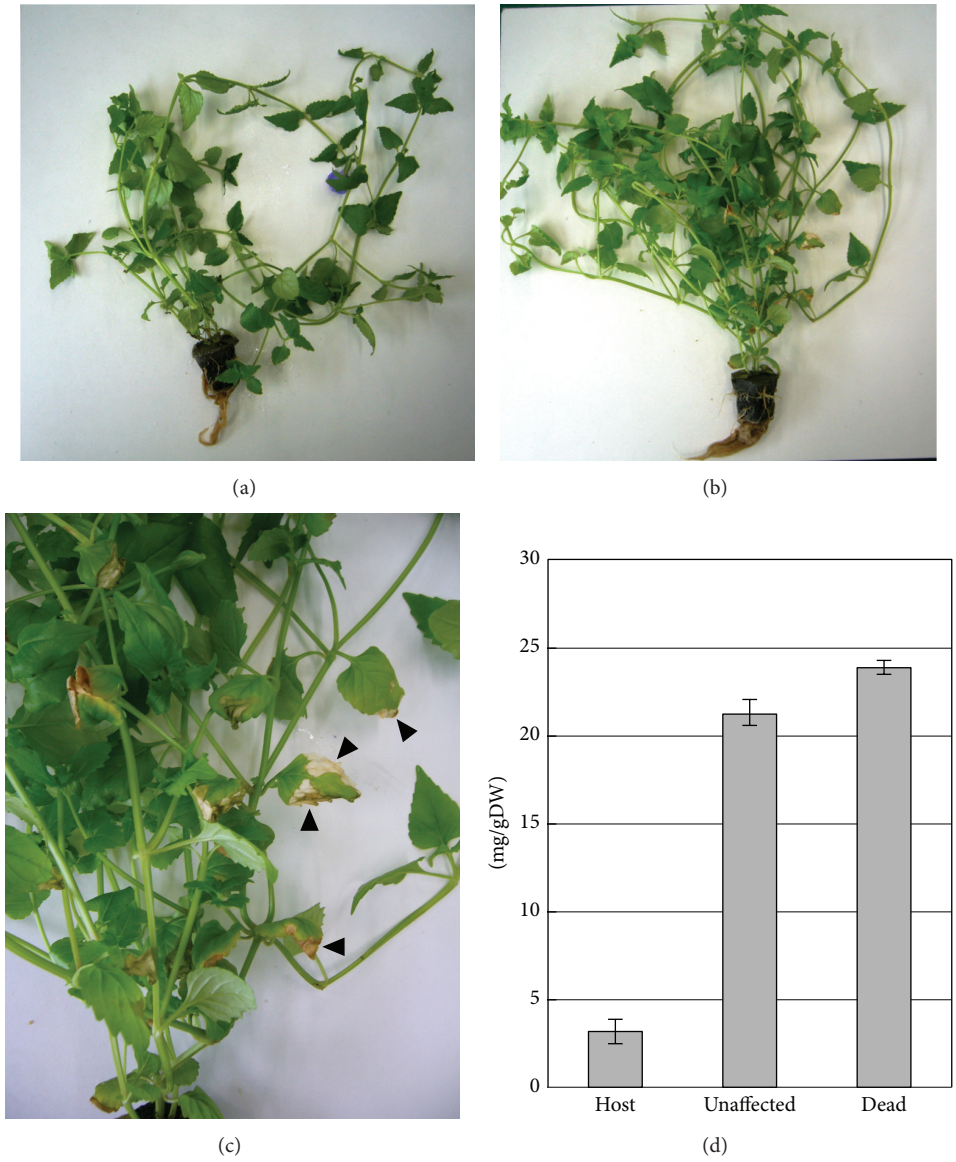
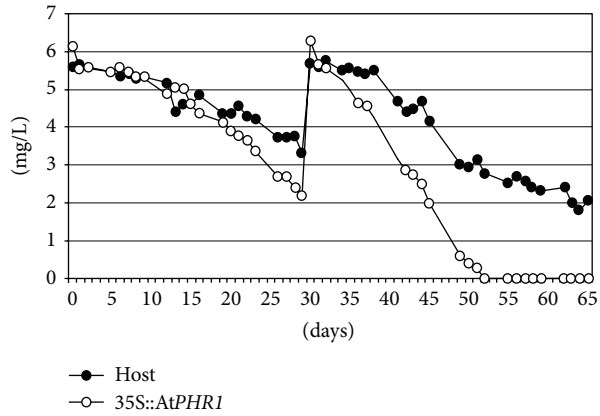


Figure 3: Dead tissue in At*PHR1* transgenic torenia. (a) Host plant at the end of hydroponic experiment. (b) At*PHR1* transgenic plant after 4 weeks of hydroponic experiment. (c) Magnified image of (b). Arrowheads indicate partially dead sections. (d) Phosphorus concentration in unaffected and dead areas from leaves of host and At*PHR1* transgenic plants. The longitudinal axis shows the phosphorus concentration per gram dry weight of sample (mg/gDW).

amounts of Pi accumulated in transgenic leaves. In *A. thaliana*, At*PHR1* gene is not transcriptionally regulated even under Pi starvation condition [17]. Since the key mechanism of the Pi starvation response is still debatable in *Arabidopsis thalinana* [17, 29], it is difficult to postulate why overexpression of At*PHR1* is effective for Pi uptake in other species. We have isolated orthologous Pi starvation-related genes (At*PHR1*, At*IPS1*, At*PHT1;1*, and At*PHO2*) in torenia and examined expression pattern of these genes (data not shown). We could not detect any differences between transgenic torenia and host plants. Overexpression of At*PHR1* may interfere with the proper posttranscriptional modification of the endogenous At*PHR1* counterpart, possibly through competitive inhibition.

Since phosphorus is expected to be exhausted as a natural resource within a hundred year [30], it is necessary to recover phosphorus from the environment, especially in polluted areas. Currently, over 90% of the produced phosphorus in the world is used as fertilizers. Therefore, it is most reasonable to recover phosphorus from fertilized soils and agricultural runoffs. Phytoremediation is a suitable method for such a recycling process, in addition to cleaning up phosphorus from the aquatic environment. One of the critical problems of phytoremediation is the cost of the disposal of the plant [31]. The plant used for phytoremediation was in many cases simply discarded without being used as a source of Pi. Ideally, plants containing high accumulation of Pi can be returned to soils of agricultural land without processing and can be directly



(a)

(b)

(c)

Figure 4: Large-scale hydroponic experiments. (a) Changes in Pi concentration in hydroponic solutions. The phosphorus concentration in a hydroponic solution in which host (filled circle) and At*PHR1* transgenic torenia (empty circle) were cultured was measured. The longitudinal axis shows the phosphorus concentration (mg/L), and the horizontal axis shows the number of days. Hydroponic solutions were fully exchanged 30 days after starting the experiment. (b) Large-scale experiment (0 day). (c) Large-scale experiment (65 days).

used as fertilizer. However, at present, absorbing ability of the existing plants used for phytoremediation is not efficient enough to be used as Pi sources for agriculture in this way. In this study, the At*PHR1* transgenic plants accumulated a high level of Pi. Therefore, applications of At*PHR1* transgenic plants for phytoremediation of water could be cost-effective. Moreover, the Pi recycling ability of flowers and ornamental plants for gardening can be increased by means of At*PHR1* gene introduction, and thereby purifying water with plants having both ornamental beauty and high purification ability.

4. Conclusions

In this study, we prove the feasibility of using At*PHR1* as an enhancer of Pi uptake in transgenic plants. By introducing At*PHR1* to garden plants, amounts of Pi accumulation and absorption of Pi were increased to rates approximately 3-fold higher than host plant. There was no significant reduction in biomass or morphology of the transgenic plant expressing At*PHR1*. Taken together, these observations indicate that the At*PHR1* gene will be valuable for production of hyperaccumulator plants for the purification of waters polluted with Pi. In addition, an improved appearance of purification sites can be provided by using ornamental plants with many flowers, as shown in Figure 4(c).

Conflict of Interests

The authors have no conflict of interests to declare.

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Review Article

Anaerobic Ammonium Oxidation: From Laboratory to Full-Scale Application

Shou-Qing Ni and Jian Zhang

School of Environmental Science and Engineering, Shandong University, Jinan 250100, China

Correspondence should be addressed to Shou-Qing Ni; sqni@sdu.edu.cn

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From discovery in the early 1990s to completion of full-scale anammox reactor, it took almost two decades to uncover the secret veil of anammox bacteria. There were three milestones during the commercialization of anammox: the development of the first enrichment culture medium, the completion of the first commercial anammox reactor, and the fast start-up of full-scale anammox plant. Till now, the culture of anammox bacteria experienced a big progress through two general strategies: (a) to start up a reactor from scratch and (b) to seed the reactor with enriched anammox sludge. The first full-scale anammox reactor took 3.5 years to realize full operation using the first approach due to several reasons besides the lack of anammox sludge. On the other hand, the first Asian anammox reactor started up in two months, thanks to the availability of anammox seed. Along with the implementation of anammox plants, anammox eventually becomes the priority choice for ammonium wastewater treatment.

1. Introduction

Conventional biological nitrogen removal from wastewater usually consists of two steps, nitrification and denitrification. During nitrification process, ammonium is biologically oxidized to nitrate, which is then reduced to nitrogen gas using organic matter as electron donor during denitrification process. When BOD/TKN ratio is low as in many ammonium-rich wastewaters, biodegradable organic matter source must be added to achieve complete denitrification [1, 2]. The operations are rather cost-intensive for both oxygen demand for aerobic nitrification and organic substrates addition for denitrification [2–4]. The surplus sludge generated in conventional biological nitrogen removal process also increases the treatment cost.

Anaerobic Ammonium Oxidation (anammox) is a novel, autotrophic, and cost-effective alternative to the traditional biological nitrogen removal process [5–7]. The existence of the bacteria was first predicted in the 1970s on the basis of thermodynamic calculations. Anammox bacteria oxidize ammonium to nitrogen gas using nitrite as an electron accepter under anoxic conditions, and their growth occurs by carbon dioxide fixation (Table 1) [8].

The discovery of anammox process brought revolutionary changes to conventional biological nitrogen removal from wastewater. Some unique characteristics make anammox process a promising and sustainable technique [9], such as low biomass yield, no need for aeration, and no addition of external carbon sources [10]. While the newly discovered anammox process opens up new possibilities for nitrogen removal from wastewater, the major obstacle for the implementation of anammox is the slow growth rate ($\mu_{max} = 0.065 d^{-1}$, doubling time ($t_{1/2} = \ln 2/\mu_{max}$) of 11 days) of anammox microorganisms [8, 11], making this process difficult for practical wastewater treatments. Meanwhile, anammox bacteria have been extremely difficult to cultivate in pure culture, even *Candidatus* Brocadia anammoxidans has only been purified to apparent homogeneity by Percoll density centrifugation [12]. In order to fulfill practical application of anammox process, researchers focus on the enrichment of slowly growing anammox bacteria. Many studies were carried out to enrich anammox organisms, either by different methods such as biofilm or granulation, or by all types of reactors. This paper reviews the development of anammox process and relative studies in the laboratory, especially the discovery and biochemistry of the bacteria responsible

Table 1: Reactions involved in the realization of anammox process.

Reaction no.	Reaction	$\Delta G^{o\prime}$ (kJ/mol NH_4^+)	N_2 composition (%)	
			$^{14\text{-}15}N_2$	$^{15\text{-}15}N_2$
1[a]	$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+$	−297	75	25
2[a]	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	−358	100	0
3[b]	$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$	−358	100	0

[a]Van de Graaf et al. [21].
[b]Strous et al. [8].

for anaerobic ammonium oxidation. Special attention was paid on the commercialization and full-scale application of anammox technique.

2. Discovery and Phylogeny of Anammox

Already in 1932, it was reported that dinitrogen gas was generated via an unknown mechanism during fermentation in the sediments of Lake Mendota, Wisconsin, USA [14]. More than 40 years ago, Richards [15] noticed that most of the ammonium that should be produced during the anaerobic remineralization of organic matter was unaccounted for. As there was no known biological pathway for this transformation, biological anaerobic oxidation of ammonium received little further attention [13]. Three decades ago, the existence of two chemolithoautotrophic microorganisms capable of oxidizing ammonium to dinitrogen gas was predicted on the basis of thermodynamic calculations [7]. It was thought that anaerobic oxidation of ammonium would not be feasible, assuming that the predecessors had tried and failed to establish a biological basis for those reactions [16]. By 1990s, Arnold Mulder's fantastic observations were just consistent with Richards' suggestion [16]. In their anoxic denitrifying pilot reactor, ammonium disappeared at the expense of nitrite with a clear nitrogen production. The reactor used the effluent from a methanogenic pilot reactor, which contained ammonium, sulphide and other compounds, and nitrate from a nitrifying plant as the influent. This process was named "anammox," and people realized that it had great significance in the removal of unwanted ammonium. Even without full understanding of anammox reaction, Arnold Mulder patented the process immediately [17, 18]. The discovery of anammox process was first publicly presented at the 5th European congress on biotechnology [19]. By the mid-1990s, the discovery of anammox in the fluidized bed reactor was published [20]. A maximum ammonium removal rate of 0.4 kg $N/m^3/d$ was achieved. It was shown that for every mole of ammonium consumed, 0.6 mol of nitrate was required, resulting in the formation of 0.8 mol of N_2 gas ((1) in Table 1). In the same year, the biological nature of anammox was identified [21]. Labeling experiments with $^{15}NH_4^+$ in combination with $^{14}NO_3^-$ showed that $^{14\text{-}15}N_2$ was the dominant product, making up 98.2% of the total labeled N_2. These findings conflicted with reaction 1 in which the percentage of $^{14\text{-}15}N_2$ and $^{15\text{-}15}N_2$ in the formed dinitrogen gas would be 75% and 25%, respectively. It was realized

