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NOTE

Quantitative Assay of Bioemulsifier by Turbidometric Method

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A quantitative method for assaying bioemulsifiers in culture broth was developed and applied to cultivation of *Pseudomonas aeruginosa* YPJ80. SED(Standard Emulsification Dilution), an indirect measure of bioemulsifier concentration, was proposed. Production of bioemulsifier and rhamnolipid reached their maximum simultaneously. However, the bioemulsifier /rhamnolipid ratio decreased with cultivation time. This indicates the presence of another bioemulsifier other than rhamnolipid. The bioemulsifier seems to be protein-like activator which showed emulsification activity in addition to rhamnolipid.

Some kinds of microorganisms are known to synthesize biosurfactants when growing on water-insoluble substrates to facilitate substrate uptake (9, 10). Biosurfactants, such as amphiphilic compounds, show various surface active properties including surface tension reduction, emulsification, detergency, foaming and wetting. Biosurfactants and bioemulsifiers can be assayed on the basis of these properties, particularly surface tension reduction and emulsification.

Many researchers have used CMC^{-1}, the reciprocal CMC (Critical Micelle Concentration), as an indirect measure of biosurfactant concentration (4, 6, 11, 13). CMC^{-1}, a dilution factor where surface tension abruptly increases, is determined from a plot of [surface tension of culture broth] vs. [log of dilution factor]. This indicates that the experimental error in determining CMC^{-1} increases exponentially as CMC^{-1} increases. In addition, surface tension is greatly affected by pH and ionic strength (15). However, pH and ionic strength can be kept constant only when dilutions are made with a sufficient buffer. Therefore, CMC^{-1} cannot be determined accurately in any case.

A few quantitative methods for measuring emulsification activity have been reported (3, 7, 8, 12, 16-20). However, few researchers have tried to assay bioemulsifiers in culture broth quantitatively.

In the present work, we developed a quantitative method for assaying bioemulsifiers in culture broth using a modified Rosenberg's emulsification test (18). SED (Standard Emulsification Dilution), which is an indirect measure of bioemulsifier concentration, was proposed and applied to bioemulsifier production by *Pseudomonas aeruginosa* YPJ80.

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The bacterial strain YPJ80 used in this study was isolated in our laboratory from the soil of a gas station. This strain was identified as *Pseudomonas aeruginosa*. The bacteria was stored at 4°C on plate count agar (Difco).

The medium contained (g/l): $(NH_4)_2SO_4$, 1; K_2HPO_4, 1.5; KH_2PO_4, 0.75; yeast extract, 1; $MgSO_4 \cdot 7H_2O$, 0.5; and 20 ml/l of the following trace elements: NaCl (5 g/l), $CaCl_2 \cdot 2H_2O$ (0.5 g/l), $MnSO_4 \cdot 5H_2O$ (0.5 g/l) and $FeSO_4 \cdot 7H_2O$ (0.5 g/l). The trace elements were dissolved in an acidic solution containing 1% (v/v) concentrated HCl. The solution was then added to the medium after separate sterilization. Glycerol as a carbon source was added to the medium at 2% (w/v). pH was then adjusted to 8.0 with 1 N NaOH. Growth was initiated with 2 ml inocula of late exponential-phase cultures into 20 ml of media in 250 ml flasks. Cultivation was carried out at 32°C with an agitation speed of 250 rpm.

The measurement of emulsification activity was based on Rosenberg's emulsification test (18). Hexadecane/2-methylnaphthalene (Sigma Chemical Co., U.S.A.) mixture (1 : 1 v/v) was prepared. The hydrocarbon mixture (0.1 ml) was added to 10 ml of 20 mM citrate-phosphate buffer (pH 5.4) containing an appropriate volume of the culture broth in a 50 ml flask. After reciprocal shaking (150 strokes per min) for 1 h at 25°C, the resulting emulsion was allowed to stand for 10 min. Its absorbance through 1 cm pathlength was then measured at 620 nm with a Hewlett Packard Spectrophotometer (HP8452, U.S.A.). The emulsification activity was expressed as the absorbance.

Rhamnolipid content was determined by multiplying

rhamnose content by a standard coefficient of 3. Rhamnose content was measured by the method described by Chandrasekaran and Bemiller (2). Protein was assayed using a BCA-1 kit (Sigma Chemical Co., U.S.A.). Ammonium concentration was measured with an Orion electrode (Model 95-12, U.S.A.). Cell growth was expressed as the absorbance at 660 nm.

