Exploring Microorganisms

Exploring Microorganisms:
Recent Advances in Applied Microbiology

Edited by
A. Méndez-Vilas

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Introduction

We are pleased to present a selection of papers presented at the VII International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2017), which was held in Madrid, Spain, from 18 to 20 October 2017.

The aims of this conference were to provide a forum for communicating current research priorities and progress in the fields of industrial microbiology, biotechnology, environmental sciences, food and medical microbiology and other related fields, as well as inspiring collaborations for future research. This edition gathered 290 participants, coming from 40 countries, and nearly 328 works were presented at the conference. Some of those research works are discussed in this book covering the topics: biofilms, BBB, industrial microbiology, microbiology of food and animal feed…

We specially thank the International Advisory Committee, a group of international experts who helped us in the development of the conference program. In addition, we also thank the authors for participating in the conference and sharing their work and findings.

This book acts as formal proceedings of the meeting. We hope that this set of papers is inspiring and stimulating enough for readers in their current research work. Finally, we would like to look forward to see another successful edition in 2018.

A. Méndez-Vilas
Editor
BioMicroWorld2017 General Coordinator

Agriculture, Soil, Forest Microbiology

Global and Mexican local diversity of mycorrhizal fungi in forests soils revealed by Next-Generation Sequencing: a preliminary approach

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Global forest soils is a vast source of unknown and useful microbial biodiversity for human sustainable future in harmony with nature. However until now scant reliable information is available concerning Mexican and Global mycorrhizal forest soil biota. We collected ~15,000 soil samples from 365 forest sites around the world. In Mexico four sites were sampled. Soil samples were processed and analyzed with 454 Life Sciences pyrosequencing. Metabarcoding analysis revealed that globally 23.2% were ectomycorrhizal fungi and 0.2% were arbuscular mycorrhizal. In Mexico there are ~3,000 species-level MOTUs mycorrhizal fungi of which the predominant were ectomycorrhizal with ~99%. The Mexican mycorrhizal biota was related with North America, Europe and East Asia biogeographic regions but idiosyncratic towards the tropics.

Keywords Next-Generation Sequencing; global mycorrhizal diversity, Mexican Neotropical forests, ectomycorrhizal fungi, Glomeromycota.

1. Introduction

Forest soils are the most biocomplex and heterogeneous ecosystem on Earth and the synergetic natural bioreactor for planetary sustainability. This below-ground synergistic activity is mediated by microbial functional groups (such as the mycorrhizal fungi) in ecosystem processes, including: nutrients cycling, plant growth regulation and carbon alleviating limitations of other soil organisms. Because its hidden existence, the mycorrhizal diversity of forest soils remains little known and poorly understood although it´s relevance as supplier of multiple ecosystem services and a valuable source of genetic resources and food for the humankind benefit and sustainable future [1, 2, 3]. In addition, large proportion of mycorrhizal species is possibly confined to few biodiversity hotspots in megadiversity countries such as Mexico, located in the "biodiversity belt" that surrounds the world [4]. Although the task of disentangling the "biomicroworld" appears to be puzzling an unattainable by using classical culture-dependent methods, recent technological advances in high-throughput sequencing and "omics" approaches could help to unravel the global forest soil microbiome [5]. In order to overcome this problem the UNITE project was established in 2001 through a free online database for high-quality reference records of ITS sequences with North European ectomycorrhizal fungi [6] and updated to process environmental samples from over the world with a new generic support for any other gene and genetic marker such as the nuclear large subunit (nLSU ⁄ 28S) gene [7]. Nowadays, UNITE is a reliable platform adapted to process samples using high-throughput sequencing technologies such as massively parallel (454) pyrosequencing and the term 'species hypothesis' (SH) was introduced for the taxa discovered in clustering on different similarity thresholds (97–99%) [8]. These reference sequences are available (http://unite.ut.ee/repository.php) for the scientific community in local sequence similarity searches and in the QIIME pipeline. As a consequence in 2011 it was launched "The Fungal Macroecology Consortium", a worldwide initiative integrated by 35 institutions from 23 developed and developing countries in 5 continents to study the global soils eukaryotic microbial diversity and contribute to disentangle the soil micobiome on local to global scales [9]. In this research we present the preliminarily results of the soil survey at global and local scales (Mexican Neotropical forest soils) of mycorrhizal fungal diversity.

2. Materials and methods

2.1 Studied sites

Representative terrestrial biomes were selected worldwide as follows: (1) Arctic tundra, (2) Boreal forests, (3) Temperate coniferous forests, (4) Temperate deciduous forests, (5) Mediterranean, (6) Southern temperate forests, (7) Tropical montane forests, (8) Tropical moist forests, (9) Tropical dry forests, (10) Tropical savannas, (11) Grasslands and shrublands. In Mexico 4 characteristic Neotropical forest sites with climate contrasting conditions and geographically distant from each other were selected as follows: 1 dry tropical forest (*Neea*)/Quintana Roo, Southeastern Mexico; 2 tropical coniferous montane forests (*Pseudotzuga-Abies-Pinus*)/Puebla, East-Central Mexico and Oaxaca in Southwestern Mexico and 1 chaparral (*Quercus-Arctostaphylos*) in Aguascalientes, North-Central Mexico.

2.2 *In situ* and *ex situ* methodology

In Figure 1 we illustrate the general methodology used in this research and described in Tedersoo *et al*. [9].

