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Selection of Microbes Producing Biosurfactants in Media without Hydrocarbons

CATHERINE N. MULLIGAN, DAVID G. COOPER, and RONALD J. NEUFELD

Biochemical Engineering Research Unit, McGill University, 3480 University Street, Department of Chemical Engineering, Montreal, PQ, Canada H3A 2A7

Microorganisms capable of synthesizing biosurfactants on non-hydrocarbon substrates were screened by their hemolytic activity on blood agar plates. Hemolytic zones around colonies could be related to the ability of the microbes to produce surfactants. The degree of clearing caused by surfactin, a biosurfactant from *Bacillus subtilis*, was proportional to the concentration applied. Microorganisms capable of producing biosurfactants on hydrocarbon-containing media only did not demonstrate lytic activity. The technique of using blood agar plates to screen for biosurfactant production on soluble substrates is shown to be quick and reliable.

Most of the known microbial surface active agents originate from bacteria growing on hydrocarbons or related substrates.[1–6] There are a few exceptions, such as the lipopeptide surfactin from *Bacillus subtilis*, produced on water-soluble substrates.[7–9] In spite of the temporary reduction in the price of crude oil, it is essential to isolate organisms which can assemble biosurfactants from cheap, renewable substrates if production is to be commercially feasible.

Attempts have been made to screen for organisms which synthesize surfactants during growth on soluble substrates or to induce surfactant production, on these substrates, from microbes known to synthesize surfactants from hydrocarbons.[3,10,11] This work has been slow because there was no quick method to evaluate which organisms were potentially useful for this purpose. It was necessary to grow each microbe for several days on the chosen medium and then test for surface activity. Similarly, experiments for strain improvement of biosurfactant producers are not feasible until a method is developed to select improved clones.

The use of blood agar plates to characterize certain hemolytic bacteria is well known.[12,13] These plates also provide a rich growth medium for many organisms. It has already been shown that surfactin, although it is not a hemolytic enzyme, can rupture erythrocytes in liquid media.[8]

Materials and Methods

The organisms were grown in media which have been described elsewhere. These included *Bacillus subtilis*,[9] *Corynebacterium lepus*,[2] *Corynebacterium fascians*[10] *Corynebacterium insidiosum*[1] and *Torulopsis bombicola*.[5]

In the screening studies, bacteria were isolated from environmental sources by inoculating a medium consisting of 4% glucose, 0.1% yeast extract, 0.1% nutrient broth, 0.4% NH_4NO_3, 0.4% KH_2PO_4, 0.4% Na_2HPO_4, and trace amounts of $MgSO_4$, $CaCl_2$, $FeSO_4$, and Na_2 EDTA. After 2 days growth, these mixed cultures were used to inoculate sheep blood agar plates obtained from the Institute Armand Frappier, Laval, Quebec. The plates were incubated for 2 days at 25°C. Single colonies were used to inoculate fresh media containing 4% glucose, 0.1% yeast extract, and the mineral salts.

Surface tensions were determined using a Fisher Autotensiomat. Surfactin was isolated from *B. subtilis* and purified using previously published methods.[7] Diffusion of surfactin through the agar was studied by placing aqueous solutions into a well in the blood agar made with a 10 μl micropipette.

Results

Spreading dilute cultures of *B. subtilis* on blood agar and incubating for 2 days resulted in colorless rings around the colonies. Typ-

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ically these were about twice as wide as the diameter of the colonies.

Figure 1 illustrates the results of adding various concentrations of aqueous solutions of surfactin to narrow wells punched into the agar. The maximum diameter was reached 4 h after the addition of the solution. As the concentration of surfactin increased the diameter of the resulting cleared area also increased.

Three other bacteria, *C. fascians*, *C. insidiosum*, and *C. lepus*, could all be induced to grow on the blood agar but caused no lysis of the red blood cells. All three bacteria produce significant quantities of biosurfactant when grown on hydrocarbon media but do not produce these products on carbohydrate media.

The yeast *T. bombicola* was also unable to clear the agar. This organism produced copious amounts of glycolipid surfactant on media containing carbohydrate and vegetable

Table 1 Results of experiments to isolate biosurfactant-producing organisms.

Source of[a] organism	Ability to cause clearing	Surface[b] tension (mN/m)	Colony morphology	Gram stain	Microscopic[c] observations
A	−	72	smooth, yellow	+	motile, spores
A	−	68	smooth, beige, small	−	non-motile
B	−	68	white, small	+	non-motile, cocci
C	−	68	smooth, bright yellow	+	motile, short chains
D	−	67	rough, beige, irregular	−	motile
B	−	63	dark yellow, small	+	small, non-motile
B	−	63	flat, clear	+	non-motile
E	+	56	rough, beigh, small	+	non-motile, spores, short chains
F	+	55	smooth, yellow	+	non-motile, short chains
D	+	55	white, large	+	motile
A	+	55	pale-yellow, small	+	non-motile, spores, short chain
G	+	48	dull, beige	+	non-motile, spores, paired
G	+	45	biege, irregular	+	non-motile, spores
D	+	43	dull, white, flat	+	motile, spores small
D	+	40	large, white, irregular	+	motile, irregular shape
C	+	38	smooth, yellow	+	motile, small
B	+	37	smooth, white, small	+	non-motile, spores, short chains
D	+	32	smooth, beige, flat	+	motile, spores short chains
A	+	29	irregular, white large	−	motile

[a] Sources include; A, water from a peat bog; B, neopeptone solution exposed to air in laboratory; C, industrial sewage sludge; D, Athabasca tar sand; E, diseased leaf from *Crassula* species; F, oil from aeration basin; G, bark used as potting medium for orchid species.
[b] Surface tension of liquid media inoculated with the colony.
[c] All rod-shaped unless noted otherwise.

