MICROBIAL CHARACTERISATION OF SOME BACTERIAL ENDOPHYTES ISOLATED FROM *Vicia faba* L. SEEDS

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Abstract. In nature, plants colonisation with microorganisms is inevitable. Seed endophytes are the first interacting with young seedlings and may impact their growth. The internal colonization of plant tissues and endophyte vertical transmission denote a very good relationship between these two partners, perhaps a constricted interdependence sustained by environmental conditions, or a very good microbial adaptation to endophytic lifestyle in a certain host. However, endophytes are defined as asymptomatic internal colonisers, thus being classified as neutral, commensal or beneficial. If we subject them to various laboratory tests, we can determine their biologic attributes. If beneficial, endophytes can be used as inoculants. This study is focused on revealing new endophytic strains isolated form *Vicia faba* seeds, and their enzymatic activity. Although the endophytic seed community was limited, each strain revealed particular biologic traits with high potential attributes for plants protection and growth promotion.

Keywords: microbial characterisation, endophyte bacteria.

Rezumat. Caracterizarea microbiologică a unor bacterii endofite izolate din semințe de *Vicia faba* L. În natură, colonizarea plantelor cu microorganisme este inevitabilă. Endofitele semincere sunt primele care interacționează cu tinerele plante, putând influența creșterea acestora. Colonizarea internă a țesuturilor vegetale și transmiterea pe verticală a endofiților, dovedesc o relație foarte bună între acești doi parteneri, sau poate o interdependență constrânsă de condițiile de mediu, sau o bună adaptare a unor microorganisme la stilul de viață endofit într-o anumită gazdă. Cu toate acestea, endofiții sunt definiți drept colonizatori interni asimptomatici, putând fi astfel clasificați ca neutri, comensali sau benefici. Determinarea precisă a caracteristicilor biologice poate fi realizată prin supunerea acestora la diferite teste de laborator. Ulterior, endofiții cu proprietăți benefice putând să fie folosiți ca inoculanți. În acest studiu sunt prezentate noi tulpini endofite, izolate din semințe de *Vicia faba*, precum și activitatea lor enzimatică. Deși comunitatea endofită prezentă în semințe s-a dovedit a fi redusă, fiecare tulpină microbiană nou izolată a dezvăluit trăsături biologice particulare cu atribute benefice pentru promovarea creșterii sau protecția plantelor.

Cuvinte cheie: caracterizare microbiologică, bacterii endofite.

INTRODUCTION

In nature, plant-microbial interactions are inevitable. Plants can be colonised by various microorganisms. The most abundant are free-living microorganisms, which first establish external contact with the plants, but there are also internal tissue colonisers, that should not be undervalued. Those microorganisms, used to live inside the plants without causing disease symptoms or apparent plant metabolic changes, are called endophytes (MALFANOVA et al., 2013). After the internal colonisation of plant tissues, endophytes could be vertically transmitted to the next plant generation through seeds and vegetative propagation (FRANK et al., 2017). This could be a beneficial aspect for the new plantlets. Studies have shown various endophyte microorganisms with plant growth promoting attributes and plant protection features, which increase host resistance to environmental stresses factors and/or pathogenic infections (AFZAL et al., 2019; LUO et al., 2019). Various biological activities were noticed in endophytic microorganisms, such as: atmospheric nitrogen fixation, phytohormone synthesis or regulation of plant hormone levels, phosphate solubilisation and nutrients uptake improvement, enzyme production, or synthesis of antimicrobial compounds (MALFANOVA et al., 2013). Due to these beneficial traits, the interest for endophyte microorganisms is increased. Studies are focused on various aspects, such as: (i) endophytes role on their natural host plant, or other potential hosts, (ii) endophytes access pathways in plants and trough the plant, (iii) environmental features influencing compositional, proportional and numerical status of endophytic communities, (iv) the variations and similarities in endophytic communities in different host plants, on in plants grown in different environmental conditions (v) endophyte activity on the ecology, health and productivity of plants, (vi) endophyte formulation and best application procedures, (vii) endophyte potential in improving plant attributes for phytoremediation, or phytotherapy purposes, and many others (MUKHERJEE et al., 2018; FADIJI & BABALOLA, 2020).

