The EuroBiotech Journal
RESEARCH ARTICLE
Environmental Biotechnology

Antifungal activity of a biosurfactant-producing lactic acid bacteria strain

Gabi Mirela Matei[1], Sorin Matei[1], Adrian Matei[2] and Elena Draghici[3]

Abstract

Lactic acid bacteria are frequently utilized in food industry and they are also recognized as antimicrobial agents due to their capability to produce metabolites such as: organic acids, biosurfactants, bacteriocins, hydrogen peroxide, cyclic dipeptides, exopolysaccharides. The main goal of this paper was to present the results of the research carried out on the strain LCM2 of lactic acid bacteria isolated from brined cucumbers, for production of biosurfactants and to assess its antifungal properties. The emulsification capacity of biosurfactant was measured using kerosene as the hydrophobic substrate. The value of emulsification index E24 was 89.04% showing a high emulsification activity of the biosurfactant. The structural characterization of biosurfactant by TLC revealed its glycolipidic nature. Assay of the ionic charge established the anionic charge of the biosurfactant revealed by the presence of precipitation lines towards the cationic surfactant dodecyl-dimethyl-ammonium chloride. The biosurfactant presented antibiofilm activity with low adherence capacity, structural damages of the hyphal net, conidiophores and delays or lack of sporulation and decreased biomass accumulation in four mycotoxigenic *Penicillium* and *Aspergillus* isolates. Results of *in vitro* assays recommend the biosurfactant produced by the new lactic acid bacteria strain LCM2 for biotechnological purposes, as alternative antifungal agent in food industry.

[1]Department of Research in Soil Science, Agrochemistry and Environment, National RD Institute for Soil Science, Agrochemistry and Environment, Bucharest, Romania

[2]Department of Nosocomial Infections Surveillance, Prevention and Control, Institute of Oncology "Prof. dr. Alexandru Trestioreanu", Bucharest, Romania

[3]Department of Bioengineering of Horti-wine Systems, UASVMB, Faculty of Horticulture, Bucharest, Romania

Corresponding author: S. Matei
E-mail: so_matei602003@yahoo.com

Published online: 20 July 2017
doi:10.24190/ISSN2564-615X/2017/03.02

Introduction

Lactic acid bacteria (LAB) have practical applications including the production of fermented foods, enhancement of its quality and as bio-preservatives (1), due to their high metabolic ability to produce compounds able to control pathogenic fungi or bacteria e.g. weak acids (lactic, acetic, phenyl-lactic, propionic), cyclic dipeptides, reuterin, exopolysaccharides, biosurfactants (2,3,4).

Biosurfactants production by lactic acid bacteria on various cheap substrates has an advantage over chemical surfactants confered by their biological origin, low toxicity and high biodegradability.

Biosurfactants can be usefull for crude oil recovery, biodegradation of hydrocarbons with application in soil or water decontamination (5), against biofilm forming microbial contaminants of food or medical instruments (6).

Fungi belonging especially to the genera *Penicillium* and *Aspergillus* play a role in damaging food and feed products stored and subsequently used in human and animal nutrition, by the synthesis of secondary metabolites known as mycotoxins and the biosurfactants produced by lactic acid bacteria could be an alternative biopreservation method.

The purpose of the present work was to investigate the biosurfactant produced by the strain LCM2 of lactic acid bacteria isolated from brined cucumbers and its antifungal properties against *Penicillium* and *Aspergillus* isolates from food products used in human nutrition.

Materials and Methods

Test microorganisms used in this study

Lactic acid bacteria strain LCM2 was isolated from brined cucumbers and grown in MRS broth (Liofilchem) at 36^0C. The strain from Laboratory of Soil Biology Collection was previously identified on the basis of Gram staining, morphology and catalase test as *Lactobacillus* sp.

Potential mycotoxigenic fungal species belonging to genera *Penicillium* and *Aspergillus* were isolated from suspensions of various plant materials and food products, infected or contaminated (**Table 1**), plated on PDA medium (Merck KGaA Germany), then isolated in pure cultures. Taxonomic identification was carried out on the basis of colony morphology and structural characteristics observed by optic microscopy.

