Screening of Philippine Actinomycetes for Biosurfactant Production

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Abstract

Biosurfactant are non-toxic and biodegradable surface active agents that can replace petroleum-based surfactants used as emulsifier, dispersants and foaming agents. In this study, twenty-eight (28) actinomycetes isolated from volcanic soil samples and distillery effluent, sludge, mud press and coco dust were screened for the production of extracellular biosurfactant. Preliminary screening was done by lipase assay and drop collapse test and showed that 21 isolates are potential biosurfactant producers. Emulsification activity (E_{24}) of the isolates positive for the preliminary tests were determined wherein several isolates exhibited high emulsification activities ranging from 43.58% -- 47.14%. This study implies that actinomycetes have a potential in biosurfactant production and can be further studied for optimization and industrial scale production.

Introduction

Surfactants are amphiphilic compounds that have the ability to lower the surface tensions between two immiscible compounds. These have hydrophobic and hydrophilic moieties, being used as emulsifiers and foaming agents as well as dispersants and wetting agents[1]. Surfactants have important pharmaceutical applications such as medium for the preparation of water and oil soluble drugs, active ingredient in soaps[2] and in the food industry primarily as foam or anti-foam agent[1]. Surfactants having these vast applications in various multimillion industries, however, are mainly derived from petroleum and are mostly toxic and non-degradable.

Biosurfactants from bacteria and yeast are good alternatives to address the deleterious effects to the environment of petroleum/chemical-based surfactants. In microorganisms, these compounds are produced in order to utilize poorly accessible substrates specifically water insoluble carbon sources such as hydrocarbons[3]. Like petroleum-based surfactants, biosurfactants are capable of reducing interfacial tensions and producing foams and emulsions. However, unlike the chemically synthesized surfactants, biosurfactants have high biodegradability, low toxicity, multi-functionality, and environmental capability making these compound a promising substitute for industrial and domestic applications[4]. Moreover, biosurfactants are known to have high activities at extreme temperatures, pH, and salinity; and can be produced at a cheaper cost using renewable substrates[5]. Thus, the production of biosurfactant having its potential multimillion dollar applications and environment compatibility, is now an emerging interest in the field of research.

In this study, biosurfactant from local actinomycetes isolates isolated from volcanic soil samples and distillery effluent, sludge, mud press and coco dust were screened for the presence of biosurfactant via lipase plate assay and drop collapse test. The emulsification index (E_{24}) of the isolates were measured in order to determine promising biosurfactant-producing actinomycetes with potential for industrial scale production.

Methodology

Sampling and Isolation of Actinomycetes

Sludge, mud press, coco dust, and effluent samples from an alcohol distillery plant in the province of Tarlac and volcanic soil samples from different barangays in Albay were collected. Isolation and enumeration of actinomycetes were done by serial dilution and spread plating on starch casein agar (SCA) (soluble starch 1%, casein 0.03%, KNO_3 0.2%, $MgSO_4 \cdot 7H_2O$ 0.005%, NaCl 0.2%, $CaCO_3$ 0.002%, agar 1.8%). The plates were incubated at 30 °C for 7 to 10 days. The cultures were observed and isolated colonies that have the characteristics of actinomycetes being rough and elevated or embedded were picked and streaked to new SCA plates. The isolated actinomycetes were also gram stained and observed under the compound light microscope (Olympus Biological microscope CX-40, Olympus Corporation, Tokyo, Japan) (10X magnification) to check for mycelia formation and purity of the isolates.

Lipase Plate Assay. Isolates were streaked on Luria Bertani agar (LBA) plates supplemented with 1% Tween 80 and 0.01% CaCl, The plates were incubated at 37 °C for 5 days and the presence halo of Ca-free fatty acid precipitates around the colonies were observed[6].

Production of Biosurfactant. Isolates were inoculated in glucose yeast extract (GYE) (peptone 1%, NaCl 0.5%, glucose 1%, yeast extract 0.3%) broth with 1 mL of spore suspension (0.25 OD) of the actinomycetes isolates, and incubated at ambient temperature (30 °C) at 150 rpm shaking for 7 days. The broth cultures were centrifuged at 4,000 rpm for 10 min to remove the cells and the resulting supernatant were used for the screening of biosurfactant production.