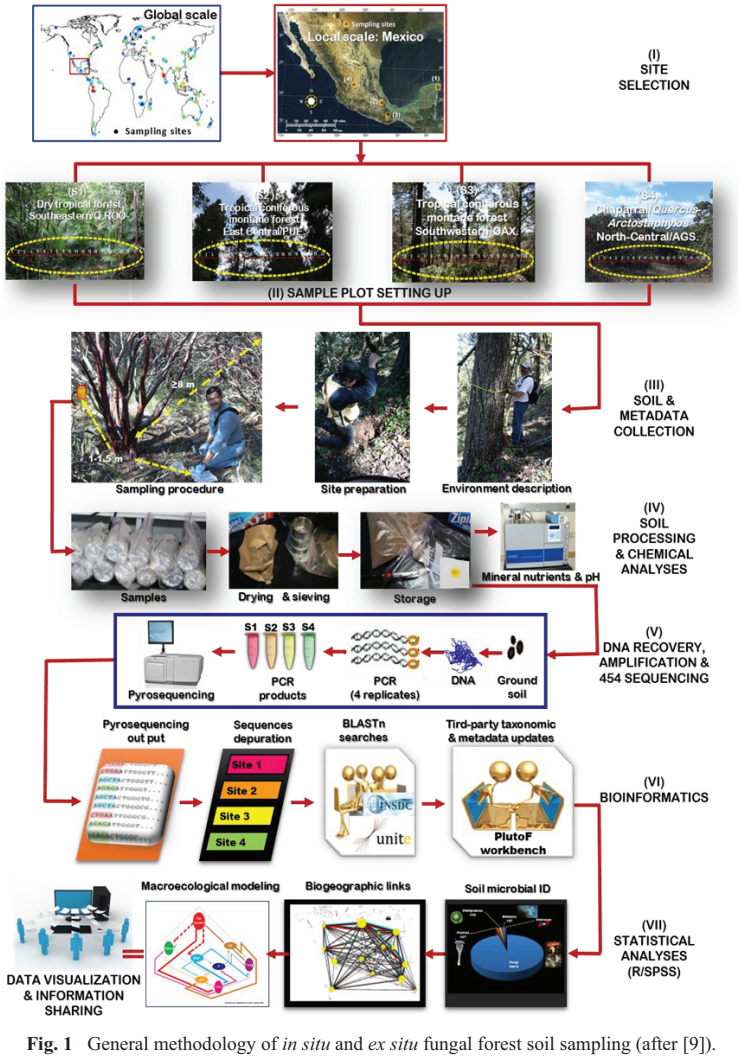

Fig. 1 General methodology of *in situ* and *ex situ* fungal forest soil sampling (after [9]).

3. Results

3.1 Mycorrhizal fungal species-level MOTUs richness.

Soil microbial metabarcoding analysis revealed at global scales 963,458 sequences and 80,486 MOTUs classified as Fungi of which 23.2 percent were ectomycorrhizal and 0.2 percent arbuscular mycorrhizal. At local scale in México the pyrosequencing analysis of 160 soil samples revealed more than 3,000 species-level MOTUs mycorrhizal fungi of which the predominant were ectomycorrhizal with 99%. Basidiomycetes was well represented with 92% followed by ascomycetes with 7%. Arbuscular mycorrhizal fungi was detected in a very low proportion with only five MOTUs (Fig2).

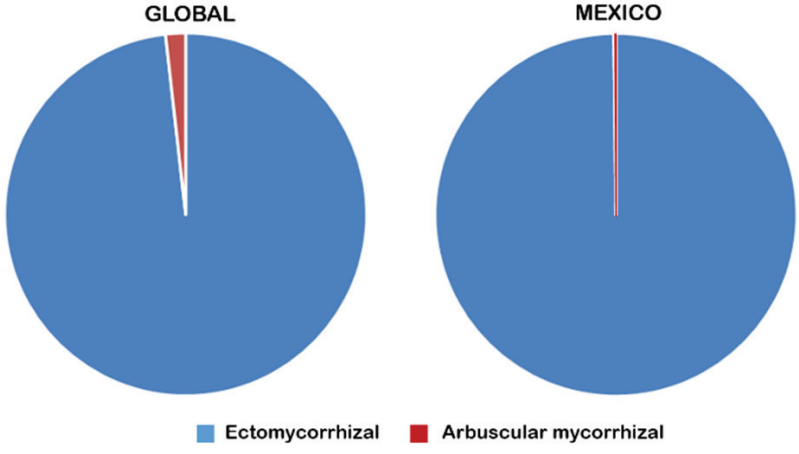

Fig. 2 Total of species-level MOTUs richness of mycorrhizal fungi at global and local (Neotropical Mexican) forests soils.

3.2 Mycorrhizal fungal species-level MOTUs richness per Mexican Neotropical forest site.

The dry tropical forest with the *Neea* sp. ectomycorrhizal tree was dominated by 99% of ectomycorrhizal fungi of which 97% was basidiomycetes and 2% ascomycetes. Arbuscular mycorrhizal fungi represented only 1% of total mycorrhizal biota. The East-Central tropical coniferous forests (*Pseudotzuga-Abies-Pinus*) dominated by *Pseudotzuga-Abies-Pinus* ectomycorrhizal trees presented a dominant proportion of ectomycorrhizal fungi with 59% basidiomycetes and 51% ascomycetes. Arbuscular mycorrhizal fungi MOTUs remained undetected. The tropical coniferous forests (*Pseudotzuga-Abies-Pinus*) from the Southwestern region of Mexico were dominated by ectomycorrhizal fungi of which 85% are basidiomycetes and 15% are ascomycetes (Fig. 4). Arbuscular mycorrhizal fungi were represented with one MOTUs.The *Quercus potosina-Arctostaphylos pungens* chaparral soil was dominated by ectomycorrhizal fungi with 94% basidiomycetes and the remaining 6% with ascomycetes. Arbuscular mycorrhizal species-level MOTUs were not detected.