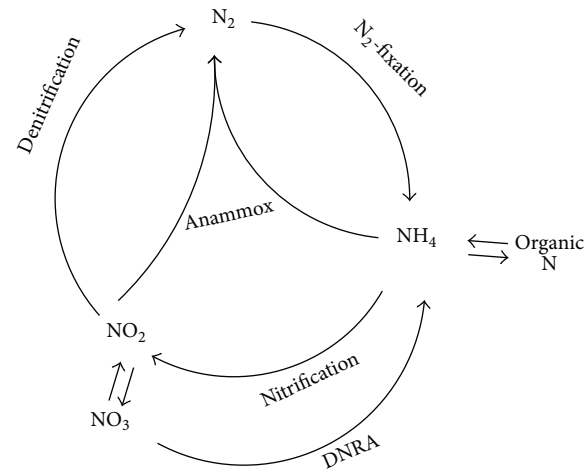
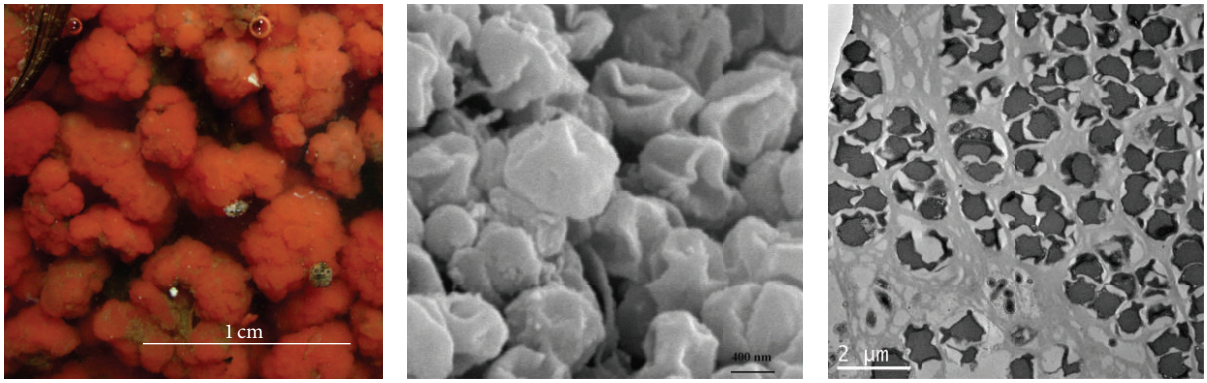


Figure 1: The biological N cycle (based in part on Arrigo [13]). DNRA, dissimilatory nitrate reduction to ammonium.

that, instead of nitrate, nitrite was assumed as the oxidizing agent of ammonium in anammox reaction ((2) in Table 1) [21]. Based on a previous study, Strous et al. [8] calculated the stoichiometry of anammox process by mass balancing ((3) in Table 1), which is widely accepted by other groups. Later, anammox bacteria were identified as planctomycetes [12], and the first identified anammox organism was named *Candidatus* "Brocadia Anammoxidans" [22]. Before 2002, anammox was assumed to be a minor player in the N cycle within natural ecosystems [23]. In 2002, anammox was found to play an important part in the biological nitrogen cycle, accounting for 24–67% of the total N_2 production in the continental shelf sediments that were studied [24]. Globally, anammox may be responsible for 30–50% of N_2 production in the ocean [25]. The discovery of anammox process modified the concept of biological nitrogen cycle as depicted in Figure 1.

The specific red color of anammox bacteria (Figure 2(a)) is due to the heme c group of the protein cytochrome c that plays an important role in anammox metabolism [27]. The irregular shapes of anammox bacteria were displayed by both transmission electron microscopy and scanning electron microscopy images (Figures 2(b) and 2(c)). The anammox species have a single membrane bound anammoxosome and riboplasm with ribosome-like particles separated from paryphoplasm by an intracytoplasmic membrane. The cells



(a) (b) (c)

Figure 2: The specific red color of anammox bacteria (a), the typical irregular shapes of anammox bacteria displayed by scanning electron microscopy (b), and transmission electron microscopy images (c).

contain three distinct membrane bound compartments: the paryphoplasm, cytoplasm, and anammoxosome.

Till now, five anammox genera have been discovered, with 16S rRNA gene sequence identities of the species ranging from 87 to 99% [27]. It is well known that all anammox bacteria belong to the same monophyletic order named the Brocadiales and are related to the Planctomycetales. Among them, four "*Candidatus*" anammox genera have been enriched from activated sludge: "Kuenenia" [28, 29], "Brocadia" [12, 22, 30], "Anammoxoglobus" [31], and "Jettenia" [32]. The fifth anammox genus, "*Candidatus* Scalindua" [33–35], has often been detected in natural habitats, especially in marine sediments and oxygen minimum zones [36–39].

3. Possible Reaction Mechanisms for Anammox

To understand the possible metabolic pathway for anammox, ^{15}N labeling experiments were first carried out in 1997 [42]. These experiments showed that ammonium was biologically oxidized with hydroxylamine, most likely derived from nitrite, as the probable electron acceptor. The conversion of hydrazine to dinitrogen gas is postulated as the reaction generating the electron equivalents for the reduction of nitrite to hydroxylamine. Generally, two possible reaction mechanisms were addressed [26]. A membrane-bound enzyme complex converts ammonium and hydroxylamine to hydrazine first, followed by the oxidation of hydrazine to dinitrogen gas in the periplasm. At the same time, nitrite is reduced to hydroxylamine at the cytoplasmic site of the same enzyme complex responsible for hydrazine oxidation with an internal electron transport (Figure 3(a)). Another possible mechanism for anammox process is concluded as follows: ammonium and hydroxylamine are converted to hydrazine by a membrane-bound enzyme complex, hydrazine is oxidized in the periplasm to dinitrogen gas, and the generated electrons are transferred via an electron transport chain to nitrite reducing enzyme in the cytoplasm where nitrite is reduced to NH_2OH (Figure 3(b)). Whether the reduction of nitrite and the oxidation of hydrazine occur at different sites of the same enzyme (Figure 3(a)) or the reactions are catalyzed by different enzyme systems connected via an electron transport chain (Figure 3(b)) remains to be investigated. The occurrence of hydrazine as an intermediate in microbial nitrogen metabolism is rare [43]. Hydrazine has been proposed as an enzyme-bound intermediate in the nitrogenase reaction [44].

A possible role of NO or HNO in anammox was proposed by Hooper et al. [45] by way of condensation of NO or HNO and ammonium on an enzyme related to the ammonium monooxygenase family. The formed hydrazine or imine could thereafter be converted by the enzyme hydroxylamine oxidoreductase to dinitrogen gas, and the reducing equivalents produced in the reaction are required to combine NO or HNO and ammonium or to reduce nitrite to NO. Environmental genomics analysis of the species *Candidatus* Kuenenia stuttgartiensis, through a slightly different and complementary metabolism mechanism, postulated NO to be the intermediate instead of hydroxylamine (Figure 4) [29]. But this hypothesis also agreed that hydrazine was an important intermediate in the process. In this pathway (Figure 4), there are two enzymes unique to anammox bacteria: hydrazine hydrolase (hh) and hydrazine dehydrogenase (hd). The hh produces hydrazine from nitric oxide and ammonium, and hd transfers the electrons from hydrazine to ferredoxin. Few new genes, such as some known fatty acid biosynthesis and S-adenosylmethionine radical enzyme genes [29], containing domains involved in electron transfer and catalysis were detected.

4. Basal and Designated Medium Development

Once nitrite was realized to be the electron acceptor with ammonium as electron donor, a basal medium containing ammonium, nitrite, bicarbonate, minerals, and trace elements was developed for the enrichment of anammox microorganisms [46]. The medium contained ammonium (5–30 mM) and nitrite (5–35 mM), as the only electron donor and electron acceptor, respectively, with bicarbonate (10 mM) as the only carbon source. Minerals and trace elements were

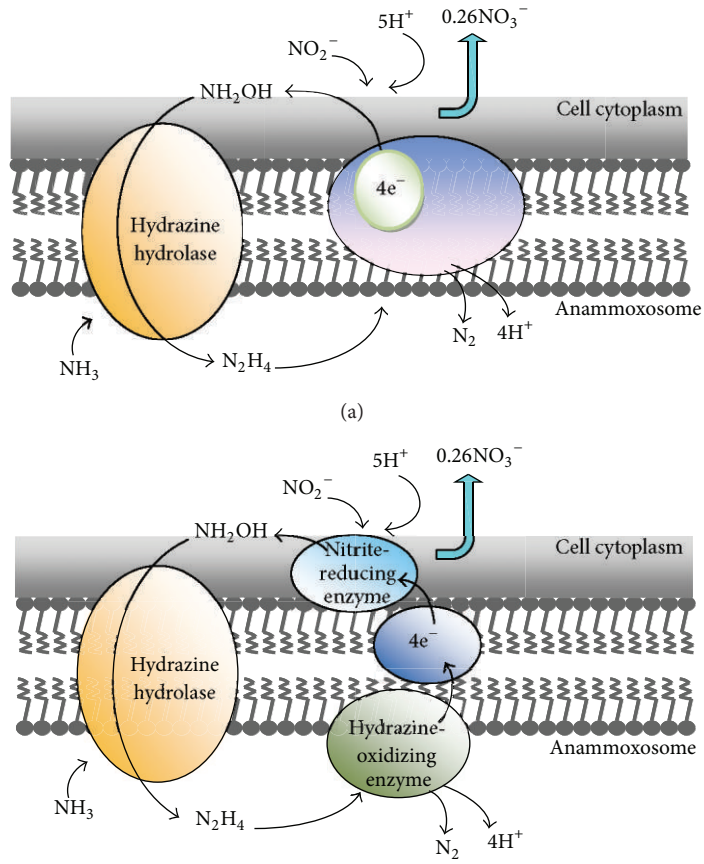


FIGURE 3: Possible biochemical pathway and cellular localization of the enzyme systems involved in anammox reaction. Figure modified, with permission, from FEMS Microbiology Reviews [26] and Process Biochemistry [2].

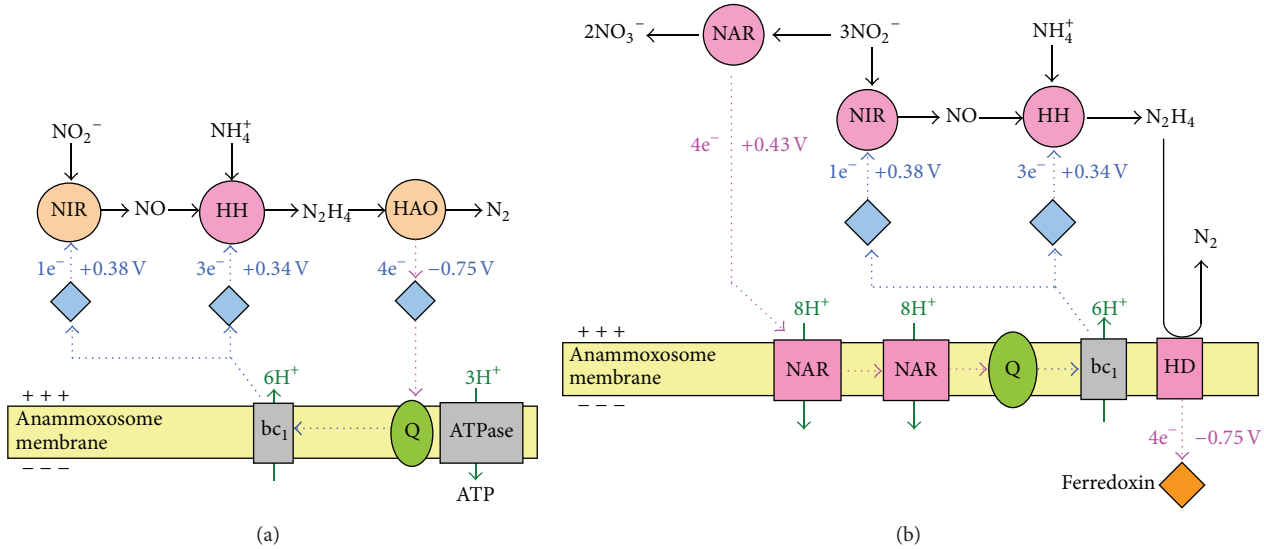


FIGURE 4: Hypothetical metabolic pathways and reversed electron transport in the anammoxosome. (a) Anammox catabolism that uses nitrite as the electron acceptor for the creation of a proton motive force over the anammoxosomal membrane. (b) Proton motive force-driven reversed electron transport combines central catabolism with nitrate reductase (NAR) to generate ferredoxin for carbon dioxide reduction in the acetyl-CoA pathway. HAO, hydrazine oxidoreductase; HD, hydrazine dehydrogenase; HH, hydrazine hydrolase; NIR, nitrite oxidoreductase; Q, quinine. Light blue diamonds, cytochromes; blue arrows, reductions; pink arrows, oxidations. Figure modified, with permission, from *Nature* [29].

also provided. Phosphate concentration of the medium was kept below 0.5 mM, in order to avoid its possible inhibitory effect on the process, and medium was flushed with argon gas to achieve anaerobic conditions. Experiments which were carried out in a fluidized bed reactor with basal enrichment medium showed that the anaerobic ammonium removal rate increased from original 0.4 kg N/m^3/day to 2.4 kg N/m^3/day [20]. The maximum specific activity of the biomass in the fluidized bed reactor was 25 nmol NH_4^+/mg VS/min. For every mol of ammonium oxidized, 0.041 mol of CO_2 was incorporated into biomass. The estimated growth rate in the fluidized bed systems was 0.001/h, equivalent to a doubling time of about 29 days. The basal medium enhanced the activities of anammox bacteria.

The development of the basal medium, the milestone of anammox enrichment, turned on the fervent zeal for this infant investigation. Since then, vast number of researchers flooded in this specific topic. As medium shows positive effects on anammox process, many studies focused their attention on this area. Unfortunately, there is no systemic medium development study like those for other bacteria [48, 49].