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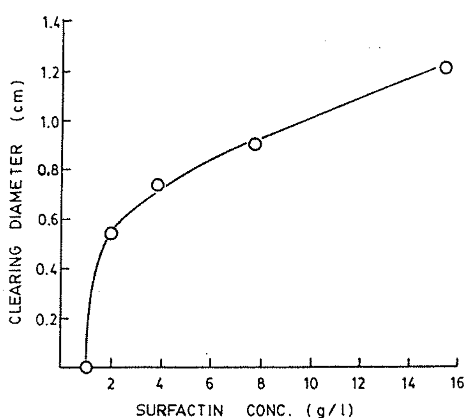

Fig. 1. A plot of the diameter of the clear zone *vs* the concentration of the surfactin solution added to the well in the blood agar.

oil but not on carbohydrate alone. The glycolipid was isolated from a fermentation using the appropriate medium and streaked on the blood agar. This compound was able to lyse the erythrocytes and cause clearing.

This method was used for a preliminary screening of a number of organisms from a variety of sources for the ability to produce biosurfactants on non-hydrocarbon media. Table 1 contains data for a number of typical isolates. The table includes results for all of the clearing colonies and some of the non-clearing colonies. Seven of the colonies which did not cause clearing were tested for surfactant production. In each case, there was no surfactant produced and the surface tension remained above 60 mNm^{-1}. Twelve isolates, from a variety of sources, lysed the erythrocytes. Half of these were not promising as the surface tension measurements when cultured in liquid media ranged from 45 to 55 mNm^{-1}. In most cases, diluting the broth samples several fold caused the initial, low surface tension to increase. This suggests that the surfactant concentration was less than or near the critical micelle concentration (CMC). The other half of the isolates which caused clearing, produced effective biosurfactants and two of these resulted in very low surface tensions of 32 and 29 mNm^{-1}. Samples from the culture broth could be diluted 200-fold before an appreciable increase was observed in the surface tension, suggesting biosurfactant concentrations greatly in excess of the CMC.

Discussion

It has been shown previously that the lipopeptide surfactin isolated from *B. subtilis* can cause the lysis of red blood cells.[8] Inoculating blood agar plates with *B. subtilis* resulted in clear rings of lysed erythrocytes around each colony. If surfactin was added to wells in the blood agar the degree of clearing was proportional to the concentration of surfactin in the solution. Thus the lysis of the red blood cells was related to the production of a highly surface active compound by *B. subtilis*.

The Corynebacteria *C. lepus*, *C. fascians*, and *C. insidiosum* all require the presence of a hydrocarbon to produce significant quantities of extracellular surfactants.[1,2,10,11] Thus it was not surprising that none of these bacteria caused clearing of the blood agar plates. *T. bombicola* produces large amounts of glycolipid only if there is vegetable oil or related compounds in the growth media, and colonies on the blood agar did not cause clearing. If the surfactant was isolated from an appropriate fermentation of *T. bombicola* and put on the blood agar, this did result in clearing.

Thus clearing of the agar around a colony could be an indication that the organism was capable of producing appreciable quantities of biosurfactant on carbon sources other than hydrocarbons. It could also indicate a lytic enzyme, however the surfactant producers are easily verified by inoculating liquid media and measuring the surface tension after a few days.

This method was used for preliminary screening of a number of microorganisms from a variety of sources for the ability to produce biosurfactants on non-hydrocarbon media. Non-clearing colonies were incapable of biosurfactant production in liquid media while clearing isolates produced biosurfactants, some greatly in excess of the CMC.

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The CMC is the minimum amount of surfactant required to cause the maximum decrease in surface tension, and excess surfactant allows proportional dilution of the mixture without an increase in surface tension. Most of the biosurfactants were produced in low yields and diluting the samples showed that in most cases the concentration present varied from a few-fold excess of the CMC to insufficient biosurfactant to reach the CMC. The most promising exception to this was the organism which produced the most effective surfactant ($29\ mNm^{-1}$). In this case the concentration produced was 200 times the CMC.

The use of blood agar provides a quick preliminary screening technique for the selection of microorganisms capable of producing biosurfactants on renewable, water-soluble substrates. There is a correlation between the amount of surfactant and the amount of lysis (Fig. 1) and potentially this method could also be used to select for superior clones in mutation studies. This technique may also select organisms producing lytic enzymes but these are quickly eliminated by growing the microbes in liquid culture and measuring surface tension.

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