4. Discussion

From a global perspective Peaey *et al*. [10] recently states that Tederso *et al*. [9] global fungal research is "The most comprehensive survey of global fungal diversity to date…" In the same line in the present research as part of Tederso *et al*. [9] global initiative, it was possible to demonstrate that Mexican Neotropical forest soils is a large repository of mycorrhizal microbial biodiversity dominated by basidiomycetes ectomycorrhizal fungal communities. We identified >3000 species of mycorrhizal fungi soil inhabitants half of MOTUs were new species for Mexican microbial biota. The impact of this soil mycorrhizal fungal metadataset can be useful as a base line at local-national-and transnational-level monitoring strategies, and with the international soil classification system for naming soils and creating legends for soil maps [11]. Also it can be relevant for better decision-making involving soil mycorrhizal fungi-forests-people as an integral part of sustainable development and the fair and equitable sharing of benefits as stated by FAO [12]) in: (1) identifying soil mycorrhizal biodiversity 'hotspots' in Mexico; (2) mapping important conservation sites with potential source of microbial genetic resources for food, human health, environment protection and improvement and to create a green economy; (3) preventing the possible impact of climate change by using native mycorrhizal fungi on land

restoration. In the particular case of Mexico the organismal, functional and genetic mycorrhizal diversity are important components of forest ecosystems, with intrinsic (option, bequest, existence) or utilitarian value (direct or indirect) from ancient to actual Mexican humans groups in which microbial communities such as fungi and mushrooms has been part of their culture and alimentary strategies (with more than 200 edible mushrooms species reported [13]) based on the sustainable use of natural forest resources [14]. Although classical microbial taxonomy is influx in Mexico as elsewhere, some mycologists speculate (on estimation figures based on plant to fungal ratio-alpha taxonomic classical approaches) that ~200,000 fungal species exists, in the Mexican territory. This figure needs to be reviewed and all Mexican mycorrhizal fungal names tested with molecular methods and species hypothesis (SH) to build a reliable fungal mycorrhizal taxonomy. Because bioprospecting, detection, genomic characterization, conservation *in situ* and *ex situ* programs are progressing slowly and are very much needed, the results we have obtained in this study using a standardized protocol will help to delineate the possible strategies to study mycorrhizal diversity as part of a National Agenda with international and interinstitutional projects.

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Improving yield and nutrients uptake of rice in the presence of plant growth promoting microorganisms

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Two plant growth promoting microorganisms (PGPM) including *Pantoea ananatis* and *Piriformospora indica* were tested for their ability to improve yield and nutrients uptake of rice (cv. 'Tahrom Mahalli') in this study. The experiment was arranged in a split plot based on a randomized complete block design with three replications. Four levels of potassium (K) sulfate fertilizer (PSF: zero, 60, 120 and 180 kg ha^{-1}) was used as the main plot and four levels of inoculation (single inoculations with *P. ananatis, P. indica*, co-inoculation and control) served as the sub-plots. The results indicated that dry weight (DW) of rice straw and grain, uptake of K in the straw and grain, HI of K, uptake of nitrogen in the grain and protein content increased by 3.64-24.3%, 12.5-31.6%, 1.81-27.4%, 10.8-39.9%, 7.04-12.5%, 12.3-36.8% and 3.24-5.56%, respectively, compared to the control (depending on the PGPM and PSF). Grain yield reached its maximum value (24.5 g $hill^{-1}$, 23% more than the control) at a declined PSF (56 kg ha^{-1} or 40% lower than the control) when applied co-inoculation treatment as a result of positive interaction between *P. ananatis* and *P. indica*. Therefore, these PGPMs can be used as biofertilizer in sustainable rice production systems as well as.

Keywords chemical fertilizer; nutrients uptake; *Pantoea ananatis*; *Piriformospora indica*; rice

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important tropical cereals in Asia, South America and Africa. It is a staple food for about 50% of the population around the world, including Iran. Rice is grown in some areas of northern and southern Iran with an annual grain production of 2.5 million tonnes from an area of 0.59 million ha^{-1} (1). It is also eaten every day in some parts of Iran. Estimates indicate that rice yield should be enhanced about 65% in the world by the year 2020, especially in developing countries where it is the main food crop (2). Soil is known as an important source of nutrients and energy for all living organisms. Plant growth promoting microorganisms (PGPMs), as a part of microbial population in the soil, are vital components in sustainable rice production systems and also are safe for the environment.

The third macro-nutrient next to nitrogen (N) and phosphorus (P) is known as potassium (K). K is absorbed by roots equal to N and or second after N in some plants like rice, cotton and banana (2). However, the ability of K uptake and or K requirement by plants is varied and depending on plant species (3). It is available to plants about 1-2% of total K in the soil (K^+, soluble forms) while 90-98% of this is unavailable for plant uptake, as a result of the strong binding force between K and other minerals such as mica and feldspar (4). K is involved in many plant metabolisms such as cell division and growth, photosynthetic process, regulates the activity of stomata cells and production of carbohydrate, protein and oil (2).

To date, many researchers reported that farmers usually use large amounts of chemical fertilizers that are required for plants which can cause harmful effects on human health and environment (5). Among PGPMs, several fungal and bacteria species called potassium solubilizing microorganism (KSM) that improve plant growth using solubilization of insoluble forms of K by various mechanisms like organic acid production (6). A range of most important KSMs were belonging to the *Bacillus*, *Burkholderia*, *Enterobacter*, *Paenibacillus*, *Pantoea*, *Pseudomonas* for bacteria and *Aspergillus* and *Glomus mosseae* for fungal genera (4, 6). In general, the interaction between KSMs and plants (e.g., rice) determines the plant health and soil fertility.