In our lab, a study was conducted towards designing an appropriate medium by investigating growth requirement of anammox bacteria with respect to amino acids. Twenty L-amino acids were added to basal medium (Table 2). After experiment set I, set II was carried out to further evaluate the enhanced effects of the selective amino acids on microorganisms growth. To quantify the growth of anammox bacteria, quantitative molecular techniques were employed. Preliminary experiments indicated that glycine, methionine, threonine, tryptophan, and tyrosine enhanced the growth of anammox bacteria. On the other hand, asparagine, aspartic acid, and histidine slightly decreased bacterial activities. While 12 of 20 L-amino acids (alanine, arginine, cysteine, glutamic acid, glutamine, isoleucine, leucine, lysine, phenylalanine, proline, serine, and valine) totally inhibited the growth of anammox bacteria, resulting in the sludge turning from reddish to blackish. Another 3 amino acid (asparagine, aspartic acid, and histidine) slowed down the growth of anammox bacteria. This unpublished study would benefit anammox study and their application.

Table 2: Growth of anammox bacteria using basal medium with L-amino acids.

Amino acid	Plate concentration (mmol/L)	OD_{600}*
Alanine	0.5	n.d.
Arginine	0.6	n.d.
Asparagine	0.3	−
Aspartic acid	0.3	−
Cysteine	0.3	n.d.
Glutamic acid	5.0	n.d.
Glutamine	5.0	n.d.
Glycine	0.1	+
Histidine	0.1	−
Isoleucine	0.3	n.d.
Leucine	0.3	n.d.
Lysine	0.3	n.d.
Methionine	0.3	+
Phenylalanine	0.3	n.d.
Proline	2.0	n.d.
Serine	4.0	n.d.
Threonine	0.3	+
Tryptophan	0.1	+
Tyrosine	0.1	+
Valine	0.3	n.d.

*Optical density (600 nm) after 7 days of incubation at 35°C, + means increase, − means decrease, and n.d. means not detected because of the color change.

5. Anammox Culture in the Laboratory

Anammox process has been recognized as being difficult to apply for practical applications. Anammox bacteria grow in a mixture of bacterial populations, and they have not been isolated in a pure culture [50]. Anammox bacteria, being strictly anaerobic and autotrophic, are difficult to enrich making application of this process limited due to unavailability of sufficient biomass required for the process. Different methods have been employed to culture and enrich anammox biomass from different types of seed sludge [51, 52]. A relative population of 88% anammox bacteria was achieved in a batch study inoculated from a rotating biological contactor (RBC) treating a landfill leachate [53]. Enrichment culture of anammox bacteria was also developed in lab-scale reactors inoculated with marine sediments [35] and paddy field soil samples and activated sludge from wastewater treatment plants [54].

The slow growth rate of anammox bacteria with the approximate doubling time of 11 days is the major obstacle for implementation of anammox process [8]. A long start-up period is thus expected in anammox process. Shortening anammox process start-up period by reducing washout potential of anammox biomass becomes an important strategy for full-scale application. Different types of reactor design have been used to minimize the washout of anammox biomass including continuous stirred-tank reactor, anaerobic biological filtrated reactor, sequencing batch reactor (SBR), upflow reactor, and biofilm reactor [8, 55–57]. Faster growth of anammox bacteria was achieved in a membrane bioreactor (MBR) (the doubling time was less than 10 days), resulting in an unprecedented purity of the enrichment of 97.6% [58]. The formation of compact aggregates was reported to maintain a large amount of active anammox biomass in a reactor [55]. Therefore, granulation is also an alternative approach for anammox enrichment.

In summary, there are two main approaches (strategies) to start up an anammox reactor: (a) to start a reactor from scratch and (b) to inoculate it with highly enriched anammox sludge. For the first strategy, the reactor configuration is very important. The SBR technique ensured over one year reliable

operation under stable conditions with efficient biomass retention (more than 90% of the biomass was maintained in the reactor) and homogeneous distribution of substrates, products, and biomass aggregates [8]. The MBR was also applied successfully for cultivation of anammox bacteria with fast growth rate (the minimum doubling time for anammox bacteria was estimated to be 5.5–7.5 days) [58]. Among different reactors, the anammox nonwoven membrane reactor (ANMR) is a novel reactor configuration to enrich anammox biomass (Figure 5) [40, 41]. The reactor was developed by connecting a set of nonwoven membrane module, which also served as an effluent port, with an anaerobic reactor. The membrane module was installed outside the reactor, which is different from the immerged membrane reactors. Unlike conventional MBR, wastewater circulated in the membrane module, and the biofilms grew on the membrane interior surface. A large amount of the suspended biomass could remain in the reactor by filtration through the nonwoven membrane and biofilms, resulting in improvement of the effluent quality and enhancement of the solid retention in the reactor. After over eight months of operation, the purity (percentage of anammox cells in the community) of anammox bacteria in the reactor was quantified to be 97.7% [40]. The cost-effective ANMR was shown to be suitable for the slowly growing anammox bacteria having the following advantages: (1) a large amount of the biomass could remain in the reactor by filtration through the nonwoven membrane and the formation of biofilm, (2) the formation of aggregates and biofilm enhanced the solid retention in the reactor, (3) the nonwoven membrane was cost efficient, and (4) the design of the anaerobic reactor could dilute the influent medium and avoid inhibition from high nitrite concentrations, leading to high tolerance ability of substrates. Recently, the upflow anaerobic sludge blanket (UASB) reactor was highly recommended for the culture of slowly growing bacteria [59–62]. This is because of not only the improvement of physiological conditions, making them favorable for bacteria and their interactions, especially syntrophisms in the anaerobic system, but also the formation of granular sludge, being the major reason of the successful introduction of the UASB reactor [63]. Hence, granulation also improves anammox application. Surprisingly, Ni and his colleagues used inactive methanogenic granules as inocula to realize fast granulation successfully [64]. The start-up nitrite concentration was significantly higher than the published toxic level for anammox bacteria and other lab-scale studies. The accommodations and proliferations of anammox bacteria in the inactive methanogenic granules might be the main reason for the high anammox purity in a short period. Anammox cells could use the skeleton of inactive methanogenic granules and proliferate from the interior as observed in TEM (Figure 6). The second approach mentioned previously significantly shortens the required time for anammox start-up under the premise of large quantity of anammox sludge but is usually limited by the lack of anammox sludge. The gradual construction of full-scale anammox plants increases the availability of anammox sludge. The introduction of the exotic anammox sludge to seed a granular reactor is a good choice [59]. The reactor was started successfully in two weeks; in addition, high nitrogen removal was achieved for a long period, showing that the inoculation of mature anammox granules was ideal to start up a new reactor.

6. Commercial Application of Anammox Process

The lack of pure cultures of anammox bacteria makes a genomic approach less straightforward. Combined with the low maximum specific growth rate of anammox bacteria and stringent operational conditions, the practical application of anammox fell far behind the research progress.

Many efforts have been made on the development of a marketable product. Here, we would like to mention the Paques BV (Balk, The Netherlands) for its unremitting efforts on the practical application of anammox process. Early in 2001, Van Dongen et al. [57] scaled up lab-scale SHARON (single reactor system for high rate ammonium removal over nitrite) reactor [3] in collaboration with the Paques BV. The effluent of the SHARON process was ideally suited as influent for anammox process, for the ammonium was oxidized by 53% to nitrite, rather than nitrate in SHARON process at 1.2 kg N load per m^3 per day without pH control [57]. The combined SHARON-anammox system could work stably over long periods, and the authors predicted that the combination process was ready for full-scale implementation.

Based on constant and successful study, in 2007, the first full-scale granular anammox reactor was accomplished at the wastewater treatment plant of Waterboard Hollandse Delta in Rotterdam, The Netherland [9, 65]. This stands for the start of the commercial application of anammox process, exhibiting to be another milestone. The first full-scale 70 m^3 reactor was directly scaled up 7000-fold from 10 l lab-scale experiment. The reactor was initially inoculated with nitrifying sludge and a total amount of 9.6 m^3; settled biomass from an anammox enrichment reactor was added from day 622 to 1033 [65]. Even with the addition of anammox sludge, the start-up took 3.5 years, 1.5 years longer than designed. Several reasons caused the long start-up time, besides the low growth rates of anammox microorganisms. Most important is that there was no anammox seed sludge available to inoculate the first full-scale reactor, and delay was caused by technical issues such as operational and temperature problems [9], as the first full-scale reactor was directly scaled up from lab scale, skipping the pilot phase. This first full-scale reactor on the other hand had a pilot plant character. In September 2006, the reactor was in full operation and the loading rate could be reached to a level of 750 kg/d, 50% higher than the design load.

Another four anammox plants were built before 2008, three in Europe and one in Asia (Table 3). The third reactor, part of a plant for the treatment of the effluent of a potato factory, exhibited a largest ammonium load rate. The capacity of the reactor is 1200 kg N/d, while only about 700 kg N/d is converted as no more nitrogen available in the wastewater. Japan built the first full-scale Asian anammox reactor at a semiconductor plant. In 2009, Paques Environmental Technology (Shanghai) released the news that an agreement had been reached to build world's largest anammox based

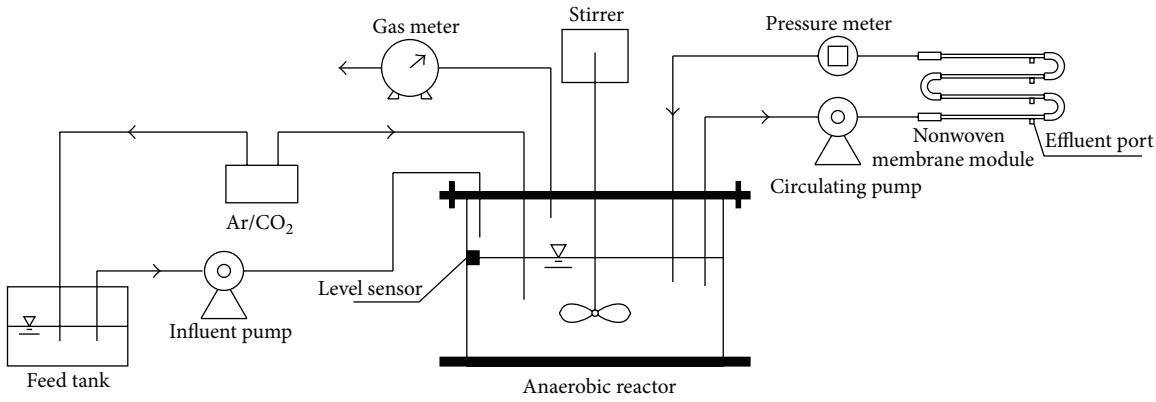
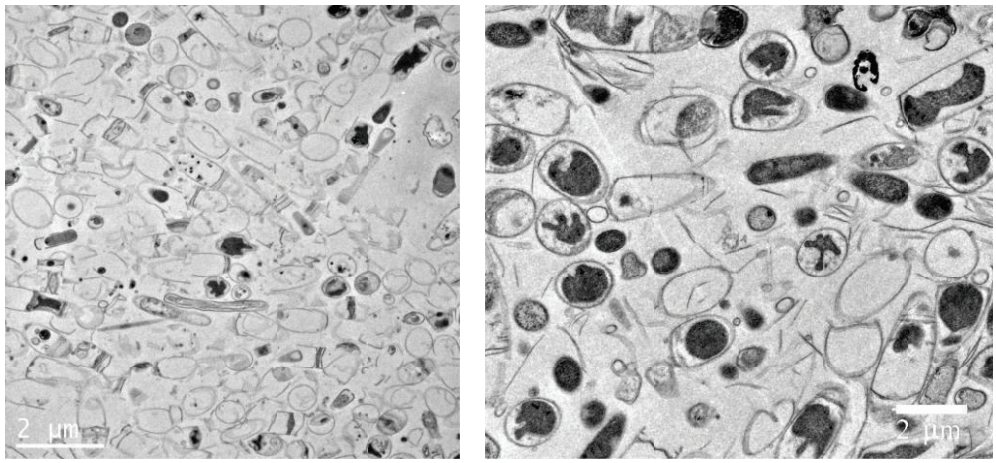


Figure 5: Schematic diagram of the anammox nonwoven membrane reactor (ANMR) [40, 41].



(a) (b)

Figure 6: (a) Transmission electron micrograph showing dormant cells in the seed granule (bar = 2 μm). (b) Transmission electron micrograph showing the anammox bacteria in the interior of granules (bar = 2 μm).

Table 3: The brief description of worldwide full-scale anammox plants implemented by Paques[a].

Process	Place	Influent	Reactor volume (m^3)	Designed load (kgN/d)	Year
SHARON-anammox	Rotterdam, NL	Reject water	72	490 (750)[b]	2002
Nitrification-anammox	Lichtenvoorde, NL	Tannery	100	325 (150)[c]	2004
Anammox	Olburgen, NL	Potato processing	600	1200 (700)[c]	2006
Nitrification-anammox	Mie prefecture, JP	Semiconductor	50	220 (220)[b]	2006
Anammox	Niederglatt, Switzerland	Reject water	180	60 (60)[b]	2008
Anammox	Tongliao, China	Monosodium glutamate (MSG)	6600	11000	2009
Anammox	Yichang, China	Yeast production	500	1000	2009
Anammox	Tongliao, China	MSG	4100	9000	2010
Anammox	The Netherlands	Reject water	425	600	2010
Anammox	Tai'an, China	Corn starch and MSG	4300	6090	2011
Anammox	Poland	Distillery	900	1460	2011
Anammox	Wuxi, China	Sweetener	1600	2180	2011
Anammox	Wujiaqu, China	MSG	5400	10710	2011
Anammox	Coventry, UK	Reject water	1760	4000	2011
Anammox	Shaoxing, China	Distillery	560	900	2011

[a] Abma et al. [47] and communication with Paques BV.
[b] Values in parentheses mean achieved loads (kg N/d).
[c] No more nitrogen available.

wastewater treatment plant in China. Anammox process was designed to have a capacity for conversion of 11 tons of nitrogen per day, almost ten times larger than the largest plant built before 2008. The two-step combination of anammox and internal circulation (IC) reactors will be the sixth full-scale application of anammox. Since 2009, anammox experienced huge development. Another 11 anammox plants were implemented by Paques, seven of which are located in China. As the world's biggest developing market, China contributes significantly towards commercialization of anammox process.