Table 1. Fungal isolates utilized for evaluation of antibiofilm effect of biosurfactant and their origin

No.	Fungal isolate	Sources
1	*Aspergillus niger*	St. Jacques scallops
2	*Aspergillus flavus*	Bread
3	*Aspergillus ochraceus*	Pickles
4	*Penicillium expansum*	Apples

Oil spreading assay

Oil spreading assay was performed using the method described by Rodrigues et al. (7) to evidence the production of biosurfactants by lactic acid bacteria strain strain LCM2. A drop of engine oil 2T (www.bervas.ro) put in a Petri plate (100 mm diameter) with water formed a hydrophobic film on water surface where 20 µl of 48 h culture filtrate of the bacterial strain LCM2 in MRS was surface applied. The diameter of clear zone formed due to the presence of biosurfactant was measured as compared with a commercial detergent as positive control (Ct+) and distilled water as negative control (Ct-).

Emulsification activity of biosurfactant

The emulsification capacity of lactic acid bacterial strain LCM2 by biosurfactant production, as an extracellular compound was measured and the emulsification index (E24) was determined by adding equal quantities of kerosene and cell-free supernatant from MRS broth in test tubes, shaking at high speed by vortex and allowing for 24 hours overnight. Percentage of emulsification index was a ratio between the high of the emulsion layer and the high of total solution as calculated according to Cooper and Goldenberg (8).

Emulsification properties of biosurfactant from lactic bacteria strain were measured for estimating of its emulsification activity. The activity of biosurfactant in the process of emulsification was tested and assayed the ability to produce kerosene suspensions with various droplet sizes into an aqueous test system.

Ionic property of biosurfactant

Ionic charge of the biosurfactant produced by lactic acid bacteria strain LCM2 was observed on 1% agar plate when the precipitation lines appeared between pure compounds with a known ionic charge and the unknown biosurfactant. Sodium dodecyl-sulfate as anionic A(-) and dodecyl-dimethyl-ammonium chloride as cationic C(+) surfactants at a concentration of 0.02M were placed on both sides of a central well with the biosurfactant and after application were kept at room temperature for 48h (9).

Cell free suspensions obtained from bacteria strain culture filtered through 0.22 µm membrane filter (Fioroni) were dialysed and after freeze-drying, the biosurfactant was purified in silica gel column eluted with gradient of chloroform and methanol ranging from 20:1 to 2:1 (v/v). The structural characterization was carried out by thin layer chromatography (TLC), according to the method described by Sharma et al. (10).

Antifungal activity of LAB strain on fungal growth and biofilm formation

Biosurfactant (2.5 mg ml^{-1} concentration) from 48 hours culture of lactic acid bacteria strain LCM2 was added in 100ml flasks with 50ml PD broth on fungal cultures of *Aspergillus niger, Aspergillus flavus, Aspergillus ochraceus* and *Penicillium expansum* (10^6 spores ml^{-1}) and monitored for a period of 14 days for biofilm development. Dry weight measurements of fungal biomass were used for direct quantification of influence of biosurfactant produced by lactic acid bacteria strain LCM2 on the fungal biofilms. The evolution of fungal biofilm was monitored for morphological appearance and sporulation, too.

Percent inhibition was calculated using formula: R_1-R_2/R_1 where, R1 represent dry weight of test fungus in control and R2 represent dry weight of fungus accumulated in the presence of biosurfactant produced by LAB strain LCM2 (11). All assays were carried out in triplicate.

Statistical analysis

Results were interpreted by one-way analysis of variance (ANOVA), the value $p<0,05$ being considered statistic significant (Student test).

Results

Oil spreading assay

Dispersion zone diameter of engine oil 2T made by biosurfactant produced by lactic acid bacteria strain LCM2 was 24mm (**Fig. 1**).