Single inoculations of rice seed with *Pantoea ananatis*, *Rahnella aquatilis* and *Enterobacter* sp. significantly increased plant height, biomass dry weight (BDW) and uptake of K (UK) in the leaves, stem and root ranged from 10.8-15.1%, 27.4-65.3%, 35.5-76.9%, 17.6-52.9% and 25.0-75.0% (depending on the bacteria strain), respectively, as compared to the control (4). In other study, *Azosprilium amazonense* inoculation with rice enhanced grain yield (GY), panicles number and N uptake in the grain by 7-11%, 3-19% and 3.5-18.5% relative to the control, respectively (7). Duarah et al. (8) showed that application of chemical fertilizers (e.g., N, P and K) along with phosphate solubilizing bacteria (PSB) reduce the nutrient runoff or leaching and enhance nutrient use efficiency (NUE) of the applied fertilizers in paddy fields. Consequently, application of PGPMs alone and or in combination with chemical fertilizers can reduce the amount of chemical fertilizers consumption (up to 50%) and support eco-friendly crop production (5, 6). Furthermore, PGPMs are able to increase nutrient

bioavailability or NUE by ~20-40% for various nutrients (9). Therefore, this study aimed to (i) determine the effects of potassium sulfate fertilizer (PSF) on rice growth, (ii) determine N and K concentration and uptake of them by rice plant and (iii) evaluate the efficiency of these PGPMs on rice growth, separately and as co-inoculation, then select the best inoculation treatment.

2. Materials and Methods

A field experiment was conducted in Mazandaran province (located at 36°33'N, 53°E and 25.7 m above sea level) for evaluating the efficiency of two PGPMs on the growth and UK of rice (cv. 'Tahrom Mahalli', known for its high grain quality which was cultivated in northern Iran) under different levels of PSF (K_2SO_4, contained at least 44% soluble K). Two PGPMs were tested: *P. ananatis* with an accession number of KM977993 as proposed by (10) and endophyte fungus *P. indica*, which were provided by Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari, Iran. Based on previous studies, *P. ananatis* was a multiple plant growth promoting rhizobacteria because it was able to solubilize insoluble P ($Ca_3(PO_4)_2$, 172 µg ml^{-1} after 5 days at 28 °C), K (mica, 38.9 µg ml^{-1} after 25 days at 28 °C) and produce indole-3-acetic acid (IAA, 8 µg ml^{-1} after 3 days at 28 °C) (4, 10). *P. indica* was also known as an effective plant growth promoting mycorrhizal fungus that its characteristics are fully described in (11). In this study, *P. ananatis* was grown in nutrient broth (NB; Scharlau, Spain; 8 g l^{-1}) medium as followed (5) to a final density of 10^7 CFU ml^{-1} and also *P. indica* was grown in Kafer medium to a final density of 10^9 CFU ml^{-1} as in (12). The experiment was carried out as a split plot arrangement based on a randomized complete block design with three replications. Four levels of PSF (zero, 60, 120 and 180 kg ha^{-1}) were used as the main plot and four PGPM inoculations, namely, single inoculations with *P. ananatis*, *P. indica*, co-inoculation (*P. ananatis* + *P. indica*) and non-inoculated as a control, served as the sub plots. Plot size was 5 m long and 2.5 m width (15 m^2) with a row spacing of 20 cm which included 12 rows. Roots of rice seedling (25 cm in height with 4-5 fully leaves) were inoculated with each PGPM suspension, which was diluted in water to a final density of 10^6 CFU ml^{-1} for *P. ananatis* and 10^8 CFU ml^{-1} for *P. indica*, and then applied by dipping the seedling roots into the suspension, for 12 h at room temperature before transplanting in the paddy field on 6 May 2016. Roots of rice seedling were also treated in the same manner with non-inoculated mediums as a control.

Some physiochemical properties of the soil (at a depth of 0-30 cm, sampled by a soil auger), measured by the Joibar Soil and Water laboratory were: 1.02% organic carbon; pH 7.75; electrical conductivity (EC) of 3.15 dS m^{-1}; 14.2 and 92 mg kg^{-1} available P and K, respectively. The soil type was sandy loam (73, 20 and 7% sand, clay and silt, respectively). Based on the results of soil test, 50 kg ha^{-1} of triple super phosphate (TSP) was used before transplanting and 150 kg ha^{-1} of urea was top-dressed in twice at transplanting time (100 kg ha^{-1}) and booting stage (50 kg ha^{-1} at 50 days after transplanting (DAT)). Different levels of PSF as mentioned above were also used before transplanting. The experiment was conducted under optimal agronomic conditions based on local recommendations. On the other hand, if necessary, weeds were controlled by hand and appropriate chemicals were used to control pests and diseases. Plots were irrigated with a water depth of ~ 3-5 cm from transplanting until two weeks before harvesting time (78 DAT).