Thanks to the experience from the established anammox plants, the start-up time of the marketable plant became shorter and shorter. This could be another milestone. The second reactor started up in 1 year and it took 2 months for the start-up of the first Asian plant. Till now, more than 30 full-scale variant plants are in operation around the world, mostly in the Austria, China, Japan, The Netherlands, and USA. All these emphasize on anammox process becoming a commercial technique.

7. Conclusion

The discovery of the green process, anammox, brings revolutionary changes to conventional biological nitrogen removal. Playing an important part in the biological nitrogen cycle, this unique process makes great contribution to our environment and economy. Anammox development experienced several important points: laboratory culture based on basal medium, full-scale reactor system implementation, and extensive engineering applications. Although starting up the reactor from scratch is universal, inoculation with highly enriched anammox sludge is more feasible. Currently, at least 30 full-scale anammox systems are operational. Thus, application of anammox process offers an attractive alternative to current wastewater treatment systems for ammonia-nitrogen removal.

Highlights

Development of anammox process from laboratory to commercialization was reviewed. There were three milestones: basal medium, first plant, and extensive applications. Seeding with enriched anammox sludge is more feasible than starting from scratch. Over 30 full-scale anammox plants are in operation around the world. Anammox eventually becomes the priority choice for ammonium wastewater treatment.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Fractionation and Purification of Bioactive Compounds Obtained from a Brewery Waste Stream

Letricia Barbosa-Pereira,[1] Ainara Pocheville,[2] Inmaculada Angulo,[2] Perfecto Paseiro-Losada,[1] and Jose M. Cruz[3]

[1] *Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Pharmacy, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[2] *GAIKER Technological Centre, 48170 Zamudio, Spain*
[3] *Department of Chemical Engineering, Industrial Engineering School, University of Vigo, 36310 Vigo, Spain*

Correspondence should be addressed to Jose M. Cruz; jmcruz@uvigo.es

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The brewery industry generates waste that could be used to yield a natural extract containing bioactive phenolic compounds. We compared two methods of purifying the crude extract—solid-phase extraction (SPE) and supercritical fluid extraction (SFE)—with the aim of improving the quality of the final extract for potential use as safe food additive, functional food ingredient, or nutraceutical. The predominant fractions yielded by SPE were the most active, and the fraction eluted with 30% (v/v) of methanol displayed the highest antioxidant activity (0.20 g L^{-1}), similar to that of BHA. The most active fraction yielded by SFE (EC_{50} of 0.23 g L^{-1}) was obtained under the following conditions: temperature 40°C, pressure 140 bar, extraction time 30 minutes, ethanol (6%) as a modifier, and modifier flow 0.2 mL min^{-1}. Finally, we found that SFE is the most suitable procedure for purifying the crude extracts and improves the organoleptic characteristics of the product: the final extract was odourless, did not contain solvent residues, and was not strongly coloured. Therefore, natural extracts obtained from the residual stream and purified by SFE can be used as natural antioxidants with potential applications in the food, cosmetic, and pharmaceutical industries.

1. Introduction

Bioactive phenolic compounds are widely distributed in nature and are the most abundant antioxidants in the diet, being common components of fruits, vegetables, and beverages [1, 2]. Numerous studies have associated the consumption of foods rich in bioactive compounds, such as phenolic compounds, with the prevention of cardiovascular diseases, certain types of cancer, and other diseases related to aging [3]. The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity. These bioactive compounds may be a major determinant of the antioxidant potentials of foods, and they may therefore be a natural source of antioxidants [4]. Antioxidants are widely used in food products to prevent or delay the oxidation of fats and oils [5]. The recent worldwide trend to avoid or at least reduce the use of synthetic additives, such as BHT and BHA, has created the need to identify natural (and possibly safer) alternative sources of food antioxidants [6, 7]. In recent years, there has been a growing interest in the use of natural antioxidants in the food industry, not only for application as preservatives but also because of their benefits to human health [8, 9].

Beer production is an extensively studied biotechnological process that generates various by-products. The most common byproducts are generated from the main raw materials used to make beer, that is, barley malt, hop, and yeast. These by-products can be used in biotechnological processes, such as fermentative processes for the production of value-added compounds (e.g., xylitol, ethanol) as substrates for culturing microorganisms and as raw material for extraction of compounds such as antioxidants [10].

Beer contains a large variety of phenolic compounds which are derived from the biotechnological fermentation of barley malt (70%) and hop (30%) and which are responsible for the overall antioxidant activity of the beverage [11, 12]. Numerous studies have shown that polyphenols

are extremely important for the physical stability (a fundamental quality parameter) of beer [12]. During storage of beer, colloidal haze forms as a result of the complexes that polyphenols form with proteins and polypeptides [13]. The negative impact of malt and hop polyphenols on haze stability can be minimized by using polyvinylpolypyrrolidone (PVPP) resin to stabilize beer and consequently extend its shelf life. Stabilization with PVPP removes a substantial portion of the haze active and nonhaze active polyphenols from beer, and these polyphenols can subsequently be recovered from the PVPP by an alkaline treatment [14]. Therefore, a natural extract containing bioactive phenolic compounds with high antioxidant activity can be obtained from the alkaline residual stream generated after cleaning the PVPP in the brewery industry, by extraction in a solvent such as ethyl acetate [15].

The composition of the extract will depend on the solvent used and also on the quality of the original material, its composition, genetic factors, environmental conditions, storage conditions, and any prior treatment. In order to obtain a high quality extract with antioxidant activity that is suitable for use in the food, cosmetic, and pharmaceutical industries, the extract must be purified to remove all inert and undesirable components, so as to improve the antioxidant activity of the extract and minimize any odour, taste, and colour [16].

A purification process that removes fractions with limited antioxidant activity enables a good level of antioxidant activity to be obtained from relatively small amounts of the original natural extract. Moreover, it is also important to obtain pure extracts to ensure the identity and safety of antioxidant compounds to be used as food additives [17].

In the present study, we evaluated two methods of purifying the crude extract—solid-phase extraction (SPE) and supercritical fluid extraction (SFE). SPE has been widely used for clean-up and purification of extracts as well as preconcentration of juices, wines, and beer. Phenolic compounds are readily fractionated by several formats of SPE in different materials of natural origin; elution with methanol on reverse-phase columns is the most popular method of separating these compounds [18–20].

Extraction and recovery of valuable compounds are the most common uses for SFE, which operate at low temperatures, in the absence of oxygen, and typically use CO_2 as extraction solvent (SC-CO_2). These features make SFE an ideal technique for extracting bioactive compounds [21]. The most obvious advantages of SFE are that it is clean and environmentally friendly. Direct SC-CO_2 extraction is not recommended for by-products obtained on a large scale and that contain small amounts of bioactive compounds [17]. However, SFE has been used to purify crude extracts yielded by organic solvents, to improve their purity and their biological properties without thermal or chemical degradation. As CO_2 is a non-toxic, inexpensive, noninflammable, volatile solvent, it can be used in a variety of different conditions [22, 23]. The extraction efficiency of SC-CO_2 can be optimized by changing the density of CO_2 (varying pressure and temperature), the modifier (e.g., organic solvent), modifier percentage, or time, among other parameters. Due to the apolar nature of CO_2, the use of modifiers (e.g., ethanol) can significantly improve the recovery of the phenolic compounds due to the polarity of these compounds [17].

The aims of the present study were (i) to evaluate the efficiency of the SPE and SFE techniques to purify natural antioxidants obtained from brewery waste and (ii) to determine the recovery yield and the radical-scavenging activity of the fractions obtained. Chemical analysis of the fractions by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to a diode array detector (DAD) was carried out to identify and quantify the polyphenols responsible for the antioxidant activity.

2. Materials and Methods

2.1. Reagents, Solvents, and Standard Phenolics. Ethyl acetate (GR for analysis), methanol (≥ 99.9%), absolute ethanol, hydrochloric acid (37%), glacial acetic acid, and acetonitrile (ACN, HPLC grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q filter system (Millipore, Bedford, MA, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH, ≥ 85%) and gallic acid (≥ 98%) were supplied by Fluka Chemie AG (Buchs, Switzerland). 2,6-Di-tert-buthyl-4-methylphenol (BHT, 99.0%) and 2(3)-tert-butyl-4-hydroxyanisole (BHA, 98%) were provided by Sigma-Aldrich (Steinhein, Germany). Supercritical carbon dioxide, CO_2 SCF (purity : 99.998%), was supplied by Air Liquide (Spain).

Polyphenol standards were supplied as follows: protocatechuic acid (≥ 97.0%), caffeic acid (≥ 98.0%), (−)-epicatechin (≥ 90%), acetosyringone (97%), resveratrol (≥ 99%), (±)-naringenin (95%), epigallocatechin (≥90%), (+)-catechin hydrate (98%), ferulic acid (99%), quercetin (≥ 98%), kaempferol (≥97.0%), gallocatechin (≥ 98%), p-coumaric acid (≥ 98.0%), and apigenin (≥ 97%) by Sigma-Aldrich (Steinhein, Germany); gallic acid (≥ 98.0%), syringic acid (≥ 97%), isoquercetin, and salicylic acid (≥ 99.0%) by Fluka Chemie AG (Buchs, Switzerland); and homovanillic acid (98%), 4-hydroxybenzoic acid (99%), and acetovanillone (98%) by Alfa Aesar (Karlsruhe, Germany).

2.2. Sampling. In beer production, a clarification step is essential to improve beer stability. As a result of this process, a PVPP sludge is obtained in the brewing industry. The PVPP sludge loaded with polyphenolic compounds was washed with a NaOH solution (2% w/w) at room temperature. After the NaOH-PVPP was filtered, a cleaned PVPP resin and a PVPP washing solution (PVPP-WS) containing phenolic compounds were obtained (see Figure 1). The residual stream generated after the PVPP cleaning process was kindly supplied by Mahou-San Miguel, Spain.

2.3. Industrial Plant Scale Extraction of the Antioxidants from PVPP Sludge. The PVPP-WS (1000 L) was acidified to pH 1.5 with HCl (37%), and polyphenolic compounds were extracted with ethyl acetate (2000 L) by stirring for 30 minutes at room temperature. The organic and aqueous phases were separated by decantation, and the organic phase

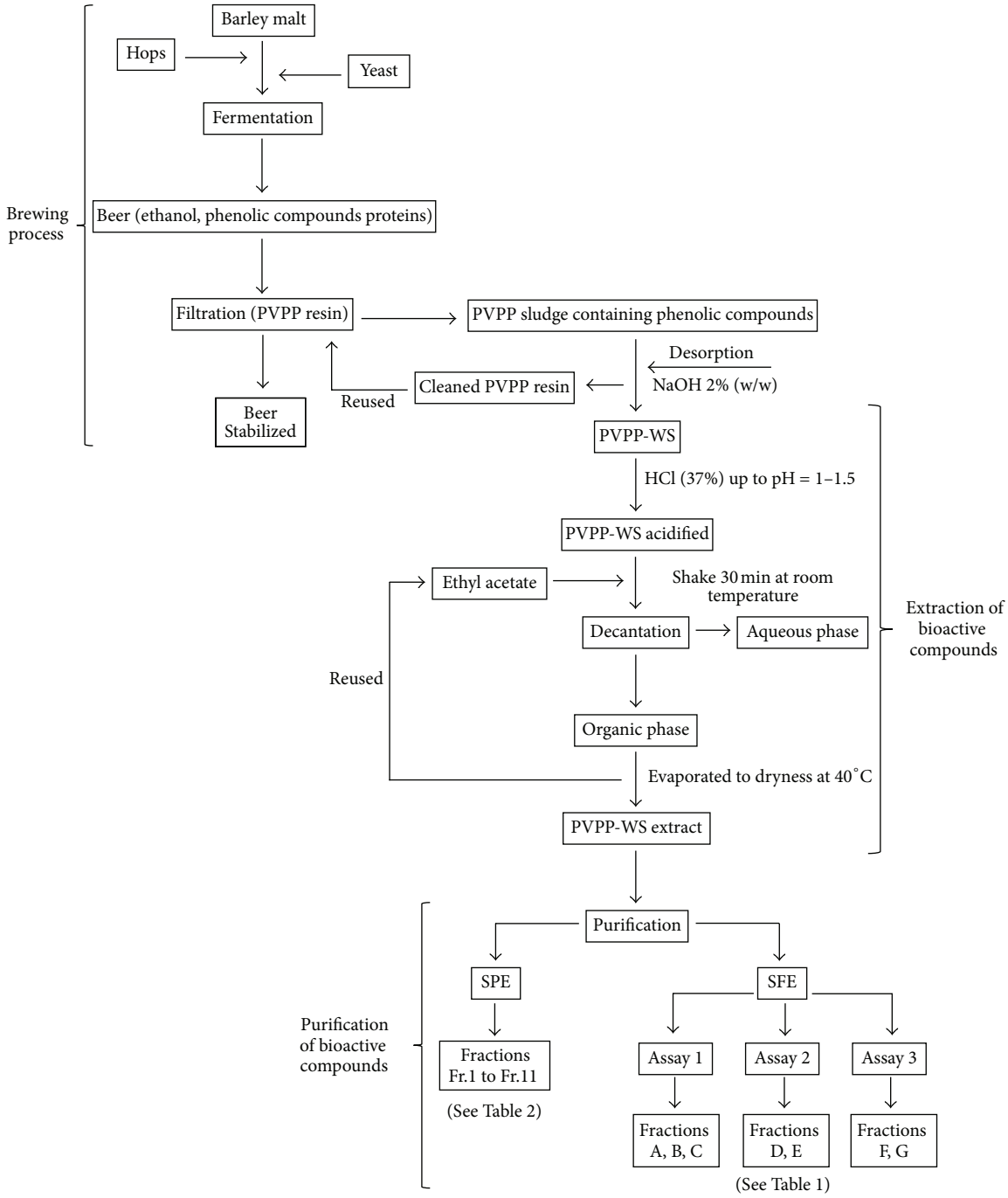

Figure 1: Schematic representation of the brewing process, extraction, and purification of the PVPP-WS extract containing bioactive compounds.

was collected and evaporated to dryness at 40°C. The residual water was removed from the extract by lyophilisation before the recovery yield was determined gravimetrically, and the dry extract was used in fractionation experiments (see Figure 1).