Commonly, cultivated leguminous plants are seed inoculated with plant beneficial microorganisms to improve the nodulation process and promote plant growth. However, such treatments are applied only for crops grown on large areas (mostly on soya, alfalfa, common bean, and pea), plants with high economic value or significance in crop rotation. *Vicia faba* L., also known as faba bean, broad bean (when used for food) or horse bean (as feed), could also be inoculated with nitrogen fixing bacteria, but such seed treatments are not very common for this crop in all countries (ADISSIE et al., 2020). Therefore, natural occurring seed endophytes are the most intimately-bound microorganisms influencing plant growth in the first stage of emergence, before other plant interaction with soil microbial community. Within this paper, the aim is to evaluate the biological activity of *Vicia faba* seeds endophytes and highlight the potential biological attributes they have on their host plant.

MATERIAL AND METHODS

Endophyte isolation. *Vicia faba* L. seeds, from the Valencia (Spain) local market of organic products, were used as endophyte isolation source. For this purpose, seeds were surface disinfected using a two-step protocol. Seeds were first immersed in 70% ethanol for at least 30 seconds under manual stirring, then rinsed for tree times with sterile distilled water (SDW). The second disinfection step was made with 5% sodium hypochlorite for 15 minutes in continuous shaking at 100rpm. Five rinses, each of 5 minutes under continuous stirring, were performed at the end. The surface disinfected seed were manually grinded with sterile mortar and pestle. Grinded seeds were mixed with SDW and then plated on Luria Bertani (LB) agar. The resulting bacterial endophytes were purified using the streak plating technique on LB agar.

Biochemical tests. Several enzymatic tests correlated with bacterial potential activity in the biological control of plant pathogens (such as: cellulase, chitinase, protease and lipase production) and plant growth promotion (such as: amylase, arginine-decarboxylase, phytase production) were performed. Several growing substrates (Table 1) were used in order to detect the enzymatic activity of the studied bacterial strains.

Table 1. Growth media for endophyte characterization.

Medium name and use purpose	Medium composition and preparation	References
Agrawal and Kotasthane medium for chitinase activity detection	4.5 g colloidal chitin, 3 g magnesium sulphate heptahydrate; 3 g ammonium sulphate; 2 g dipotassium phosphate; 1 g acid citric; 0.15 g bromcresol purple; 200 µl Tween 80; 20g agar to one litre of distilled water; final pH 5.5	AGRAWAL & KOTASTHANE, 2009, 2012; SICUIA et al., 2015
CMC medium for cellulase activity evaluation	10 g carboxymethyl-cellulose (CMC); 0.5 g sodium chloride; 1 g dipotassium phosphate; 0.5 g magnesium sulphate heptahydrate; 0.01 g manganese sulphate monohydrate; 0.3 g ammonium nitrate; 0.01 g ferrous sulphate heptahydrate; 12 g agar to one litre of distilled water; final pH 7.0	CONSTANTINESCU et al., 2010; SICUIA et al., 2015
Casein blue agar or CAM-BG for protease activity evaluation	Two solutions must be prepared and separately autoclaved. One solution should include 25mM Tris (pH 7.2), 150mM sodium chloride, and 0.6% casein. For CAM-BG medium, the casein solution must be supplemented with 0.0015% (w/v) bromocresol green dye. The other solution should have 1.8% agar and pH 7.0. After sterilisation the solution are aseptically mixed under the laminar flow.	VIJAYARAGHAVAN & VINCENT, 2013; URSAN et al., 2018.
Tween supplemented medium to confirm lipolytic activity	10 g peptone, 5 g sodium chloride, 0.1 g calcium chloride, 20 g agar and 10 ml (v/v) Tween 80 to one litre of distilled water. The Tween 80 is autoclaved separately and aseptically added in the medium.	RAMNATH et al., 2017; SICUIA et al., 2015
Starch supplemented medium for amylase detection	nutrient agar medium (containing 0.5% peptone, 0.3% beef/yeast extract, 0.5% sodium chloride and 1.5% agar) supplemented with 0.4% soluble starch	CONSTANTINESCU et al., 2010; SICUIA et al., 2015
Arginine supplemented medium for arginine ecarboxylase detection	2 g L-arginine, 5 g bacto-peptone, 5 g beef extract, 0.5 g glucose, 5 mg pyridoxine, 20 mg phenol red and 18 g/L agar to one litre of distilled water, final pH 6.0.	SICUIA et al., 2012; 2015
TS medium for phytase detection	0.5% glucose, 1% peptone, 0.5% yeast extract, 0.1% magnesium sulphate, 0.1% calcium chloride, 0.2% sodium phytate, final pH 7.0	DEMIRKAN et al., 2014
	TS medium could also be supplemented with pH sensitive dyes such as bromophenol blue 0.01% or bromocresol purple 0.01 %.	KUMAR et al., 2012
Pikowskaya (PVK) medium for phosphate solubilization	10 g glucose; 0.5 g yeast extract; 0.5 g ammonium sulphate; 0.2 g potassium chloride; 0.01 g magnesium sulphate heptahydrate; 0.0001 g ferrous sulphate heptahydrate; 0.0001 g manganese sulphate monohydrate; 5 g tricalcium phosphate; 15 g agar to one litre of distilled water, final pH 7.0	KAUR & REDDY, 2013; DOBRE et al., 2016
CTAB - methylene blue agar medium for screening of biosurfactant-producing strains	20 g glucose; 10 g peptone; 1 g beef extract; 0.78 g CTAB; 0.002 g methylene blue; 0.5 g yeast extract; 17 g agar to one litre of distilled water; final pH 7.2	LIU et al., 2013