At harvest maturity (HM), five rice $hill^{-1}$ were randomly selected and the following parameters were measured; K concentration into the rice plant organs (straw (leaves + stems) and grain, separately) measured by a flame photometric method suggested by the International Center for Agricultural Research in the Dry Areas (13). In this method, 0.5 g of dried rice organs, separately, was oxidized in a furnace at 550 °C and then use hydrochloric acid 2 normal to digest the organs. Furthermore, N content in the grain was measured by a near-infrared (NIR) composition meter (model KJT270, Japan). Briefly, five g of dried rice grain was powder in an electric mill (Hamilton GH-107 model), then two g of powder was placed into NIR instrument to measure the content of N. We used the data of 30 samples of rice grain that their N were measured by Kjeldahl method, to calibrate NIR instrument before measurement started. Protein content (PC) of rice grain was estimated using multiplying the total N content (%) with 5.95 as a conversion factor (14). Finally, the uptake of N and K in rice organs was determined by the following equation [N and/or K uptake (kg ha^{-1}) = (Nutrient concentration (%) × DW of the organ (kg ha^{-1}))/100]. We also used the following equation to calculate harvest index (HI) of K [KHI= ((K uptake in grain)/(K uptake in grain + straw))×100]. All statistical analysis were conducted using the statistical analysis system (SAS) ver. 9.4 (15). To compare the means of treatments, a two-way analysis of variance by GLM procedure was used based on the least significant difference (LSD) test.

3. Results and discussion

DW of rice straw and grain were significantly increased with the increasing PSF ($P \leq 0.01$, ~ 24.3 and 31.6% more than the control, respectively) (Table 1). Similarly, DW of straw and grain significantly increased by 3.64

and 20.8% when the co-inoculation treatment was applied in comparison with the control, respectively ($P \leq 0.01$, Table 1). Similarly, a significant increase in DW of rice organs (e.g., leaves, stems, roots) was reported by (5) when the plant inoculated with various PSB in competition with the control. In other studies, (16) in *Brassica campestris* and (17) in rice obtained higher DW of roots and shoots in the presence of *P. indica*.

Variation in K concentration was ranged from 3.75 to 6.78 g kg^{-1} in the straw and from 1.47 to 2.13 g kg^{-1} in the grain (depending on the PSF and PGPM). The simple effects of PGPM and PSF were significant on UK, although their interactions were not statistically significant (Table 1). Rice plants reached to the maximum value of UK (averaged 210.4 and 46.48 mg $hill^{-1}$ for the straw and grain, respectively) at 120 kg ha^{-1} PSF. The amount of increase was 22.6 and 33.4% when compared to the control, respectively (averaged for all levels of PGPM) (Table 1). The UK in the straw was ~4.5 times greater than the grain as a result of higher DW accumulation and K concentration in straw. The lowest and highest amount of UK in the grain was also observed in the control (37.8 mg $hill^{-1}$) and the co-inoculation treatment (46.25 mg $hill^{-1}$) which was 22.1% more than control. K concentration in rice grain and straw ranged from 1.87 to 3.68 and 3.30 to 9.42 g kg^{-1}, respectively (depending on K fertilization). Furthermore, the UK in rice straw and grain were ranged from 33.34 to 291.9 and 11.75 to 48.8 mg $hill^{-1}$, respectively (2).

PGPM significantly increased HIK ($P \leq 0.05$) but PSF had no significantly effect on HIK ($P \geq 0.05$). In general, the increase in HIK was ranged from 6.7% for the control to 12.5% for the co-inoculation treatment as compared to the control (depending on the PGPM and PSF) (Table 1). Yaghoubi et al. (18) also indicated that HIK was not affected by chemical and biological fertilizer and its value was about 16% in rice which was lower than that reported in this study (ranged 16.8-18.9%). Grain N concentration ranged from 1.12 to 2.16% and increased by 5.1% with the increasing PSF from zero to 180 kg ha^{-1} (data not shown). The influence of PSF, PGPM and their interactions were statistically significant on the uptake of nitrogen (UN) and PC of rice grain ($P \leq 0.01$, Table 1). The UN and PC were varied from 206.8 (41 kg ha^{-1}) to 563.4 (113 kg ha^{-1}) mg $hill^{-1}$ and 6.68 to 12.85%, respectively (Table 1). The increase in UN and PC were 36.8 and 5.56% more than the control at 180 kg ha^{-1} PSF, respectively (Table 1). Among PGPMs, the highest UN and PC was observed in the co-inoculation treatment (23.6 and 4.81% more than the control, respectively) (Table 1). In fact, UN in grain was 73 kg ha^{-1} for zero, 100 kg ha^{-1} for 180 kg ha^{-1} PSF, 80 kg ha^{-1} for the non-inoculation and 99 kg ha^{-1} for the co-inoculation treatment. The values for single inoculations with *P. ananatis* and *P. indica* were also intermediate to the control and the co-inoculation treatment in all traits (Table 1). Similar our results, the N concentration and UN in rice grain were ranged from 0.94 to 1.36% and 279.1 to 535.0 mg $hill^{-1}$, respectively (depending on cultivars and P fertilization) (19). A similar result was reported by (20) who indicated UN in rice grain varied from 76 to 124 kg ha^{-1} (depending on cultivars). They also reported that GY was positively correlated with BDW and UN and a higher value of them in rice plant resulted in a greater DW and N translocation to grain.

GY reached its maximum value (24.5 g $hill^{-1}$, 23% more than the control) at a declined PSF (56 kg ha^{-1} or 40% lower than the control (140 kg ha^{-1})) when applied co-inoculation treatment as a result of positive interaction between *P. ananatis* and *P. indica*. Similarly, single inoculations of rice seed with *Pantoea ananatis*, *Rahnella aquatilis* and *Enterobacter* sp. significantly increased plant height, BDW and UK in the leaves, stem and root ranged from 10.8-15.1%, 27.4-65.3%, 35.5-76.9%, 17.6-52.9% and 25.0-75.0% (depending on the bacteria strain), respectively, as compared to the control (21). In other study, *Azosprilium amazonense* inoculation with rice seedling enhanced GY, panicles number and UN in the grain by 7-11%, 3-19% and 3.5-18.5% relative to the control, respectively (7). Our results show that, for the first time, a positive interaction between *P. ananatis* and *P. indica* as a good combination which can be applied as biofertilizer in sustainable rice production systems.