Emulsification activity of biosurfactant

Emulsification assay was used as an indirect method to screen lactic acid bacteria for biosurfactant production. It was assumed that the cell-free lactic acid bacterial culture filtrates in MRS

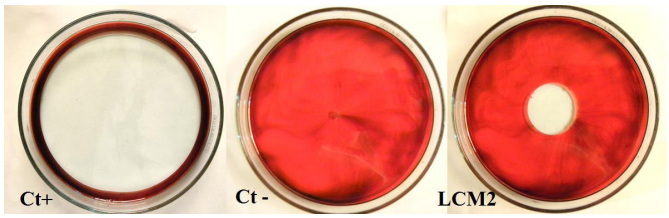

Figure 1. Clear dispersion zone of engine oil 2T, formed by the biosurfactant produced by lactic acid bacteria strain LCM2 compared with commercial detergent (Ct+) and distilled water (Ct-).

broth used for evaluation contains biosurfactants which will emulsify the hydrocarbons introduced in the test tubes. LCM2 strain showed intense positive emulsification activity when Kerosene was used as the hydrophobic substrate in the test.

The results of emulsification activity analysis showed that biosurfactant produced by LCM2 strain had a high emulsification activity (emulsification index E24 of 89.04%).

The result of emulsification process by biosurfactant ability to form emulsions in certain conditions was related to the droplet size. It is well-known that the droplet size is inversely proportional with the emulsification activity.

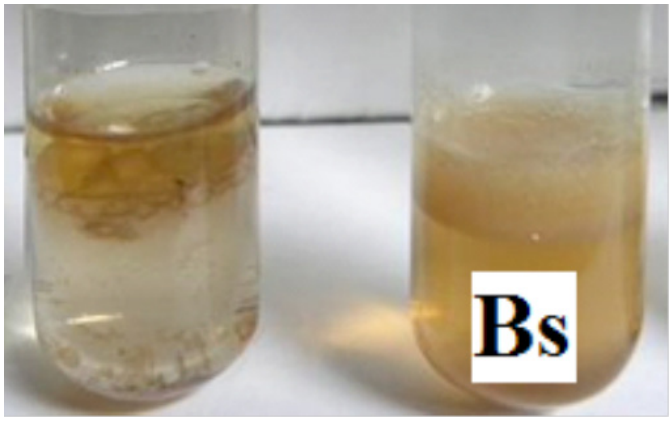

Figure 2. Emulsification pattern for biosurfactant (Bs) producing strain LCM2 of lactic acid bacteria.

Lactic acid bacterial strain LCM2 presented a great ability to release biosurfactants able to produce droplets with small size and acted as a good disperser of the kerosene (**Fig. 2**).

The emulsification activity of biosurfactant remained at the same intensity up to 7 days, as observed by the aspect of droplets layer.

Ionic property of biosurfactant

Assay of the ionic charge of biosurfactants produced by the strain LCM2 revealed the formation of precipitation lines towards the cationic surfactant (dodecyl-dimethyl-ammonium chloride) and established the anionic charge of this biosurfactant (**Fig. 3**).

After dialysis, the biosurfactant was purified in silica gel column eluted with gradient of chloroform and methanol and the structural characterization by TLC revealed its glycolipidic nature.

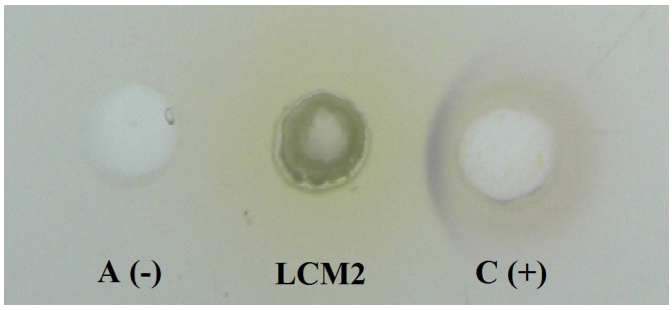

Figure 3. The anionic charge of biosurfactant produced by LAB strain LCM2; C(+) = dodecyl dimethyl ammonium chloride and A(-) = sodium dodecyl sulfate.

Antifungal activity of LAB strain on fungal growth and biofilm formation

Effect of biosurfactant produced by LAB strain LCM2 on fungal growth and biofilm developed by *Penicillium* and *Aspergillus* isolates was analyzed by quantitative changes in biomass accumulation, in conditions of fungal development on culture broth in the presence of biosurfactant.