The growth media were inoculated in spots with fresh bacterial biomass obtained on LB agar. Cultures were incubated at 28°C for 3 to 7 days and examined on a daily basis. For each test, positive known bacterial strains were tested as control.

Chitinase activity was evaluated on Agrawal and Kotasthane medium. Due to the pH indicator dye (bromocresol purple), the enzymatic breakdown of chitin can be recognised based on a colour change from yellow (in acid pH) to purple (in alkaline pH). To evaluate the bacterial cellulolytic potential, the isolated were grown on a CMC medium. Afterwards, the incubation plates were stained for 15 minutes with 0.1% Congo red, and then washed with 1M sodium chloride. The saline solution is used to remove the dye from the broken CMC matrix. Therefore, CMC-ase producing strains should have a lighter colour halo around their colonies. The protease activity is similarly revealed by a clear lighter halo around the colonies able to hydrolyse casein. The production of lipolytic enzymes was evaluated on Tween 80 supplemented medium. The enzymatic hydrolysis of Tween 80 was correlated with calcium salt precipitation and the appearance of white soap deposits around the colonies.

The amylase activity was tested for bacteria grown on starch supplemented medium. After incubation, plates were flooded with iodine solution, which stains the non-hydrolysed starch in violet-indigo colour. If the strains are amylase producing, a clear halo can be seen around their colonies. The arginine decarboxylase activity was revealed by a change of colour from yellow to pink due to the pH acidification of the arginine-based substrate, in the presence of the pH indicator dye (phenol red). Phytase activity was tested in TS and TS with two different indicator dyes on which phytase producing strains are revealing a clear halo or a halo associated with a change of colour due to pH change. Phosphate solubilisation was studied on Pikowskaya agar, containing tricalcium phosphate, on which positive strains are surrounded by a clear halo.

Biosurfactant production. The screening for biosurfactant-producing strains was performed on the CTAB - methylene blue agar medium. On this substrate, positive strains are surrounded by a coloured halo revealed by the pH-sensitive indicator dye. The oil spreading technique was performed similarly to OLTEANU et al. (2011). For this, the bacteria were grown in LB broth and the culture supernatant was forwardly used. For this test, PP plates of 5 cm in diameter were half filled with distilled water faintly coloured with Congo red. Formerly, 30 µl of oil were slowly added on top and let to spread in a thin layer. Then, 10 µl culture supernatant were added to the oil surface. The oil spreading zone appearing was considered an indication of biosurfactant production. Two reference solutions were used for comparison: a positive control made of 5% (w/v) SDS, and a negative control of distilled water.