Drop Collapse Test. A micro titer plate with 96 wells were coated with 5 uL Pennzoil motor oil (USA). A droplet of supernatant (5 uL) was then placed to each of the wells and were observed after 1 minute[7]. Droplets that collapsed, which got bigger or flat, were positive for biosurfactant production.

Emulsification Activity. Equal volume of kerosene and supernatant (3mL) were added in a dram vial and mixed using a high speed vortex mixer for 2 minutes[8]. The vials were allowed to rest for 24 hours and the resulting emulsion layers were measured using a digital caliper. Emulsification activity of the biosurfactant was expressed as emulsification index (E_{24}) computed as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm).

Results

The isolated actinomycetes were screened for biosurfactant production by lipase plate assay, drop collapse test, and emulsification index (E_{24}) (Table 1).

Table 1. Screening for biosurfactant production of actinomycetes isolates using three tests: lipase plate assay, drop collapse test, and emulsification index of isolated actinomycetes.

Actinomycetes Isolate	Lipase Plate Assay	Drop Collapse Test	Emulsification Activity
CADT 1	+	+	+
CADT 2	+	+	+
CADT 3	+	-	-
CADT 4	+	+	+
CADT 5	+	+	+
CADT 6	+	+	+
CADT 7	+	+	+
CADT 8	+	+	+

CADT 9	-	+	+
CADT 10	+	+	+
CADT 11	+	-	+
CADT 12	-	+	+
APJ 1	-	+	+
APJ 2	+	+	+
APJ 3	-	-	+
APJ 4	+	+	+
APJ 5	-	-	+
VAA 1	-	+	+
VAA 2	+	-	+
JSM 8	-	-	-
CGS A9	+	+	+
CGS B11	+	+	+
BDY A4	+	+	+
CGS A10	+	+	+
UNO B2	+	+	+
BDY B13	+	+	+
PURO B2	+	+	+
UNO C14	+	-	-

The following figures show the actual images of actinomycetes isolates (Figure 1) as well as their ability to form Ca-fatty acid precipitates (Figure 2) and collapsed drops (Figure 3) indicating biosurfactant production as supported by their emulsification activities (Figure 4).

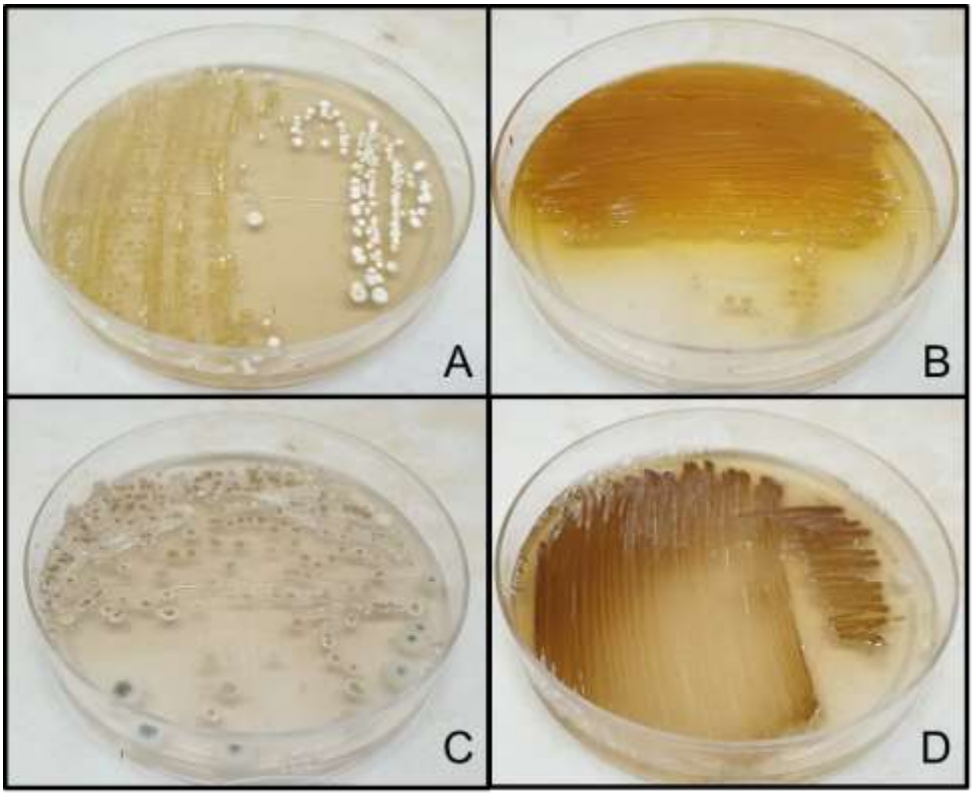

Figure 1. Some of the isolated actinomycetes from soil compost, sludge, mudpress, coco dust, slops, waste water and effluent samples. A) CGS A10, B) CADT 1, C) VAA 1, D) CADT 11.