Table 1 Analysis of variance and means comparison on the studied traits related to nutrients uptake in rice (cv. 'Tarom Mahali') under different levels of potassium sulfate fertilizer (PSF, kg ha^{-1}) and inoculation with various plant growth promoting micro-organisms (PGPM).

Sources of variation	Degree of freedom	Straw DW (g $hill^{-1}$)	Grain DW (g $hill^{-1}$)	UK in straw (mg $hill^{-1}$)	UK in grain (mg $hill^{-1}$)	Harvest index of K (%)	UN in grain (mg $hill^{-1}$)	Protein (%)	Grain yield (mg $hill^{-1}$)
Descriptive parameters									
Observation		48	48	48	48	48	48	48	48
Minimum		38.40	22.62	136.66	30.95	13.0	206.78	6.68	21.37
Maximum		43.35	26.79	244.62	55.52	24.0	563.39	12.85	31.72
Average		32.76	18.05	194.63	42.30	18.0	451.55	11.78	26.85
Standard deviation		3.77	2.92	25.96	6.95	2.00	69.93	0.90	9.92
F-test									
PSF	3	**	**	**	**	ns	**	**	**
PGPM	3	**	**	ns	**	*	**	**	**
PGPM×PSF	9	**	**	ns	ns	ns	**	**	**
CV (%)		5.40	4.21	8.17	7.79	10.6	4.04	4.03	5.20
Means comparison									
PSF (kg ha^{-1})	0	33.72 c	18.95 d	171.65 b	34.85 c	17.0 a	364.69 c	11.33 b	17.96d
	60	36.71 (+8.87) b	22.10 (+16.6)c	181.28 (+5.61) b	41.38 (+18.7)b	18.7 (+10.1)a	439.8 (+20.6) b	11.85 (+4.59)a	20.94 (+16.6)c
	120	41.28 (+22.4)a	24.49 (+29.2) b	206.02 (+20.0)a	44.18 (+26.8)a b	18.1 (+6.72)a	492.33 (+35.0)a	11.91 (+5.12)a	23.43 (+30.5)a b
	180	41.91 (+24.3)a	24.93 (+31.6)a	218.59 (+27.4)a	48.77 (+39.9)a	18.2 (+7.14)a	498.82 (+36.8)a	11.96 (+5.56)a	23.63 (+31.5)a
PGPM	Control	37.13 b	20.29 c	192.10 a	37.81 c	16.8 b	399.53 c	11.42 b	19.46 c
	P. ananatis	39.15 (+5.45)a	22.83 (+12.5) b	195.57 (+1.81)a	41.87 (+10.8)b	18.0 (+7.04)a b	448.86 (+12.3) b	11.79 (+3.24)a b	21.63 (+11.2)b
	P. indica	38.85 (+4.63)a	22.83 (+12.5) b	193.43 (+0.69)a	43.26 (+14.4)b	18.2 (+8.54)a b	455.09 (+13.9) b	11.87 (+3.94)a	21.63 (+11.2)b
	P. ananatis + *P. indica*	38.48 (+3.64)a	24.52 (+20.8)a	197.27 (+2.69)a	46.25 (+22.1)a	18.9 (+12.5)a	493.81 (+23.6)a	11.97 (+4.81)a	23.24 (+19.4)a

** and ns values significant at 0.01 probability level and non-significant based on the least significant difference test (LSD), respectively. CV is the coefficient of variation which was related to overall data. DW, dry weight; UK, uptake of potassium, UN, uptake of nitrogen.

† Means with the same letter are not significantly different at the probability level of 0.05. Values in parentheses indicate percent change in the respective studied traits in comparison with the control condition.

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Environmental, Marine, Aquatic Microbiology. Geomicrobiology

Biosurfactant production on crude glycerol medium

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Biodiesel production is continuously increasing as large amounts of crude glycerol, a by-product of this process, are made available. Crude glycerol contains many impurities and is unsuitable for refined applications. Still, new biotechnological process using crude glycerol can be proposed as an economically viable carbon source. In this study, the kinetics of crude biosurfactant production by *Bacillus subtilis* ATCC 6633 was investigated in a low-cost medium using crude glycerol. *B. subtilis* was able to grow and produce biosurfactant in a minimum medium with glycerol as carbon source. The highest concentration of biosurfactant was 0.34 g L^{-1} at 48 h. Results indicated that biosurfactant production occurred during exponential cell growth. However, after 96 h of incubation, the biosurfactant concentration decreased to 0.19 g L^{-1}. The decrease in biosurfactant production can possibly be linked to glycerol metabolites from the fermentation pathways. Such cost reduction in downstream processes contributes to the debate of using this product in oil spills and hydrocarbon contamination. The compounds produced simultaneously in the medium could also be recovered to maximize the commercial value of these biomolecules.

Keywords *Bacillus subtilis*; biodiesel; agroindustrial; low-cost

1. Introduction

Biosurfactants are molecules produced by microorganisms and constituted of a hydrophobic and hydrophilic portion. The main biosurfactants described in the literature are rhamnolipids produced by genus *Pseudomonas* and surfactin produced by the genus *Bacillus* [1, 2, 3, 4]. However, other microorganisms of the genus *Rhodococcus*, *Candida, Acetinobacter* are also described as producers of biosurfactants or bioemulsifying molecules [1]. The emulsifying activity and surface tension reduction are desirable properties of biosurfactants due to their many applications in pharmaceutical industries, food processing industries and bioremediation processes of oil spills.