RESULTS AND DISCUSSION

Five bacterial endophytes were harvested from the analysed *Vicia faba* seeds. These newly isolated strains were the only ones able to grow *in vitro* conditions (Fig. 1). Three of the strains (no. 1, 3 and 4) developed semi-translucent creamy biomass on LB agar, strain no. 2 produced rough, beige colonies and strain no.5 developed faintly yellow colonies.

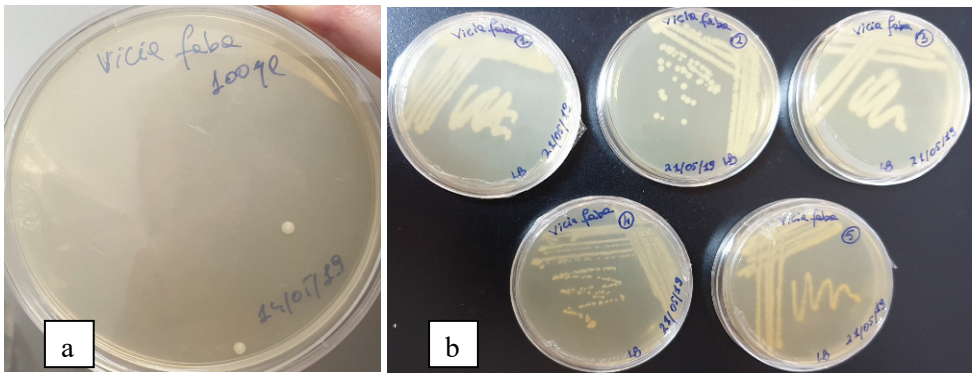

Figure 1. New strains of bacterial endophytes isolated from *Vicia faba* seeds
a. endophytes isolation *in vitro*; b. endophytes growth in pure cultures.

The biochemical characterisation of the strains was performed through several enzymatic tests on agar slants. The chitinase assay was performed on colloidal chitin from crab shell in the presence of an indicator dye. The chitinase activity can be important in biological control due to the fact that it can contribute to the cell wall degradation of fungal phytopatogens and arthropods exoskeleton of plant pests, such as: deleterious insects, mites, nematodes (VELIZ et al., 2017). The results were negative for all newly isolated strains (Fig. 2). However, SHEHATA et al. (2016) have showed that in some endophytic bacteria chitinase production is induced only in the presence of a certain substrate or fungal pathogens.

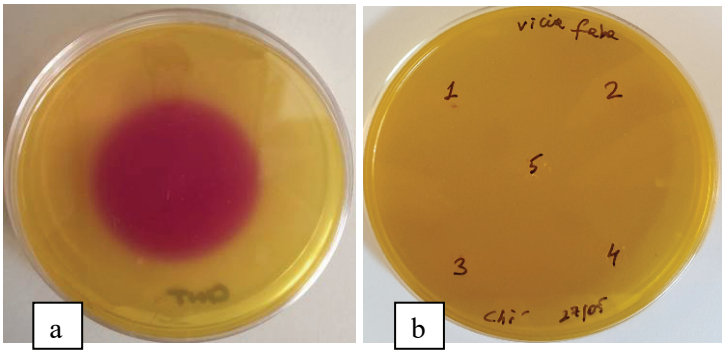

Figure 2. Chitinase evaluation a. positive reaction; b. newly isolated strains all with negative reaction after 5 days of incubation.

The cellulase activity was also tested for biocontrol purposes, since cellulose can be found in the cell wall of Oomycetes. Therefore, cellulase production can contribute to the suppression of *Pythyum* and *Phytophtora* growth (SICUIA, 2013). However, none of the newly isolated strains were able to degrade carboxymethyl-cellulose in 3 days of incubation on such substrate (Fig. 3).