2.4. Fractionation and Purification of PVPP Crude Extract

2.4.1. Solid-Phase Extraction (SPE). SPE was performed with super clean cartridges (LC-18 20 mL, from Supelco, Germany) and 5 g of reversed-phase sorbent (modified silica with octadecyl groups). The crude extract (50 mg) was dissolved in 10 mL of water and loaded on the cartridge. The natural extract was eluted with different percentages of methanol (v/v): 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and finally 100% of methanol, so that eleven separate fractions were obtained at the end of the process. All fractions were evaporated to dryness, under vacuum at 40°C, in a rotary evaporator, and finally redissolved in methanol for further analysis. The recovery yield of each fraction was

Table 1: SFE operational conditions tested.

Assay	T (°C)	Time (min)	CO_2 flow (g min^{-1})	Pressure (bar)	Modifier	Modifier (%)	Modifier flow (mL min^{-1})	Fractions obtained
1	40	30	—*	100, 120, 140, 160, 200, 250, 300	None	None	None	A_{100}, A_{120}, A_{140}, A_{160}, A_{200}, A_{250}, A_{300}
					Ethanol	—*	0.1	B_{100}, B_{120}, B_{140}, B_{160}, B_{200}, B_{250}, B_{300}
						—*	0.2	C_{100}, C_{120}, C_{140}, C_{160}, C_{200}, C_{250}, C_{300}
2	40	30	3	100, 120, 140, 160, 200, 250, 300	Ethanol	3	0.1	D_{100}, D_{120}, D_{140}, D_{160}, D_{200}, D_{250}, D_{300}
						6	0.2	E_{100}, E_{120}, E_{140}, E_{160}, E_{200}, E_{250}, E_{300}
3	40	30	3	140	Ethanol Methanol	0, 0.5, 1, 1.5, 2, 2.5, 3	0.0, 0.02, 0.03, 0.05, 0.06, 0.08, 0.1	F_0, $F_{0.5}$, F_1, $F_{1.5}$, F_2, $F_{2.5}$, F_3 G_0, $G_{0.5}$, G_1, $G_{1.5}$, G_2, $G_{2.5}$, G_3

*In pressure mode, neither CO_2 flow nor modifier percentage were controlled.

determined gravimetrically, and the antioxidant activity of each fraction was measured by the DPPH radical-scavenging test. The phenolic compounds responsible for the antioxidant activity were determined by HPLC-DAD.

2.4.2. Supercritical Fluid Extraction. The crude extract was fractionated using a supercritical fluid SCF R100 system (Thar Technologies, Inc.) equipped with a 5 mL SFE cell (Thar Technologies, Inc.).

Different extraction conditions were tested in three different assays. In each assay, 1 g of sample was submitted to the fractionation procedure. The assay conditions are shown in Table 1.

Assay 1—Pressure Mode (Pressure Range). The experiment was run at 40°C, and CO_2 was automatically fed into the system by the CO_2 pump to maintain a constant pressure. For fractionated separation of the different compounds present in the crude extract, pressure of between 100 and 300 bar (100, 120, 140, 160, 200, 250, and 300 bar) was applied. The extraction time was 30 minutes at each pressure tested. Experiments were carried out with and without modifier. Ethanol was used as a modifier, and two different flow rates (0.1 mL min^{-1} and 0.2 mL min^{-1}) were tested.

Assay 2—Flow Mode (Pressure Range). The trial was run at 40°C, and CO_2 was fed into the system at a flow rate of 3 g min^{-1}. The pressure range and extraction time at each pressure tested were the same as described earlier. The modifier (ethanol) was applied under different conditions: 3% and 6% of modifier at flow rate of 0.1 mL min^{-1} and 0.2 mL min^{-1}, respectively.

Assay 3—Flow Mode (Percentage of Modifier Range). The experiment was run at 40°C, and CO_2 was fed into the system at a flow rate of 3 g min^{-1}. In this test, pressure was maintained constant at 140 bar. Two modifiers, ethanol and methanol, were tested within a range of 0 and 3% (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%).

Single extracts obtained under the three different test conditions were evaporated to dryness under nitrogen steam. The recovery yield of each fraction was determined gravimetrically. Fractions were characterized by HPLC-DAD, and the antioxidant activity of each was determined by the free radical method DPPH.

2.5. Separation and Quantification of Bioactive Phenolic Compounds (RP-HPLC-DAD). Chromatographic analysis was performed on an HPLC system model 1200 HP (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector (DAD) and controlled by HP Chemstation chromatographic software.

Chromatographic separation of polyphenols was carried out on a reverse phase Kromasil C18 column (250 × 3.2 mm internal diameter, 5 μm particle size) (Phenomenex, Barcelona, Spain). The solvents constituting the mobile phase were milli-Q water 0.1% acetic acid (solvent A) and 100% ACN (solvent B). The gradient program was as follows: 0–5 min , 90% A and 10% B; 5–35 min, linear gradient until reaching 50% B at 35 min; 35–43 min, 50% B isocratic; 43–45 linear gradient from 50% to 10% B; and finally, the column was washed and reconditioned. The mobile phase flow rate was 0.5 mL min^{-1} during the entire analytical run, the column temperature was set at 38°C, and the sample injection volume was 20 μL. A scan in the range of 190 to 700 nm was continuously performed, by DAD.

Individual phenolic compounds were identified by comparing their retention time and their UV spectrum with those obtained by injecting standards in the same HPLC conditions. Phenolic acids were monitored and quantified at 225 nm, flavan-3-ols, flavanones, flavones, and acetophenone derivates at 280 nm, hydroxycinnamic acids and resveratrol at 325 nm, and flavonols at 372 nm.

2.6. Antioxidant Activity, DPPH Assay. The antioxidant activity of phenolics in the crude extract and its fractions, obtained during the purification processes, were determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method described by von Gadow et al. (1997) with slight modifications [24].

Standard solutions of the different antioxidant fractions and of two synthetic compounds with antioxidant properties,

BHA and BHT, which are commonly used in the food industry, were prepared in methanol. An aliquot of antioxidant (50 μL) was added to 2 mL of DPPH radical methanolic solution (3.6×10^{-5} M), shaken vigorously on a vortex shaker (MS2 Mini Vortex Shaker IKA), and left to stand in the dark at room temperature. The absorbance was measured at 515 nm, after 16 min at room temperature, in a dual-beam spectrophotometer (Uvikon XL, Bio-Tek Instruments, Milan, Italy). All determinations were performed in triplicate. The decrease in absorbance was converted to inhibition percentage of the DPPH (IP), according to the following equation:

$$\text{IP} = \frac{A_0 - A_{16}}{A_0} \times 100, \quad (1)$$

where A_0 is the absorbance of the control at initial time; A_{16} is the absorbance of the sample after 16 minutes.

The concentration of antioxidant compound or fraction required to achieve 50% inhibition of the radical DPPH (equivalent concentration = EC_{50}) was determined from the linear regression curve obtained by plotting the different concentrations of antioxidant compound or fraction used (within the range 0.1 to 3.5 g L^{-1}) against the inhibition percentage of the DPPH (IP).

3. Results and Discussion

In this study, a new by-product (PVPP-WS) was considered as a natural source of antioxidant-rich bioactive compounds with several potential applications.

The extraction procedure used to obtain the crude extract has already been tested at laboratory scale and pilot plant scale in previous studies. With the overall aim of enabling the brewing industry to implement this extraction process in industrial plants, the present study investigated the scaling-up of the extraction process and the purification of the bioactive phenolic compound extracted. The extraction yield was approximately 0.1%. In the brewery industry, around one litre of this waste stream (PVPP-WS) can be generated from every 138 L of beer produced. Approximately 403 million hectolitres of beer were produced in Europe in 2010, which means that up to 400 tons of this crude extract could be obtained in Europe every year [25].

The crude extract must be processed (by purification and fractionation) as its brown colour would hinder its use as a food additive. In addition, more information about the composition of the crude extract in bioactive phenolic compounds could be obtained from different fractions to determine the correlation between the antioxidant activity and the phenolic compounds or group of phenolic compounds present in the fractions.

3.1. Solid-Phase Extraction Results. Solid-phase extraction is generally used for sample clean-up, fractionation, purification and/or preconcentration of natural extracts. In this study, eleven differently coloured fractions containing phenolic compounds were obtained (see Figure 1).

3.1.1. Extraction Yield and Antioxidant Activity. The recovery yield and the radical scavenging activity were determined for each fraction obtained at each solvent ratio applied to the cartridge. The results are shown in Table 2, along with the colour of each fraction. The antioxidant activity of the synthetic antioxidants commonly used in the food industry was also evaluated to compare the potential of the natural compounds as food additives. The crude extract was also tested to evaluate whether the fractionation process yielded fractions that were more or less active than the crude extract.

The most active fractions and the best yields obtained in the SPE process corresponded to the first seven fractions eluted with a solvent mixture from 0%–60% of methanol. This showed that the natural extract and the polyphenolic compounds are water soluble but the addition of methanol yielded the most active fractions. Therefore, the fraction obtained with 30% (v/v) of methanol exhibited the highest antioxidant activity. All the fractions that display a notable level of antioxidant activity (fractions 1–7) were coloured, particularly fractions Fr. 4, Fr. 5, and Fr. 6, which also displayed the highest degree of antioxidant activity against the free radical DPPH. This is consistent with the results described by Woffendem et al. in a study evaluating the relationship between antioxidant activity and colour of crystal malt extracts [26]. Fractions 8, 9, 10, and 11 were colourless, and the antioxidant activity was lower than that of the other extracts.

The EC_{50} values of Fr. 3, Fr. 4, Fr. 5, and Fr. 6 were similar to that of the synthetic antioxidant BHA used in food industry. Except for the last 4 fractions yielded, all the fractions obtained from the crude extract showed a higher DPPH radical scavenging capacity than the antioxidant BHT, also commonly used in food industry. In this study, the antioxidant capacity of the crude extract ($EC_{50} = 0.32$ g L^{-1}) was also calculated: (a) to compare the capacity of this extract and the fractions obtained; and (b) to evaluate whether the antioxidant activity increased as a result of the purification process. Purification of the crude extract yielded fractions with higher antioxidant activity than the crude extract.

3.1.2. HPLC-DAD-UV Analysis of the Antioxidant Fractions. The results of the identification and quantification of major polyphenolic compounds present in each fraction obtained in the fractionation process (SPE) by HPLC-DAD-UV are shown in Table 3.

The different ratios of solvents yielded different fractions. These fractions displayed different levels of antioxidant activity because the polyphenolic content varies considerably with solubility.

The first fractions, which exhibited the highest level of antioxidant activity (see Table 2), contained the highest amounts of polyphenolic compounds (Table 3). Fractions 1 and 2 contain large amounts of gallocatechin, which largely accounts for the high radical scavenging activity exhibited by these two fractions. Compounds with flavonoid structure such as catechin generally display a higher level of antioxidant activity than nonflavonoid compounds [27].

Fraction 4 displayed the highest level of antioxidant activity, mainly due to the high content of ferulic acid, a phenolic compound. However, Fraction 3, which displayed a similar level of antioxidant activity to Fraction 4, also

Table 2: Recovery yield, radical scavenging activity (DPPH), and colouration of each fraction (Fr.) obtained by SPE with different % of methanol.

Sample	% of methanol	Recovery yield (% w/w)[a]	DPPH (EC_{50})[b]	Colour
Fr. 1	0	10.4	0.44	Wheat
Fr. 2	10	18.1	0.30	Burnt orange
Fr. 3	20	16.2	0.27	Brown
Fr. 4	30	19.4	0.20	Maroon
Fr. 5	40	18.3	0.26	Dark brown
Fr. 6	50	8.19	0.23	Dark brown
Fr. 7	60	6.71	0.89	Ochre
Fr. 8	70	1.59	7.02	Colourless
Fr. 9	80	0.324	6.64	Colourless
Fr. 10	90	0.615	8.25	Colourless
Fr. 11	100	0.194	14.3	Colourless
Crude extract	—	—	0.32	Dark brown
BHA	—	—	0.24	—
BHT	—	—	2.67	—

[a] Values expressed as % of dry crude extract.
[b] Values expressed as g L^{-1} of extract (fraction).

Table 3: Phenolic compounds present in the different fractions, obtained with a LC-18 column, identified and quantified by HPLC-DAD.