Bacillus subtilis produces a biosurfactant called surfactin able to reduce surface tension, thus forming and stabilizing emulsions. Surfactin is a cyclic lipopeptide that contains a chain of fatty acids covalently linked to a sequence of seven amino acids arranged in a closed structure. Surfactin is a mixture of isoforms that can be differentiated by the length and branching of the fatty acid chain or substitutions in the amino acid residues of the peptide chain [5]. These differences depend on the strains of *B. subtilis* and the environmental and nutritional conditions to which these microorganisms are exposed.

The biosurfactant properties have been highlighted according to their low toxicity, biodegradability, capacity to be produced in renewable substrates and stability in extreme conditions of pH, temperature and salinity [6]. Despite such advantages, the carbon sources and the downstream processing (e.g.: purification) makes the final product more expensive. For this reason, many agroindustrial wastes have been recently considered as sources to valuable products. This strategy can reduce the cost of the final product while providing a sustainable and economical way to dispose agroindustrial waste. Furthermore, agroindustrial wastes are widely available, often in large quantities, at a very reduced price - if valued. Frying oils, molasses, effluent from olive oil production and waste of processing of cassava and potatoes are examples of such waste material [6, 7, 8, 9].

The biofuel production has been introduced in the economic scenario of several countries to achieve renewable energy goals, consequently reducing greenhouse gas emission. Biodiesel is a biofuel produced by the transesterification reaction, in which triglycerides react with alcohol, producing fatty acid methyl ester (biodiesel), and glycerol as a residue. It is estimated that approximately 10 m^3 of crude glycerol is produced for every 90 m^3 of biodiesel produced by transesterification method [10]. Considering that worldwide biodiesel production is continuously increasing [10], even larger amounts of crude glycerol are now made available. This crude glycerol contains impurities, including methanol and free fatty acids, resulting in a product that is unsuitable for refined purposes [11]. For this reason, expanding the applications of crude glycerol is an important issue. Fortunately, crude glycerol is easily metabolized by many microorganisms [12, 13, 14]. Moreover, this carbon source can be converted into high-value products such as biosurfactants, 1,3-propanediol and lipids [12, 15, 16, 17]. This study aimed to produce biosurfactant using *Bacillus subtilis* ATTCC 6633

strain in a glycerol-based low-cost medium, which was later evaluated according to the emulsifying activity on petroleum hydrocarbons and biodiesel.

2. Material and methods

2.1 Growth conditions

B. subtilis ATCC 6633 was first cultivated in Erlenmeyer flasks containing 50 mL nutrient broth and 5 % (v/v) of crude glycerol. Erlenmeyer flasks were maintained in an oscillatory (rotatory) shaker at 180 rpm 35 ºC for 24 h, as showed in Figure 1. Afterward, the medium was centrifuged at 1500 *x* g for 20 min and the pellet was washed with a sterile saline solution 0.85 % (w/v). The cell density was adjusted to 0.3 a.u. (~1.66 g L^{-1} dry weight). A calibration curve was built to relate the absorbance with cell dry weight. Amounts of 1 mL were inoculated to the production medium containing 50 mL of Bushnell-Haas broth as recommended by Sigma-Aldrich and 5 % of crude glycerol. The fermentation was run in triplicate.

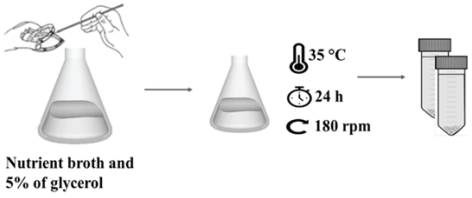

Fig. 1 Growth conditions of *B. subtilis* in Nutrient Broth with 5 % of glycerol.

2.2 Production of biosurfactant

The production medium was incubated in the same conditions as described previously in item *2.1* (180 rpm/ 35 ºC) for 96 h. Every 24 h of incubation, three flasks were collected and the following parameters were determined: biomass (dry weight), substrate consumption, pH and concentration of crude biosurfactant. Procedures for determination of biomass were identical to the described for inoculum preparation. Crude biosurfactant was precipitated at pH 2.0 by the addition of HCl 6 M following overnight at 4 °C. The biosurfactant was recovered by centrifugation at 1500 *x g* and then it was solubilized in phosphate buffer and dried at 37 ºC. The consumption of glycerol was measured by colorimetric tests according to Bondioli and Bella [18]. The substrate of conversion was calculated according to Fogler and Gürmen [19] as follows in equation 1.

$$\Delta S\ (\%) = \frac{S_0 - S}{S_0}\ x\ 100 \quad (1)$$

Where S_0 is the concentration of glycerol added in the medium and S is the glycerol concentration in the samples at each time. The specific rates of cell growth (μx), product formation (μp) and substrate consumption (μs) were performed according to Schmidell et al. [20], as showed in equation 2, 3 and 4, respectively.

$$\mu x = \frac{1}{X} . \frac{dx}{dt} \quad (2)$$

$$\mu p = \frac{1}{X} . \frac{dp}{dt} \quad (3)$$

$$\mu s = \frac{1}{x} . \left(- \frac{ds}{dt}\right) \quad (4)$$

2.3 Emulsifying activity

Emulsifying activity of the crude biosurfactant was determined by mixing 2 mL of biodiesel/diesel fuel blends with the same volume of a solution of crude biosurfactant at 1 mg mL^{-1}. The blends were: B5 (5 % biodiesel/95 % diesel), B10 (10 % biodiesel/90 % diesel), B50 (50 % biodiesel/50 % diesel, B100 (only biodiesel) and D100 (only diesel fuel). The tubes were vortexed for 2 min, and the resulting emulsion was allowed to settle for 24 h at room temperature. The emulsifying activity (E24) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm).