The protease activity was found to be produced by all strains tested, although the clear halos revealed wide differences among the strains in terms of enzyme production. The highest production of caseinase was shown by strain no. 2, followed by strains no. 5, and 4. Based on the calculated enzymatic index (CARRIM et al., 2006) the newly isolated strains are high producers of caseinase comparing with other endophytes (Table 2).

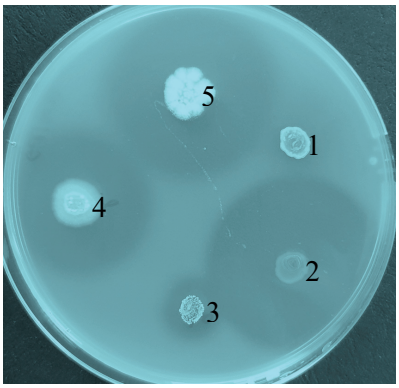

Figure 3. Protease activity after 5 days of incubation.

Table 2. Enzymatic activity of faba beans endophytes.

Enzymatic activity	**Chitinase**	**Cellulase**	**Protease**	**Lipase**	**Amylase**	**Arginine decarboxylase**	**Phytase**
Days of incubation	**5 days**	**3 days**	**5 days**	**3 days**	**3 days**	**1 day**	**5 days**
Bacterial seeds endophytes	Enzymatic index*						
Isolate no.1	0	0	1.25	0	0	2.5	0
Isolate no.2	0	0	5.75	0	0	2.4	0
Isolate no.3	0	0	2	1.75	0	3	0
Isolate no.4	0	0	3.11	2.25	0	2.5	N.A.
Isolate no.5	0	0	3.28	2.3	0	3.4	3.13
Note: * The enzymatic index represents the halo diameter of degradation/diameter of colony in cm; N.A. = not available							

The lipolytic activity was evaluated on a Tween 80 supplemented medium. If tested bacteria are lipase producers, the enzyme is catalysing the reaction between Tween 80 and the calcium chloride found in the substrate, the result involves calcium oleate formation (POHANKA, 2019) which appears as a white precipitate around positive colonies (Fig. 4).

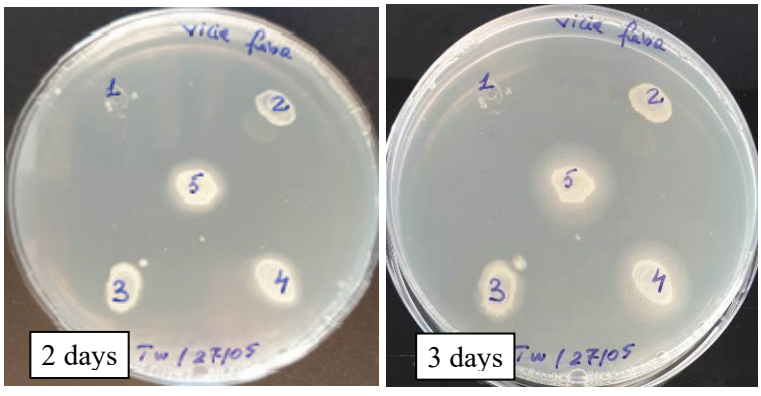

Figure 4. Lipase activity after different incubation time.

Among the newly isolated strains only tree expressed lipolytic activity. The best results were obtained with isolate no. 5 and 4, which expressed a similar enzymatic index (Table 2). The isolate no. 3 revealed obvious lipase activity only after 3 days of incubation. Comparing these results with other bacterial strains, the isolates no 5 and 4 from faba beans are moderate lipase producers, expressing enzymatic index values of 2.3 and 2.25, respectively. CARRIM et al. (2006) mentioned an enzymatic index of 2.8 to 3.2 to their positive strains, and only in a single endophytic *Corynebacterium renale* strain they obtained an index value of 6.2. However, if comparing our endophytic strains with

bacteria isolated from waste vegetable oil contaminated soil, the endophyte isolates no 4 and 5 (which revealed a precipitation halo of 12 and 13 mm in diameter after two days of incubation) were higher producers than the LECHUGA et al. (2016) isolates which developed a halo not higher than 10.7 mm in diameter, in similar incubation conditions.