	Fraction											Crude extract
Antioxidant compound	1	2	3	4	5	6	7	8	9	10	11	—
	mg g^{-1} of fraction											mg g^{-1}
Gallic acid	193	—	—	—	—	—	—	—	—	—	—	20.1
Gallocatechin	178	641	—	—	—	—	—	—	—	—	—	132
Protocatechuic acid	—	72.9	9.51	—	—	—	—	—	—	—	—	15.5
Epigallocatechin		86.2	96.4									30.6
Catechin	—	114	75.5	—	—	—	—	—	—	—	—	29.9
4-Hydroxybenzoic acid	—	6.51	7.49	—	—	—	—	—	—	—	—	2.54
Caffeic acid	—	13.7	66.0	—	—	—	—	—	—	—	—	14.1
Epicatechin	—	—	132	—	—	—	—	—	—	—	—	21.4
p-coumaric acid	—	—	73.9	1.83	—	—	—	—	—	—	—	11.4
Isoquercetin	—	—	166	—	—	—	—	—	—	—	—	28.3
Ferulic acid	—	—	29.6	138	—	—	—	—	—	—	—	33.7
Acetosyringone	—	—	35.2	50.9	—	—	—	—	—	—	—	14.6
Resveratrol	—	—	—	—	—	51.6	14.5	—	—	—	—	5.35
Quercetin	—	—	—	—	31.7	117	—	—	—	—	—	14.5
Apigenin	—	—	—	—	—	58.8	59.7	—	—	—	—	8.10
Kaempferol	—	—	—	—	—	25.3	23.4	120	—	—	—	6.06
Naringenin	—	—	—	—	—	38.7	171	11.8	—	—	—	14.6
Total (mg g^{-1})	371	934	692	191	31.7	292	269	132	—	—	—	403

contains large amounts of antioxidant compounds such as epigallocatechin, caffeic acid, p-coumaric acid, and isoquercetin [11]. Hydroxycinnamic and hydroxybenzoic acids are known antioxidants that act as free radical acceptors and chain breakers [28]. Caffeic, ferulic, and p-coumaric acids have been widely studied and reported to be major contributors to the antioxidant activity of beer [27, 29]. In the present study, the natural extract was obtained after the treatment of the beer, and, as expected, this crude extract was loaded with these bioactive compounds. Fractions 2 and 3, which contain these compounds, were the most active free radical scavengers. These findings may be attributed to a combined, synergistic, and/or additive action. This type of action has previously been observed to result in an increased antioxidant potential [30].

The recovery yields and the phenolic contents of fractions 6 and 7 were lower than those of the other fractions. However, both of these fractions displayed some antioxidant activity, mainly due to the flavonols. Fractions 6 and 7 contain the flavonols quercetin and kaempferol at similar concentrations.

Table 4: (a) SFE yield determined gravimetrically (w/w) and colour of fractions A, B, and C. (b) SFE yield determined gravimetrically (w/w) and colour of fractions D and E. (c) SFE yield determined gravimetrically (w/w) and colour of fractions F and G.

(a)

P (bar)	A		B		C	
	Yield (%)	Colour	Yield (%)	Colour	Yield (%)	Colour
100	0.02	Colourless	0.24	Wheat	0.02	Colourless
120	0.02	Colourless	7.71	Dark brown	23.04	Dark brown
140	0.02	Colourless	4.27	Burnt orange	6.39	Brown
160	0.00	Colourless	2.77	Burnt orange	0.06	Wheat
200	0.02	Colourless	12.7	Maroon	0.01	Colourless
250	0.02	Colourless	8.09	Dark brown	0.07	Ochre
300	0.02	Colourless	3.29	Maroon	0.03	Colourless
Total	0.12%		39.1%		29.6%	

(b)

P (bar)	D		E	
	Yield (%)	Colour	Yield (%)	Colour
100	0.89	Brown	3.27	Burnt orange
120	0.73	Brown	19.83	Dark brown
140	0.76	Brown orange	5.67	Burnt orange
160	1.42	Burnt orange	3.65	Burnt orange
200	1.13	Burnt orange	3.84	Burnt orange
250	0.79	Burnt orange	2.56	Burnt orange
300	0.98	Burnt orange	2.64	Burnt orange
Total	6.7%		41.5%	

(c)

% Modifier	F		G	
	Yield (%)	Colour	Yield (%)	Colour
0	0.04	Orange	0.13	Wheat
0.5	0.07	Coral	0.08	Wheat
1.0	0.33	Wheat	0.31	Wheat
1.5	0.58	Wheat	0.28	Wheat
2	0.44	Wheat	0.58	Wheat
2.5	0.61	Wheat	0.41	Wheat
3	1.08	Wheat	0.59	Wheat
Total	3.15%		2.38%	

Fractions 8, 9, 10, and 11 were extracted using high contents of methanol, and no phenolic compounds were detected in the fractions.

3.2. Supercritical Fluid Extraction. SC-CO_2 has been used successfully to purify crude extracts by concentrating the bioactive compounds (e.g., antioxidants) in the extract and also by removing contaminants. In the present study, several fractions were obtained from the SFE fractionation assay under different test conditions (see Figure 1).

3.2.1. SFE Fractions (Extraction Yield, Colour, and Antioxidant Activity of Each Fraction). The extraction yield of the different antioxidant fractions obtained under the different operational conditions (see Table 1) and the colour of each fraction are shown in Table 4. The extraction yield ranged from 0.12% (fractionation test A), for the extraction without cosolvent (see Table 4(a)), to 41.5% (fractionation test E) for the extractions in which ethanol was used as a modifier (see Table 4(b)). The results showed that the use of a modifier is mandatory essential for successful fractionation of the crude extract. This is consistent with data reported by various authors who obtained high extraction yields from diverse natural matrices by using modifiers [17].

Results showed that the increase in the percentage of modifier increases the amount of extract (see Table 4(b)). The volume of the extract collected at each pressure is more constant in the flow mode (see Table 4(b), tests D and E) than in pressure mode (see Table 4(a), tests B and C). Moreover,

Table 5: Antioxidant activity of SFE extracts determined by DPPH. Results are expressed as EC_{50} (g L^{-1}).

					Fraction				
P (bar)	Pressure mode			*P* (bar)	Flow mode		Flow mode		
	A	B	C		D	E	% modification	F	G
Cell	—	—	0.23	**Cell**	0.20	0.22	**Cell**	0.26	0.20
100	n.d.	2.65	37.73	**100**	0.33	0.74	**0**	21.99	0.64
120	n.d.	0.36	0.41	**120**	0.51	0.36	**0.5**	1.57	0.68
140	n.d.	0.27	0.25	**140**	0.62	0.23	**1.0**	0.59	3.52
160	n.d.	0.29	0.28	**160**	0.46	0.21	**1.5**	0.97	1.42
200	n.d.	0.34	1.00	**200**	0.74	0.22	**2**	0.88	0.80
250	n.d.	0.21	0.20	**250**	0.34	0.23	**2.5**	0.64	0.67
300	n.d.	0.10	3.12	**300**	0.42	0.34	**3**	0.54	0.42

Table 6: Phenolic profile of each fraction obtained under SFE conditions of test E: temperature 40°C, extraction time 30 minutes, modifier ethanol (6%), and modifier flow 0.2 mL min^{-1}. Phenolic compounds are expressed as mg g^{-1} of fraction.

Peak no.	Phenolic compound	SFE fractions (mg g^{-1})							
		E_{Cell}	E_{100}	E_{120}	E_{140}	E_{160}	E_{200}	E_{250}	E_{300}
1	Gallic acid	1.04	0.24	1.05	1.68	2.61	3.02	3.44	3.07
2	Gallocatechin	171	10.7	14.8	33.9	41.0	33.3	29.8	20.9
3	Protocatechuic acid	—	1.99	4.12	9.19	12.8	14.9	16.7	16.0
4	Epigallocatechin	1.43	10.7	10.2	—	—	—	—	—
5	Catechin	6.10	3.40	3.36	6.88	11.8	11.7	15.7	16.7
6	4-Hydroxybeinzoic acid	—	3.58	1.96	4.78	4.22	2.72	2.36	1.55
7	Caffeic acid	—	7.29	7.38	17.1	19.65	14.8	13.3	9.52
8	Epicatechin	—	—	—	—	0.23	0.14	0.17	0.40
9	p-coumaric acid	—	7.97	5.89	13.5	13.9	9.66	8.45	6.26
10	Isoquercetin	2.51	—	—	—	—	3.71	3.75	1.43
11	Ferulic acid	—	35.7	27.6	69.0	70.1	47.0	38.9	22.0
12	Acetosyringone	—	2.92	0.83	3.62	2.07	1.18	3.10	2.55
13	Resveratrol	—	—	0.54	1.68	0.37	0.46	0.60	0.80
14	Quercetin	0.57	0.65	0.98	1.91	2.61	2.83	3.13	2.96
15	Apigenin	—	—	0.04	0.66	0.78	0.72	0.79	0.93
16	Kaempferol	1.48	0.94	1.65	3.17	4.42	3.18	6.24	6.64
17	Naringenin	0.39	0.40	0.78	1.69	2.45	2.75	3.26	3.52
	Total (mg g^{-1})	11.0	88.9	82.5	172.7	192.8	155.9	152.3	117.0

when higher amounts of the modifier are used, less pressure is required, as observed in tests C (flow rate of 0.2 mL min^{-1} twice that of B) and E (6% of modifier twice that of D), in which almost all compounds were yielded at a pressure of 120 bar. These results indicated that the optimal conditions for the extraction process of the crude extract are those used in test E, which yielded most phenolic compounds and fractions with the highest antioxidant activities (see Table 5) similar to BHA and higher than that of BHT (see Table 2). Moreover, under these conditions, the fractions were paler, which is an advantage as the strong brown colour of the crude extract may hinder its use as a food additive. However, the fractions obtained in high amounts were always darker, as, for example, E_{120} (see Table 4(b)).

The darkest fractions, which contained more phenolic compounds per weight of extract fraction (see Tables 4(a), 4(b) and 4(c)), displayed a high radical scavenging capacity (see Table 5). Colourless fractions, which did not appear to contain phenolic compounds, did not display any scavenging activity of the radical DPPH. This is further indication that fractions colour depends on the content of active compounds, which are also responsible for the antioxidant activity of the fractions.

In the present study, methanol was also evaluated as a modifier in test G (see Table 4(b)). The results showed that ethanol is more suitable for the extraction process because the phenolic compounds of interest were obtained with a lower percentage of ethanol (1.5%), while 2% methanol was required to obtain compounds such as ferulic acid and gallocatechin (data not shown), the main compounds present in the crude extract. Selection of a suitable modifier and/or adjustment of the modifier percentage is essential for successful fractional separation of the antioxidant compounds present in the crude extract.

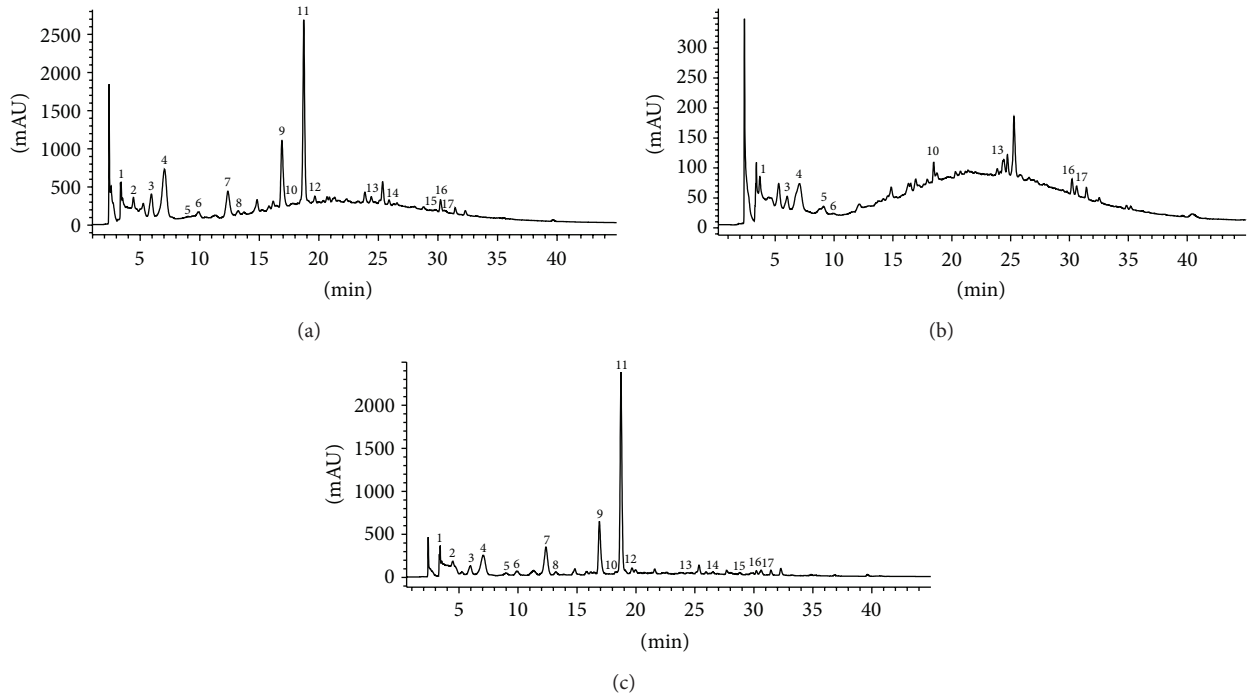

Figure 2: Chromatograms acquired at 280 nm by HPLC-DAD-UV. (a) Chromatogram of the crude extract without fractionation process. (b) Chromatogram of the residue of the crude extract that remains in the extraction cell after the fractionation process (E_{Cell}). (c) Chromatogram of the fraction obtained under optimal conditions (E_{140}).

3.2.2. HPLC-DAD-UV Analyses of the Antioxidant Fractions. The fractions obtained by SFE were analysed by HPLC-DAD-UV to the characterization of bioactive compounds in the crude extract.

Figure 2 shows, as a representative example, the chromatogram of the crude extract before fractionation by SFE (Figure 2(a)) and the chromatograms obtained after the fractionation procedure under optimal conditions, the extraction cell (Figure 2(b)) and the fraction E_{140} (Figure 2(c)). Chromatogram C (Figure 2(c)) shows that SFE fractionation improves the purity of the phenolic compounds (clearly observed from the base line of the chromatogram) relative to the crude extract (Figure 2(a)). It is also evident that the impurities in the crude extract remain in the extraction cell, as verified by the peak obtained in chromatogram B. However, phenolic compounds and probably other compounds not identified in the present study remain active in the extraction cell (E_{Cell}), which confers antioxidant activity to the residue in the extraction cell (see Table 5).