3. Results and discussion

3.1 Biomass, glycerol consumption, and biosurfactant production

The parameters of fermentation were shown in Figure 2. It was observed that the *B. subtilis* could grow and produce biosurfactant in a minimum medium using 5 % of glycerol as a carbon source. Cruz et al. [21] indicated that *B. subtilis* could not growth in the medium with 7 % and 9 % of glycerol. However, the addition of manganese salts in the medium with 5 % of glycerol enhanced the biosurfactant production.

The concentration of biomass was 1.49 g L^{-1} at 96 h of incubation. The highest biosurfactant concentration was 0.34 g L^{-1} at 48 h of fermentation. The substrate conversion (ΔS %) was 49 % at the end of 96 h.

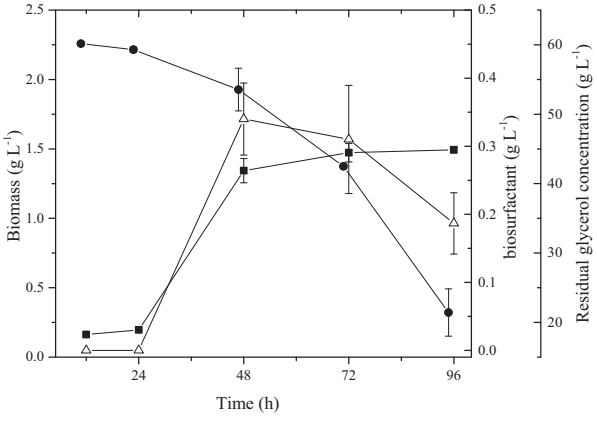

Fig. 2 Biomass dry weight (■), residual glycerol concentration (●) and crude biosurfactant production (Δ). The averages and standard deviations (error bars) were calculated from three independent experiments.

The crude biosurfactant recovered at 96 h was lower than recovered at 48 and 72 h. The results suggest that proteases in the medium at 96 h could break the hydrophilic portion formed by amino acids. Nitschke [22] also observed 73 % of the reduction in biosurfactant production after 72 h of fermentation, whereas the protease in the fermentation medium increased 5-fold. The glycerol concentration was decreased of 58.0 g L^{-1} to 21.4 g L^{-1} after 96 h of fermentation.

The specific growth rates were represented in Figure 3. The peaks of specific biosurfactant production overlapped the exponential growth phase. Therefore, the biosurfactant was produced in the exponential growth phase and it is should be growth-associated. Sousa et al. [23] founded that the biosurfactant produced by *B. subtilis* is a primary metabolite due to the association of growth and production. Some species, such as *Pseudomonas* and *Rhodococcus* produce biosurfactant during the stationary growth phase [8, 23, 24, 25]. However, the biosurfactant production by *B. subtilis* occurs in the exponential phase as founded in this study and corroborated with described in the literature [23, 26].

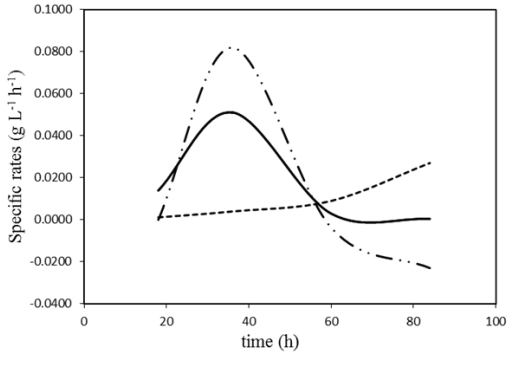

Fig. 3 Specific rates of growth (· ·—· ·) biosurfactant production (—) and glycerol consumption (---) in g L^{-1} h^{-1}. The experiments were performed in triplicate.

3.2 Emulsifying activity

The crude biosurfactant solution at 1mg mL^{-1} showed the emulsifying activity of 28 % (±5) against diesel fuel as showed in Table 1. However, as the biodiesel concentration in the blend increase, the emulsifying activity decreased. Therefore, the emulsifying activity against B5 and B10 was around 12 % and 5.4 % for B50. The biosurfactant did not show any emulsifying activity against pure biodiesel.

Table 1 Emulsifying activity against fuel

Fuel	Biosurfactant E_{24}(%)
Biodiesel 100%	0 ±0.00
B50	5.48 ±1.91
B10	12.5 ±1.78
B5	12.66 ±0.66
Diesel 100%	28.33 ±5.00

* Solution of biosurfactant at 1 mg mL^{-1}
± standard deviation

The crude biosurfactant produced without any purification process showed emulsifying activity against diesel fuels. This emulsifying activity is an important property of biosurfactants to environmental applications, because the emulsification enhances the bioavailable of hydrophobic molecule as diesel fuel. The purification of biosurfactant could increase the emulsifying activity, on the other hand, also could increase the cost of the final product, making inviable for environmental applications.

The present study was an attempt to use glycerol as a carbon source to produce biosurfactant. However, to achieve production economically viable, it is necessary to propose a strategy to produce other biomolecules from the culture medium that are produced simultaneously to the biosurfactant, such as fengycin, iturin and even proteases. Also, other agroindustrial waste should be studied as potential carbon source that promotes higher production with lower cost.

4. Conclusion

The glycerol was a carbon source used to *B. subtilis* to produce biosurfactant. The biosurfactant production was growth-associated. The highest concentration of biosurfactant was detected at 48 h, after this time, the biosurfactant might be degraded in the medium. The crude biosurfactant showed emulsifying activity against diesel fuel, but no emulsifying activity was observed against biodiesel.

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