Amylase activity was not detected in neither of the isolates. However, all strains revealed intense arginine decarboxylase production (Fig. 5). This aspect is very important for abiotic stress resistance. The arginine decarboxylase pathway conducts to polyamine formation which increases plant resistance to cold weather, temperature variations, and other abiotic stress factors (ALCÁZAR & TIBURCIO, 2018). Considering that the studied bacteria are endophyte colonisers, the polyamine production can take place *in situ*, thus providing a higher efficiency for the plants.

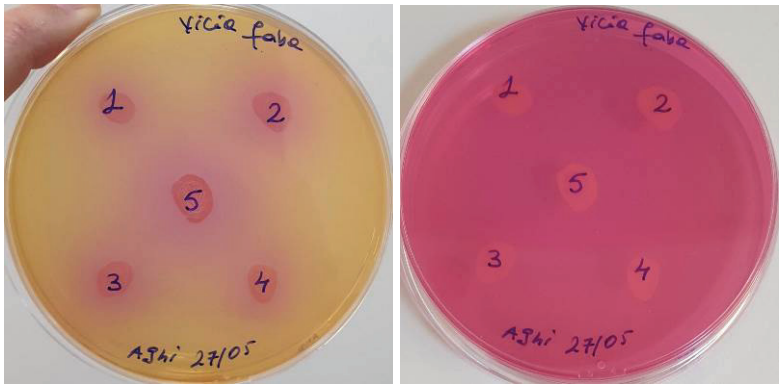

Figure 5. Arginine decarboxylase activity after 1 to 3 days of incubation.

Phosphorus (P) is an essential nutrient for plants. Growth and development processes depend on phosphorus availability. Although the phosphorus resources are abundant in soil, its' uptake in plants is limited due to its low solubility, and therefore the need for fertilizer supplies. Phosphorus resources can be organic or inorganic. In most soils, the organic phosphorus amount was evaluated to comprise 30 to 65% of total P (CONDRON et al., 2005). Microbial phosphatase and organic acid release are important for increasing the availability of organic P for plants.

Among the tested endophytic bacteria, only isolate no. 5 presented phytase activity (figure 6), revealing a 5 mm halo ring around the bacterial colony, with an enzymatic index evaluated as 3.13 (Table 2).

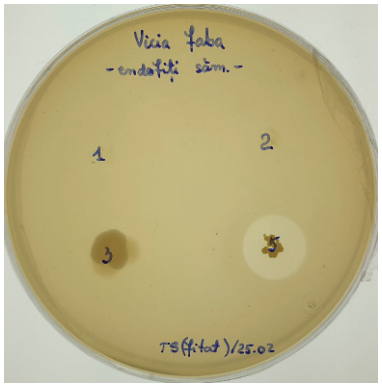

Figure 6. Phytate solubilisation after 5 days of incubation on Pikowskaya agar.

Phosphate (PO_4^{3-}) is the main form of inorganic phosphorus resource. Plants can absorb the needed P only as orthophosphate ions: $H_2PO_4^-$ and $H_2PO_4^{2-}$ (DODD & SHARPLEY, 2015). Their concentration in the soil is influenced by complex chemical and biological processes (PIERZYNSKI et al., 2005). Due to the low solubility of phosphate, microbial activity is very important for plant nutrition. Phosphate solubilizing microorganisms are thus beneficially influencing plant growth and nutrient uptake. Among the newly isolated endophytes, three of them (isolate no. 5, 4 and 1) expressed inorganic phosphorus solubilizing activity (Fig. 7).

Many plant-associated microorganisms are biosurfactant producers. For the environment, these biomolecules are considered eco-friendly. They have various implications in nature, but most importantly, they play a vital role in microbial motility, biofilm formation, signaling and therefore in plant-microbe interaction. Biosurfactants can trigger several beneficial attributes for plant protection and growth promotion. These biomolecules can reduce plant pathogen viability and increase nutrients bioavailability for beneficial plant-associated microorganisms (SACHDEV & CAMEOTRA, 2013). Therefore, exploring biosurfactants producing microorganisms is relevant for agriculture and environmental purposes.