The phenolic compounds present in the fractions obtained under optimal conditions in this study (fractionation E) are shown in Table 6. The number of phenolic compounds shown in the table corresponds to the numbers in the chromatograms in Figures (2(a), 2(b), and 2(c)). The phenolic compounds were identified and quantified in this study for all the fractions obtained under the different assay conditions (data not shown). The qualitative composition of phenolic compounds in the factions yielded by SFE is the same in almost every fraction obtained under the different test conditions assayed (see Table 6). The main bioactive phenolic compounds identified in the fractions were as follows: ferulic acid, p-coumaric acid, caffeic acid, protocatechuic acid, catechin, gallic acid, gallocatechina, and epigallocatechin. These results are consistent with those obtained with the SPE procedure (see Section 3.1.2.).

The phenolic compounds were not successfully separated by the SFE fractionation procedure as in the SPE fractionation (LC-18 column). However, the purity of the fractions yielded by the SFE was greater than that of the fractions yielded by SPE. Moreover, large amounts of pure fractions containing the main bioactive phenolic compounds were obtained by SFE, which makes this technique the most promising for purification of the crude extract.

Regarding the antioxidant activity, the fractions with the highest contents of polyphenolic compounds are those with the highest antioxidant activity. However, the antioxidant activities of the different fractions did not differ significantly because the composition of bioactive compounds was also very similar.

4. Conclusions

Industrial extraction of a natural extract containing bioactive compounds was successful and could be implemented in the brewing industry to recover a residue with added value. The dried ethyl acetate crude extract was purified by two alternative procedures to improve its quality (antioxidant activity and organoleptic properties) for potential use as a food additive.

Both purification procedures yielded fractions with better organoleptic properties (odour and colour) and with higher antioxidant activity than the crude extract. The fractions that display strong antioxidant activity may be suitable for use as food additives (to increase the shelf life of food by preventing lipid peroxidation and protecting from oxidative spoilage during storage). Moreover, these bioactive compounds may be a good source of compounds with several applications in the food industry, as food ingredients and nutraceuticals, in the cosmetics, and in pharmaceutical industries.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Partial Characterization of Biosurfactant from *Lactobacillus pentosus* and Comparison with Sodium Dodecyl Sulphate for the Bioremediation of Hydrocarbon Contaminated Soil

A. B. Moldes,[1] R. Paradelo,[2] X. Vecino,[1] J. M. Cruz,[1] E. Gudiña,[3] L. Rodrigues,[3] J. A. Teixeira,[3] J. M. Domínguez,[1] and M. T. Barral[2]

[1] *Chemical Engineering Department, School of Industrial Engineering (EEI), University of Vigo, Campus As Lagoas, Marcosende, 36310 Vigo-Pontevedra, Spain*
[2] *Edaphology and Agricultural Chemistry Department, Facultad de Farmacia, University of Santiago de Compostela, Campus Universitario Sur. 15782, Santiago de Compostela, La Coruña, Spain*
[3] *Institute for Biotechnology and Bioengineering (IBB), Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*

Correspondence should be addressed to A. B. Moldes; amoldes@uvigo.es

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The capability of a cell bound biosurfactant produced by *Lactobacillus pentosus*, to accelerate the bioremediation of a hydrocarbon-contaminated soil, was compared with a synthetic anionic surfactant (sodium dodecyl sulphate SDS-). The biosurfactant produced by the bacteria was analyzed by Fourier transform infrared spectroscopy (FTIR) that clearly indicates the presence of OH and NH groups, C=O stretching of carbonyl groups and NH nebding (peptide linkage), as well as CH_2–CH_3 and C–O stretching, with similar FTIR spectra than other biosurfactants obtained from lactic acid bacteria. After the characterization of biosurfactant by FTIR, soil contaminated with 7,000 mg Kg^{-1} of octane was treated with biosurfactant from *L. pentosus* or SDS. Treatment of soil for 15 days with the biosurfactant produced by *L. pentosus* led to a 65.1% reduction in the hydrocarbon concentration, whereas SDS reduced the octane concentration to 37.2% compared with a 2.2% reduction in the soil contaminated with octane in absence of biosurfactant used as control. Besides, after 30 days of incubation soil with SDS or biosurfactant gave percentages of bioremediation around 90% in both cases. Thus, it can be concluded that biosurfactant produced by *L. pentosus* accelerates the bioremediation of octane-contaminated soil by improving the solubilisation of octane in the water phase of soil, achieving even better results than those reached with SDS after 15-day treatment.

1. Introduction

The extensive production and use of hydrocarbons have resulted in widespread environmental contamination by these chemicals. Contaminated sites must be cleaned because of the toxicity and persistence of these compounds and the associated negative effects on living organisms. Hydrocarbons, which are hydrophobic organic compounds, are poorly soluble in groundwater and tend to partition to the soil matrix. The partitioning can account for as much as 90–95% or more of the total contaminant mass. As a consequence, hydrocarbon contaminants are moderately to poorly recovered by physic-chemical treatments, display limited bioavailability to microorganisms and limited availability to oxidative and reductive chemicals when applied *in situ* and/or *ex situ* [1].

On the other hand, lignocellulose residues like the pruning waste generated in vineyards are usually burnt in the field,

releasing greenhouse gases and cancerous compounds such as polycyclic aromatic hydrocarbons. Therefore, the use of vineyard pruning waste as a carbon source in biosurfactant production could decrease the environmental impact associated with burning this type of waste in the field [2, 3].

Bustos et al. [3] showed that *Lactobacillus pentosus* produces lactic acid and biosurfactants by utilizing hemicellulosic sugars in vineyard pruning waste, and Portilla et al. [2] demonstrated that *Lactobacillus acidophilus* produces biosurfactants by utilizing the cellulosic fraction of vineyard pruning waste. Moreover, Moldes et al. [4] produced biosurfactants using *L. pentosus* grown on synthetic media, composed by glucose and xylose and they used the biosurfactant obtained for the bioremediation of octane contaminated soil. These authors found that biosurfactant from *L. pentosus*, grown on synthetic media, reduced the concentration of octane in the soil to 58.6% and 62.8%, for soil charged with 700 and 70,000 mg Kg^{-1} of hydrocarbon, respectively. However, there are no comparative studies about the use of biosurfactants produced by *L. pentosus* and chemical surfactants, such as sodium dodecyl sulfate (SDS).

Moreover, it is important to point out that although biosurfactant from *L. pentosus* has been proposed, in previous works [4], as surfactant for the bioremediation of contaminated soil, in the literature there are no studies about the composition of this biosurfactant. Thus, thinking about the further application and commercialisation of this biosurfactant it would be interesting to elucidate its composition.

In the current work, in order to know the composition of the biosurfactant produced by *L. pentosus* during the fermentation of hemicellulosic sugars, which can be obtained from vineyard pruning waste, this was analysed by Fourier transform infrared spectroscopy (FTIR) and the corresponding spectrum was compared with those obtained for other biosurfactants, also produced by lactic acid bacteria. Moreover, soil samples contaminated with octane were treated with the biosurfactant produced by *L. pentosus* or with SDS and incubated for several days, in order to test the comparative efficacy of the two types of surfactants in the bioremediation of hydrocarbon-contaminated soil.

2. Materials and Methods

2.1. Microorganism. *Lactobacillus pentosus* CECT-4023 T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown on the complete media proposed by Mercier et al. [5], at 31°C for 24 h. Inocula were prepared by solubilisation of cells from plates, with 5 mL of sterilized hemicellulosic sugars.

2.2. Biotechnological Production of Bisurfactant. Hemicellulosic sugars containing 18 g/L xylose, 10.6 g/L glucose, and 3.9 g/L arabinose, that could be obtained by acid hydrolysis of vineyard pruning waste at 130°C with 3% sulphuric acid for 15 minutes using a liquid/solid ratio of 8 g/g [2, 3]; were supplemented with 10 g L^{-1} of yeast extract (YE) and 10 g L^{-1} of corn steep liquor (CSL) and used directly as fermentation media. The chemostat fermentation was carried out in a 2 L Applikon fermentor at 250 rpm, with a working volume of 1.4 L, at 31°C and with pH adjusted to 5.85.

2.3. Extraction of Biosurfactant. The bacterial cells were recovered by centrifugation, washed twice in demineralized water, and resuspended in 50 mL of phosphate-buffer saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCL, pH adjusted to 7.0). The suspensions were maintained at room temperature for up to 2 hours, with gentle stirring to encourage release of biosurfactants. The bacteria were removed from the solution by centrifugation, and the remaining supernatant liquid (containing the biosurfactants) was filtered through a 0.22 μm pore-size filter (Millipore) for analysis and evaluation.

2.4. Surface Activity Determination. The surface activity of the biosurfactant was determined by measuring the surface tension of the samples with the ring method. The surface tension of the PBS extract containing the biosurfactants produced by *L. pentosus* was measured using a KRUSS K6 Tensiometer was equipped with a 1.9 cm Du Noüy platinum ring. To increase the accuracy of the measurements, the measurements were made in triplicate.

The concentration of biosurfactant (mg L^{-1}) was determined from a calibration curve: concentration (mg L^{-1}) = (surface tension (mN/m) − 76.9)/−8.65 [6]. The calibration curve was calculated for a commercial biosurfactant produced by several bacilli (surfactin). Surfactin is a biosurfactant produced by a Bacillus strain, similar to *Lactobacillus pentosus* and it is commercially available. Different concentrations of biosurfactant solution were tested, below the critical micelle concentration (CMC) of known surface tension. The decrease in surface tension is linear in this range of concentration and it is therefore possible to establish a relationship between the concentration of biosurfactant and the surface tension [7]. Nevertheless, in order to estimate the concentration of biosurfactant, the solution was diluted to the CMC.

2.5. Fourier Transform Infrared Spectroscopy. Fourier transform infrared spectroscopy (FTIR) is particularly useful for identifying different types of chemical bonds (functional groups) and can therefore be used to identify the components of mixtures of unknown composition. Molecular characterization was performed with a crude biosurfactant mixture extract, which was dialyzed against demineralized water at 4°C in a Spectrapor membrane tube (molecular weight cut off 6000–8000, Spectrum Medical Industries Inc., CA, USA), and then freeze-dried. One milligram of freeze-dried crude biosurfactant was ground with 100 mg of KBr and with a pressure of 7500 kg was applied for 30 s in order to produce translucent pellets, which were then analysed by spectrometry (FT/IR-4200, JASCO). All spectra were obtained from 180 scans with a resolution of 4 cm^{-1} in the range of 550–4000 cm^{-1}. A KBr pellet was used as background reference. Moreover, previously to FTIR analysis total soluble protein and reducing sugars content of biosurfactant was analysed by Lowry and the phenol sulphuric method, respectively.

2.6. Relative Emulsion Volume and Stability of the Biosurfactant Produced by L. pentosus. Two mL of octane was mixed with an equal volume of the PBS containing the biosurfactant produced by *L. pentosus*, or with sodium dodecyl sulfate (SDS) at its critical micelle concentration (CMC), following the protocol published by Portilla et al. [2]. The hydrocarbons and surfactants were mixed and shaken vigorously for 2 min., and left for 1 h. The relative emulsion volume (EV, %) and stability (ES, %) were measured at this time (i.e., at time 0 h), and 24 h later, and the EV and ES were calculated from (1), proposed by Das et al. [8] as follows:

$$\text{EV}, \% = \frac{\text{Emulsion height, mm} \times \text{Cross section area, mm}^2}{\text{Total liquid volume, mm}^3},$$
$$\%\text{ES} = \frac{\%\text{EV, at time h}}{\text{EV, at 0 h}} * 100. \quad (1)$$

2.7. Soil Samples. The soil samples were sieved (2 mm) prior to analysis. The water content was estimated by drying the soil at 105°C until constant weight, as described by Guitián and Carballas [9]. The pH was determined either in water or 0.1 N KCl, at a soil:solution ratio of 1 : 1.5, and measured after 10 min and 2 hours, respectively. Total organic carbon (TOC) and organic matter (OM) were determined by oxidation with a mixture of $K_2Cr_2O_7$ and H_2SO_4 and titration with Mohr Salt, following the method proposed by Guitián and Carballas [9]. The particle size distribution (coarse sand, 2–0.2 mm; fine sand, 0.2–0.05 mm; coarse silt, 0.05–0.02 mm; fine silt, 0.02–0.002 mm, and clay <0.002 mm) was determined by the Robinson pipette method, after wet sieving, as described by Guitián and Carballas [9]. The nitrogen (N) content was determined by wet digestion with H_2SO_4, using the Kjeldhal method as described by Guitián and Carballas [9]. Dehydrogenase activity (DHA) was measured by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF), following the method described by Tabatabai [10]. The octane content of the soil was analysed in triplicate, by headspace gas chromatography. Table 1 shows the soil composition assayed in this work.

2.8. Incubation Experiments. The soil was contaminated up to 7,000 mg Kg^{-1} of octane and then incubated in the presence and absence of sodium dodecyl sulfate (SDS) or the biosurfactant produced by *L. pentosus*. The octane concentration selected, for contaminating soil, is in the range of those used in a previous work [4]. The surfactant/soil ratio was 1 : 5 (liquid : solid), and surfactants were added to soil, in Erlenmeyer flasks, at the CMC. The flasks were then incubated at 25°C for 30 days, without shaking. In order to study the effect of the biosurfactant on the bioremediation of octane, soil contaminated with octane in absence of biosurfactant was included in the set of experiments as a control. Moreover, in order to evaluate the effect of microbial activity on the bioremediation of hydrocarbon-contaminated soil, samples of soil were contaminated with 7,000 mg Kg^{-1} of octane and sterilized. The octane concentration of soil was analyzed in triplicate by headspace gas chromatographic.