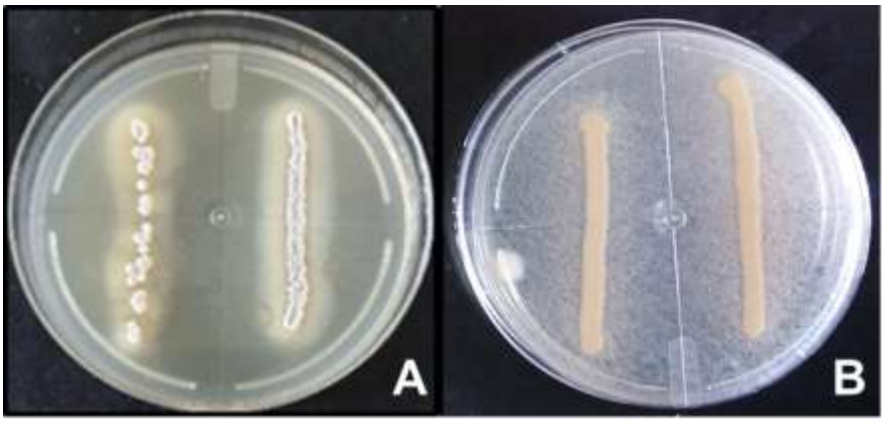

Figure 2. Lipase plate assay. A) CGS B11, B) Negative control. Opaque halo are precipitates from free fatty acid complexed with calcium indicating the presence of biosurfactant.

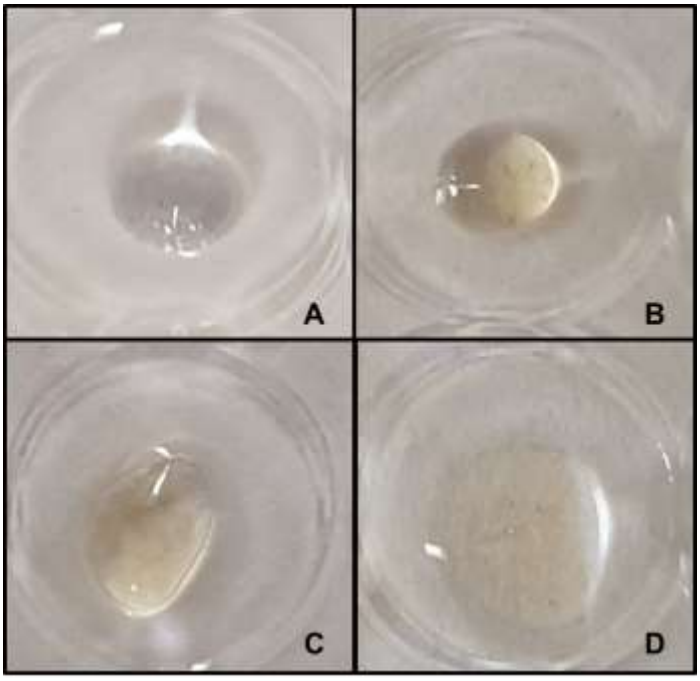

Figure 3. Drop collapse test. (A) Water, (B) Broth medium, (C) Biosurfactant, (D) Tween 80 (chemical surfactant)..

Figure 4. Biosurfactant activity forming emulsion layer. A) Negative, B) Positive, stable emulsion.

Discussion

A total of 28 actinomycetes were isolated and purified from soil and distillery samples. Colonies grown on starch casein agar (SCA) were characterized as rough in texture with varying colors and were flat or embedded having white to black aerial spores. Most of the isolates had extensive formation of mycelia as observed under the compound light

microscope (10X magnification) and were gram positive. The isolates were observed to have an earthly smell due to production of geosmin[9], which is a known characteristic of actinomycetes.