Biosurfactants producing strains was screened on CTAB methylene blue agar. Among the newly isolated endophytes, only isolate no. 3 revealed a positive reaction (Fig. 8).

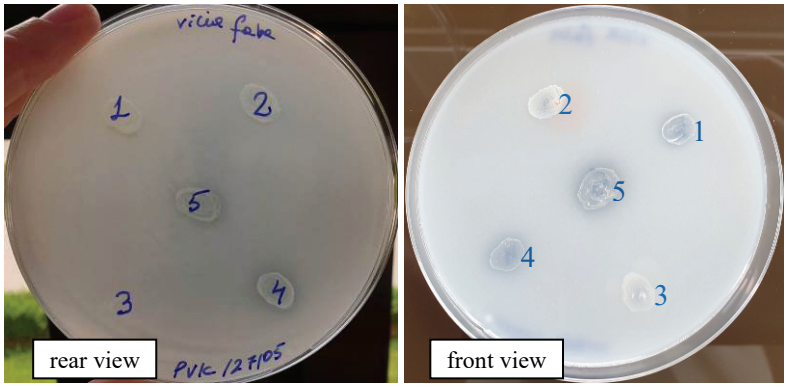

Figure 7. Phosphate solubilisation after 2 days of incubation on Pikowskaya agar.

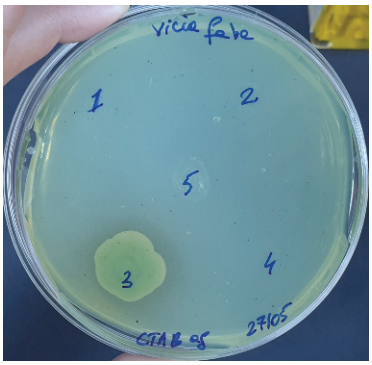

Figure 8. Bacterial growth on CTAB-methylene blue agar correlated with biosurfactant production.

Considering the results from the colorimetric CTAB agar assay, isolate no. 3 was forwardly tested for oil spreading potential (Fig. 9), compared to a positive control made of 0.1% SDS (Fig. 9a), and a negative control of distilled water (Fig. 9c). The culture supernatant of the tested bacterial endophyte revealed an oil spreading potential of 42.1% (Fig. 9b) of the chemical control activity.

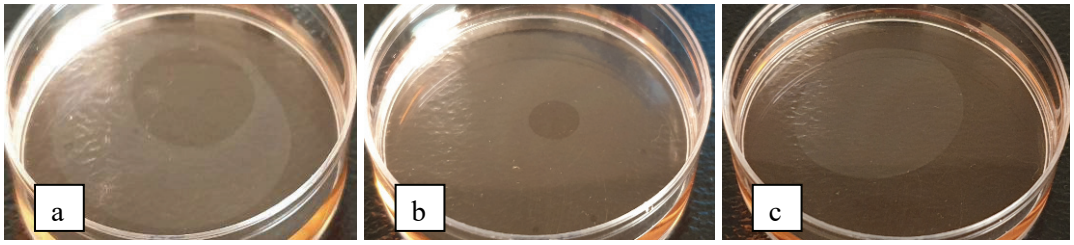

Figure 9. Oil spreading activity a. chemical control; b. culture supernatant of endophyte isolate no. 3; c. negative control.

The biosurfactants' activity can be useful for various purposes. Besides environmental and agricultural reasons, biosurfactants can be used in cleaning products, pharmaceuticals, cosmetics and food industries (SACHDEV & CAMEOTRA, 2013).

CONCLUSIONS

The microbial characterisation of *Vicia faba* endophytic bacteria revealed that seeds can possess internal colonisers with various beneficial traits. The enzymatic and biologic activity of the newly isolated strains showed that vertically transmitted endophytes possess plant beneficial attributes than could contribute to plant growth promotion and protection. All isolated strains revealed at least two plant beneficial traits.

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