Table 1: Physicochemical characterization of the soil assayed in the present study.

Properties	Units	Value
pH_{H_2O}		5.1
pH_{KCl}		4.0
Sand	%	69.7
Coarse Silt	%	3.0
Fine Silt	%	6.6
Clay	%	20.7
Texture		Loam-clayey-sandy
TOC (Total organic carbon)	g/Kg	11.2
N	g/Kg	0.9
C/N		12.4
OM	g/Kg	19.3
Octane	mg/Kg	185
DHA (Dehydrogenase activity)	mg·TPF/kg·day	334

Table 2: Correspondence between IR spectra and functional groups detected in the biosurfactant produced by *L. pentosus* and in biosurfactants produced by other lactic acid bacteria (*L. lactis*, *L. paracasei* , and *S. thermophilus A* and *B*).

Absorbance band (cm^{-1})	Region
3200–3600	OH and NH stretching
2900–2950	C–H (stretching) groups CH_2 and CH_3
1725, 1675	C=O (stretching)
1520	N–H bending in proteins
1400–1460	C–H bending vibrations of CH_3 and CH_2 groups; CH (scissor)
1100–1090	OH deformation vibrations/CN
1000–1300	C–O sugar stretching

3. Results and Discussion

3.1. Characterization of Biosurfactants Produced by L. pentosus, by Fourier Transform Infrared Spectroscopy. The FTIR method has been widely used to characterize the surface groups, since infrared (IR) transmission spectra present peaks characteristic of specific chemical bonds [11]. Comparison of the peaks and corresponding chemical groups for the biosurfactant produced by *L. pentosus* and biosurfactants produced by other lactic acid bacteria (*Lactococcus lactis*, *Lactobacillus paracasei*, *Streptococcus thermophilus A*, and *Streptococcus thermophilus B*) are shown in Figure 1 and Table 2 [12–14]. The presence of a 3200–3500 cm^{-1} peak in the *L. pentosus* biosurfactant spectrum clearly indicates the presence of OH and NH groups in glycoproteins, structures proposed for the biosurfactants produced by *L. lactis* and *L. paracasei* [6, 14, 15]. A peak at 1725 and 1675 cm^{-1} corresponding to C=O stretching of carbonyl groups and NH bending (peptide linkage) was also observed in the spectrum obtained

Table 3: Comparison of relative emulsion volume (EV) and stability (ES) after 24 h, of octane/water emulsions formed and stabilized with the biosurfactant produced by *L. pentosus* during fermentation of sugars in vineyard pruning waste and sodium dodecyl sulfate (SDS). Data are compared with EV and ES values reported for gasoline or kerosene and surfactin, SDS or biosurfactants produced by *L. pentosus*.

Hydrocarbon	EV (%)	ES (%)	Surfactant	Reference
Gasoline	38.9–45.5	85.0–94.7	Biosurfactant produced by *L. pentosus* with lignocellulosic residues as substrate	[16]
Gasoline	22.3	64.6	Surfactin produced by *Bacillus subtilis*	[16]
Gasoline	66.2	96.1	SDS	[16]
Kerosene	21.7–49.0	84.9–99.0	Biosurfactant produced by *L. pentosus* with lignocellulosic residues as substrate	[16]
Kerosene	30.4	73.1	Surfactin produced by *Bacillus subtilis*	[16]
Kerosene	62.3	87.7	SDS	[16]
Octane	39.8	85.7	Biosurfactant produced by *L. pentosus* using hemicellulosic sugars that could be obtained from trimming vineyard.	Present study
Octane	64.0	94.0	SDS	Present study

for the *L. pentosus* biosurfactant. Furthermore, important peaks were also observed at 2900 cm^{-1} (CH_2–CH_3 stretching) and at 1000–1200 cm^{-1} (C–O stretching in sugars). A glycolipid-like structure has previously been proposed for the biosurfactants produced by strains of *S. thermophilus* [12, 13], although some characteristic protein/peptide groups were observed in the FTIR spectra. Comparison of the spectrum obtained for the *L. pentosus* biosurfactant with those reported for the other biosurfactants revealed that the *L. pentosus* biosurfactant is more closely related to those produced by *L. lactis* and *L. paracasei*, suggesting that could be a glycoprotein or a glycolipopeptide (Figure 1).

Moreover it was found that the bisurfactant from *L. pentosus* was composed by 44.7 ± 1.5% soluble protein and 13.4 ± 2.9% total sugars that confirm the results found in the FTIR analysis, although in the future it will be necessary to determine the lipid content in order to clarify if biosurfactant from *L. pentosus* is a glycoprotein or a glycolipopeptide.

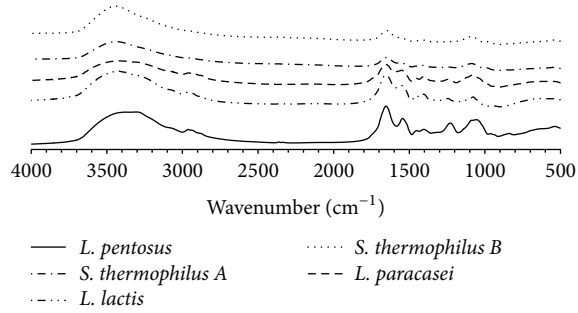

Figure 1: FTIR spectra of biosurfactant produced by *L. pentosus* in comparison with the spectra obtained for biosurfactants produced by other lactic acid bacteria (*L. lactis*, *L. pentosus*, and *S. thermophilus A* and *B*).

3.2. Study of the Emulsifying Capacity of Biosurfactants Produced by L. pentosus. Biosurfactants have often been used to enhance the bioavailability and biodegradation of hydrophobic compounds, but knowledge of the effect of biosurfactants on the biodegradation of complex hydrocarbon mixtures is limited [1]. In previous works [16] the emulsifying capacity of bisourfactants produced by *L. pentosus* was evaluated and it was found that when this strain is grown on sugars from agricultural residues, it produces biosurfactants with emulsifying properties, which could facilitate the bioremediation of hydrocarbon-contaminated sites.

In the present study, the capacity of biosurfactants produced by *L. pentosus* to stabilize emulsions octane/water was evaluated; it was found that the biosurfactants obtained after growing this strain on hemicellulosic sugars that can be obtained by vineyard pruning waste, yielded a relative emulsion volume (EV) of about 40.0%, and 85.7% stability (ES) after 24 h. The relative emulsion volume (EV) and stabilizing capacity value (ES) for emulsions of octane/water stabilized by biosurfactants produced by *L. pentosus* or SDS, in comparison with the results reported in previous works [16] are shown in Table 3. The capacity of the biosurfactant produced in the present study to stabilize octane/water emulsions is similar to that of the biosurfactants assessed in previous works [16], which were produced by the same strain (although grown on a different culture media), to stabilize gasoline/water emulsions. In previous works [16] it was found that among the biosurfactants assayed, those produced by bacteria grown with distilled grape marc hydrolyzate as a substrate yielded the highest EV (45.5%) after 24 h, followed by the biosurfactants produced with hazelnut as a substrate. The EV values were higher than those reported for commercial surfactin (22.3% for gasoline and 30.4% for kerosene) and lower than those reported for SDS (66.2% for gasoline and 62.3% for kerosene). The same was observed in the present study for the *L. pentosus* biosurfactant and SDS when used to stabilize octane/water emulsions.

3.3. Bioremediation of Hydrocarbon-Contaminated Soil. The physicochemical characteristics of the soil assayed in the present study are shown in Table 1. The soil comprised 69.7% sand and 20.7% clay, and the pH was 5. The organic matter content was 11.2 g/Kg, the total nitrogen concentration, 0.9 g Kg^{-1} with a C/N ratio about 12.4. The dehydrogenase activity (DHA) was approximately 334 mg TPF Kg^{-1}, accounting for the microbial activity of the soil.

On the other hand, the surfactants tested in the present study for the bioremediation of hydrocarbon-contaminated soil were sodium dodecyl sulfate and the biosurfactant produced by *L. pentosus*. Sodium dodecyl sulfate (SDS or NaDS), or sodium lauryl sulfate (SLS) ($C_{12}H_{25}SO_4Na$) is an anionic surfactant used in many cleaning and hygiene products. The CMC of SDS is about 0.0082 M in pure water at 25°C. SDS was applied to the soil at its CMC (0.0082 M in pure water). The biosurfactant was obtained by the fermentation of hemicellulosic sugars (18 g/L xylose; 10.6 g/L glucose and 3.9 g/L arabinose) that can be obtained by hydrolysis of vineyard pruning waste. The biosurfactant produced by *L. pentosus* reduced the surface tension of PBS from 72 mN m^{-1} to 54 mN m^{-1}, and this was diluted to its CMC (2.65 mg L^{-1}) before being added to the soil samples.

Regarding the utilization of surfactants for the bioremediation of hydrocarbon-contaminated soil, Urum et al. [17] investigated the efficiency of different surfactant solutions to remove crude oil from contaminated soils, by a soil washing process. The authors demonstrated that the synthetic surfactant-sodium dodecyl sulphate (SDS) and rhamnolipid biosurfactants were more efficient at removing the crude oil than natural surfactants saponins. However, no studies have compared the ability of SDS and biosurfactants produced by lactic acid bacteria to biodegrade hydrocarbons in soil.

In this work soil was contaminated up to 7,000 mg Kg^{-1} of octane. After 15 days of treatment, the contaminated soil reduced the octane concentration by 65.1% and 37.2% when was treated with the biosurfactants produced by *L. pentosus* or the SDS, respectively, whereas in the untreated soil, consisting of soil contaminated with octane in absence of biosurfactant or surfactant, the octane concentration was only reduced by about 2.2% (Figure 2). However the greatest reduction in the octane concentration was observed after 30 days of incubation (92 and 94% for soil containing biosurfactant or SDS, resp.). Figure 2 shows the kinetic profile of octane biodegradation in the soil after 30 days of treatment, in presence and absence of biosurfactant. Regarding the effectiveness of the biosurfactant, the greatest differences in the biodegradation of octane were achieved after 15 days of treatment. After this time, the octane concentration remained at around 6,000 and 6,900 mg Kg^{-1} in untreated soils (sterilized and unsterilized soil, resp.), whereas in the treated soil samples, the octane concentration was reduced to 2,469 and 4,400 mg Kg^{-1} by the *L. pentosus* bisurfactant and SDS, respectively. However, after 30 days, the octane concentration decreased to 591 mg Kg^{-1} and 430 mg Kg^{-1} in samples incubated with the *L. pentosus* bisurfactant and SDS, respectively.

These data demonstrate that biosurfactant accelerates the solubilisation of octane in water, improving the degradation of this contaminant by the microbial biomass of soil. Moreover, in Figure 2 it can be observed that, in the sterilized soil in absence of biosurfactant, the octane concentration remains almost stable during 30 days of treatment in comparison with the nonsterilized control (nonsterilized soil in absence of biosurfactant). This fact can be explained on the basis that in nonsterilized soil exists microbial biomass that after 15 days

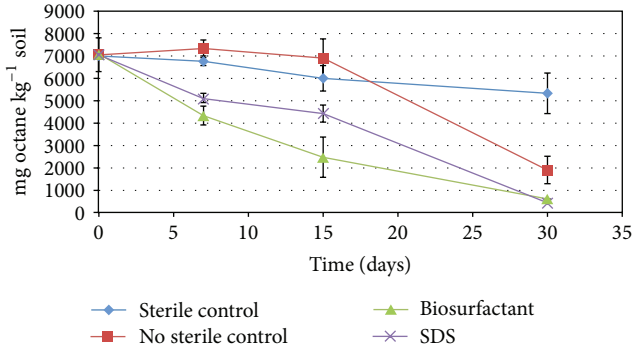

Figure 2: Kinetic profile of the biodegradation of octane in soil, in the presence and absence of surfactants (SDS or biosurfactant produced by *L. pentosus*).

of adaptation to the medium is able to metabolize the octane contained in the soil, whereas in the sterilized soil there are no microbial biomasses that can metabolize this hydrocarbon. In this case the advantage of using biosurfactant from *L. pentosus* for the bioremediation of octane contaminated soil focuses on the ability of the biosurfactant to accelerate the biodegradation process in presence of microbial biomass.

SDS is one of the most typical surfactants proposed in the literature for the bioremediation of hydrocarbon-contaminated soil [18, 19]; for example, it has been demonstrated that enhances desorption and biodegradation of phenanthrene in soil-water systems. It is not carcinogenic when applied directly to skin or consumed (CIR 1983) [20]. However, it has been shown to irritate facial skin after prolonged and constant exposure (more than an hour) in young adults [21]. Thus it is interesting to look for more friendly environmental surface-active compounds.

In comparison with SDS, biosurfactants produced by *L. pentosus* could be considered nontoxic since they are produced by a generally regarded as safe (GRAS) microorganism. In fact, lactic acid bacteria, most of which are biosurfactant producers, are widely consumed in food products. It can therefore be speculated that these cell-bound biosurfactants would not be toxic to humans or animals, and therefore could be used in many applications such as the bioremediation of contaminated sites, in place of other chemical surfactants.

4. Conclusion

Owing to their biodegradability, low toxicity and effectiveness, biosurfactant produced by *L. pentosus* is a very promising compound for use in the bioremediation of contaminated soil, because it was able to increase the solubilisation of octane in the aqueous-soil systems and thus improved its biodegradation, showing even higher capability for the bioremediation of hydrocarbon-contaminated soil than SDS. Moreover, from the FTIR analysis it can be concluded that biosurfactant from *L. pentosus* could be a glycoprotein or a glycolipopeptide and the use of hemicellulosic sugars from inexpensive substrates (such as vineyard pruning waste) may enable the large-scale production of biosurfactants.

Conflict of Interests

The authors declare no conflict of interests.

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