Luria Bertani (LB), which is a carbon limited medium[10] was used for lipase plate assay. The addition of 1% polyethylene sorbitan monooleate (Tween 80) allows lipase producing isolates to utilize this anionic surfactant as a major carbon source. Consequently, the free fatty acids from the hydrolysis of Tween 80 can form insoluble complex with the calcium ions present in the medium as shown in Figure 2. This complex can be observed as opaque halo regions surrounding the colonies. Previous study emphasized the significance of lipase and biosurfactant producing bacteria with useful applications in the cosmetics, pharmaceutical, agriculture and food industries[11]. As indicated in Table 1, a total of 21 isolates had positive result for lipase plate assay.

Biosurfactants are low molecular weight glycolypids or lipopeptides, which are amphiphatic in nature and capable of dissolution in polar and non-polar compounds[12]. These amphiphatic compounds are known to reduce surface and interfactial tensions between non-identical phases. In microbiological systems, these compounds solubilize hydrophobic molecules such as in oil contaminated soil and water and also increase cell surface hydophobicity for easy uptake of hydrophobic substrates[13]. In the drop test, the isolated actinomycetes were tested qualitatively for its ability to reduce or lower surface tension. In this test, a droplet of cell free supernatant was added in each of the wells of an oil-coated microtiter plate. Collapsed droplets signified lowered surface tension and positive for biosurfactant production while intact droplets (drop beaded up) implied the absence of biosurfactant. Biosurfactant-producting isolates had droplets size that were bigger compared to water (negative control) and comparable with Tween 80 (positive control) as illustrated in Figure 3. Addition of the biosurfactant and Tween 80 caused the drops to spread due to the lowering of surface and interfacial tension between the oily surface and the water. On the other hand, the drop lacking surfactant remains beaded because the oily surface is hydrophobic and therefore the force causes aggregation. A total of 21 isolates showed positive results for drop collapse test as indicated in Table 1.

Biosurfactants being amphiphatic are also known for their ability to form stable emulsions of immiscible liquids through the formation of micelles. These compounds are capable of emulsifying even at very low concentrations having high kinetic stability[13]. In bioremediation, biosurfactant play a major role in extracting oil contaminants by tightly binding to the hydrocarbons for easy removal[14]. In the determination of emulsification activity, emulsions were primarily formed by the binding of kerosene to the hydrophobic moiety of the amphiphilic biosurfactant molecules that in turn form micelles. Emulsification activity varied among the actinomycetes isolates used in this study (data not shown). The emulsification indices were used as basis to choose the best isolate for the optimization of biosurfactant production study . However, it is to note that the consistency of the emulsions were also considered as to being loose, compact or intense. Isolates that had dense emulsions (intense) were considered as better compared to those emulsions containing few (compact) to many (loose) bubbles.

Isolate CGS B11 was chosen to be the best biosurfactant producing actinomycetes, having an emulsification index of 43.58% with intensely dense emulsion consistency. Moreover, CGS B11 had comparatively the fastest observable growth among the isolates that gave intense emulsions being able to grow in GYE standard medium at about 48 hours. Although some isolates such as CGS A10 (E_{24}=46.43%) and BDY B13 (E_{24}=45.71%) had higher intense emulsions, CGS B11 was still chosen because of its faster growth and ability to form finer cells and inoculum size can be easily standardized. With these considerations, CGS B11 is the best isolate for biosurfactant production study to done in the future. Despite the differences in the E_{24}, all the emulsions were stable at room temperature wherein there were no changes in E_{24} after several hours. Emulsions of this type are applicable in the food, pharmaceutical, agrochemical, personal care and cosmetics industries. There is a rising application of emulsion in food processing industries where emulsions are used to encapsulate, deliver, and protect food components such as oil-soluble flavors, vitamins, colorants, preservatives, and other bioactive ingredients[15].

Conclusion

Based from the results of the biosurfactant screening experiments, twenty-one (21) actinomycetes isolates were determined to be biosurfactant producers. The isolated actinomycetes had varying emulsification activities wherein several isolates showed emulsification indices of 47.14% - 43.58%. These isolates are promising isolates for optimization studies that can be applied in large industrial scale production of biosurfactant.

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