Biomass accumulation measurements revealed that the strain LCM2 influenced the processes involved in the formation of fungal biofilm, causing an inhibition of growth and of mycelia development in the test fungi. The biosurfactant obtained by LAB strain LCM2 presented the highest antibiofilm activity against *Aspergillus ochraceus* (by 79.87% reduction of dry weight of biomass accumulated) and *Penicillium expansum* (by 76.43% reduction of dry weight of biomass accumulated). The weakest anti-biofilm activity of biosurfactant was reported in the case of *Aspergillus niger* (**Fig. 4**).

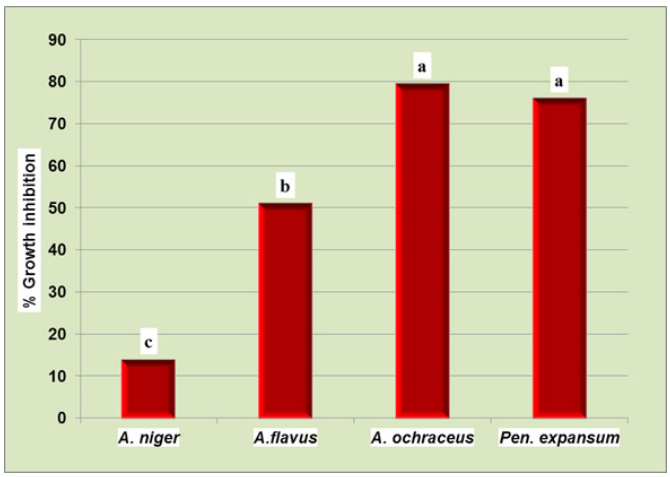

Figure 4. Inhibition of fungal growth and biofilm formation by LAB strain LCM2. Values followed by the same letter are not significantly different for $p<0.05$ (Student test).

The images revealed various morphologic changes of biofilm aspect induced by the presence of the biosurfactant produced by LAB strain LCM2 (**Fig. 5**) as compared with fungal biofilm at control variants and also, the different sporulation capacity of the fungal species as influenced by biosurfactant.

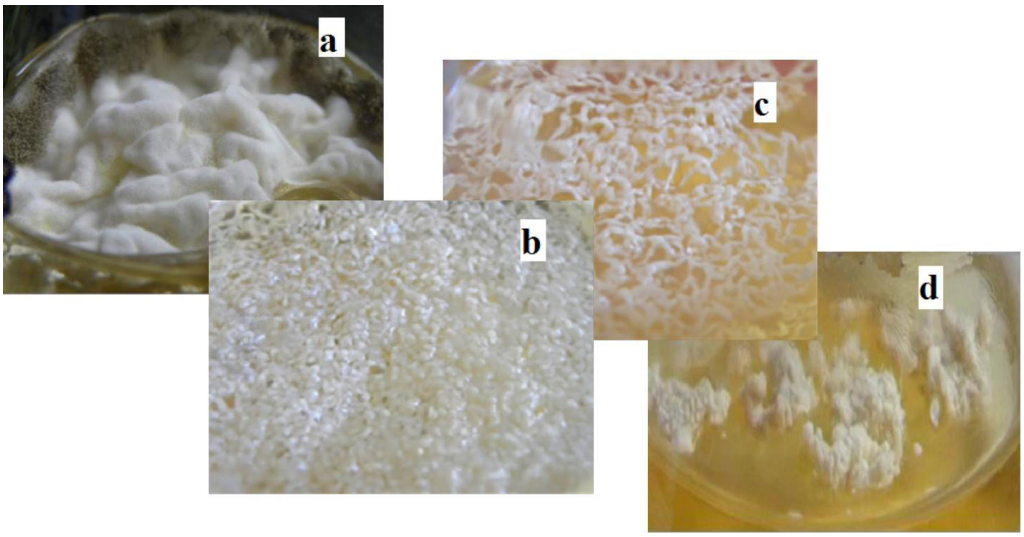

Figure 5. Influence of biosurfactant on biofilm of *Aspergillus niger (a), Aspergillus flavus (b), Aspergillus ochraceus (c), Penicillium expansum (d).*

Results of *in vitro* assay of antibiofilm activity of LAB strain against fungal species are synthesized in the **Table 2**.

Table 2. Antibiofilm activity of LAB strain LCM2

	Fungal isolates			
	A.niger	*A.flavus*	*A.ochraceus*	*P.expansum*
LAB strain LCM2	±	+	+	+

(+) inhibition of fungal biofilm; (±) weak inhibition of fungal biofilm

The influence of biosurfactant on fungal biofilm of *Aspergillus niger* consisted in weak delay in sporulation comparatively with control, visible in the first three days but not later and with non-significant effect on biomasss accumulation and adherence to jar walls, as mentioned above.

The biofilm of *Aspergillus flavus* presented modifications of hyphal net morphology, with small gaps visible at 5 days, swollen hyphae and delay in sporulation until the 14th day.

The most important changes of biofilm aspect induced by the biosurfactant were recorded for *Aspergillus ochraceus* and *Penicillium expansum*. Large gaps were formed in mycelial net, the biofilm adherence to jar walls was weak, the hyphal layer was thin and scarce traces of sporulation appeared after 21days only in *Penicillium expansum*.

Discussion

The results of the assays carried out demonstrated the emulsification activity and effectiveness of antifungal activity of anionic biosurfactant-producing LAB strain LCM2.

Other LAB strains were reported as anionic biosurfactant producers as in the case of *Enterococcum faecium* strain MRTL9 producing glycolipid biosurfactant (10) and *Lactococcus lactis* producing xylolipid biosurfactant (12).

Results from literature reported formation of clear dispersion zones with diameters variable as a function of species of microorganism producing the biosurfactant or the concentration used in the assay (13).

Literature showed various values of E24 index (from 7.8% to 63.3%. %) for bacteria strains able to produce biosurfactants, a good emulsification activity being correlated with maintaining stable emulsions with small droplets over 7 days (14), similarly with our results.

Recent work on supernatant culture of *Pseudomonas aeruginosa* strain PG1 reported E24 index values between 83% and 100% when assayed against various hydrocarbons, maximum emulsification activity of biosurfactant being registered against crude oil and a high antibacterial and antifungal activity, too (15).

Biosurfactant properties and the dispersion process could be influenced by genetic potential of the strain but, also, by non-biological factors such as: ionic charge of bio-surfactant, temperature, pH, the organic or aqueous phase composition (5).

Our results are in concordance with data from research carried out on LAB strains showing that 16% of investigated bacteria from genus *Lactobacillus* produced antifungal compounds active against *Aspergillus, Penicillium, Fusarium, Trichoderma* and *Stemphylium* (16).

Similar results were reported for biosurfactant-producing *Lactobacillus acidophilus* strain that inhibited over 59% biofilm formation in bacteria strains of *Shigella* and *Klebsiella* (17).

Other research (18) reported that fungal growth of *Aspergillus nidulans* was also affected by co-cultivation in liquid media with *Lactobacillus plantarum* MiMAB393 when 36% of the control dry weight was determined and morphological changes in mycelia (vacuolization, disturbed hyphal branching and swollen tips).

Present work evidenced that the new biosurfactant-producing LAB strain LCM2 exhibited antibiofilm activity

by inducing changes in biomass accumulation, adherence to surfaces and recommend it for utilization against mycotoxigenic fungi from genera *Penicillium* and *Aspergillus* contaminants of food products.

Conclusions

The strain LCM2 presented high emulsification ability (E24=89.04%) by releasing biosurfactant with anionic charge, able to disperse engine oil 2T and to form clear zone (24 mm diameter).

Preliminary research on structural characterization of biosurfactant revealed its glycolipidic nature.

Biosurfactant-producing LAB strain LCM2 presented antifungal activity against mycotoxigenic fungal isolates from genera *Penicillium* and *Aspergillus*.

Antifungal effect included dry weight loss, antibiofilm effect with low adherence capacity, structural damages of the hyphal net, conidiophores and delays or lack of sporulation.

Results of *in vitro* assays recommend the biosurfactant produced by lactic acid bacteria strain LCM2 for biotechnological purposes, as alternative antifungal agent in food industry or in various bioremediation processes.

Acknowledgements

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNDI-UEFISCDI, project number PN-III-P2-2.1_PTE-2016-0084, (40-PTE), acronym ECOREMTEH.

Conflict of Interest Statement

The authors declare that there is not any conflict of interest and that they alone are responsible for the content and writing of this article.

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