Rosenberg *et al.* (18) developed a convenient and sensitive method for measuring emulsification activity. They diluted emulsions with water so that the final absorbances at 620 nm (1-cm light path) were to be between 0.245 and 1.212. Emulsification activities were then determined by multiplying the final absorbances by the dilution factors. However, the absorbance of an emulsion diluted with water by two times is not likely to be the same as half the absorbance of the initial emulsion before dilution. In order to confirm this hypothesis, an emulsion with an absorbance between 0.245 and 1.212 was prepared. As the emulsion was diluted with the buffer, the absorbances were measured (Fig. 1). Fig. 1 shows that the relationship between absorbances and dilution factors is approximately parabolic. This indicates that the emulsion tends to collapse with dilutions. It was reported that the absorbance of an emulsion diluted with water by two times was similar to that for an emulsion prepared with half the bioemulsifier concentration and half the amount of oil when the oil was perfectly emulsified in the aqueous phase (13). Therefore, in determining emulsification activity, dilutions with water must be made before emulsification not after.

Thus, in this paper, SED (Standard Emulsification Dilution), an indirect measure of bioemulsifier concentration, was proposed and defined as the dilution factor where the emulsification activity of the culture broth was one. SED is the analogue of CMC^{-1}. With the serial dilution of the culture broth, the emulsification activities were measured. It was found that emulsification activity up to about 1.1 was proportional to the reciprocal of dilution factor, or bioemulsifier concentration (Fig. 2). Therefore, dilutions of culture broth were prepared so that emulsification activity was to be between 0.2 and 1.1. SED was then calculated from the reciprocal dilution factor and the emulsification activity using the standard curve.

The bioemulsifier produced by *Pseudomonas aeruginosa* YPJ80 was assayed quantitatively using SED. Fig. 3 shows the time course of bioemulsifier and rhamnolipid production. Both bioemulsifier and rhamnolipid production increased substantially when nitrogen was exhausted, as observed by other researchers (1, 15, 17). Bioemulsifier and rhamnolipid concentrations reached their maximum simultaneously. However, bioemulsifier / rhamnolipid ratio decreased dramatically from 0.29 to 0.20 during early incubation (18 h) and decreased gradually to 0.12 thereafter. On the other hand, extracellular protein concentration increased dramatically during early incubation (18 h) and increased gradually thereafter. This implies that extracellular protein plays an important role in emulsification. It may act as bioemulsifier itself or as a cofactor for emulsification. It was reported that *Pseudomonas aeruginosa* S7B1 synthesized the protein-like activator which showed emulsification activity in addition to rhamnolipid (5).

Generally, bioemulsifier concentration necessary for emulsification is significantly lower than CMC. SED is affected little by the pH and the ionic strength of culture broth because the culture broth is diluted sufficiently with the buffer. In addition to that, SED is calculated from linear plot so that it can be determined much more accurately than CMC^{-1}. Therefore, when the biosurfactants with emulsification activity (or bioemulsifiers) were to be produced, SED is a much more accurate and

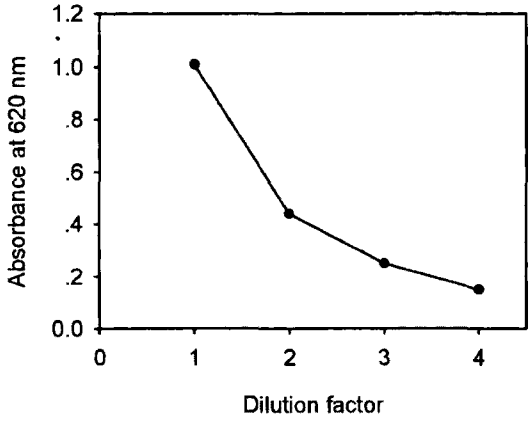

Fig. 1. Absorbances of an emulsion with dilution.
Dilution factor of one indicates the initial emulsion.

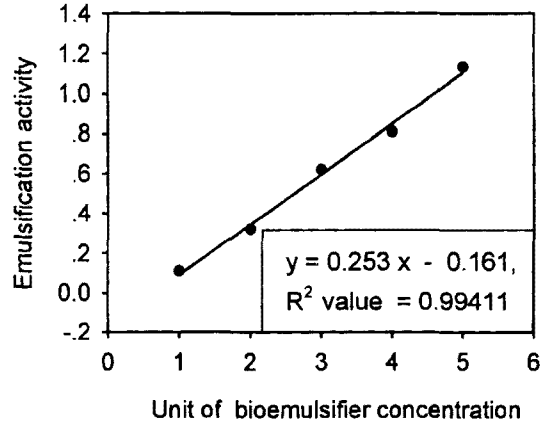

Fig. 2. Standard curve for emulsification activity and bioemulsifier concentration.
Bioemulsifier concentration was represented as reciprocal of dilution factor of culture broth which was rescaled to integer.

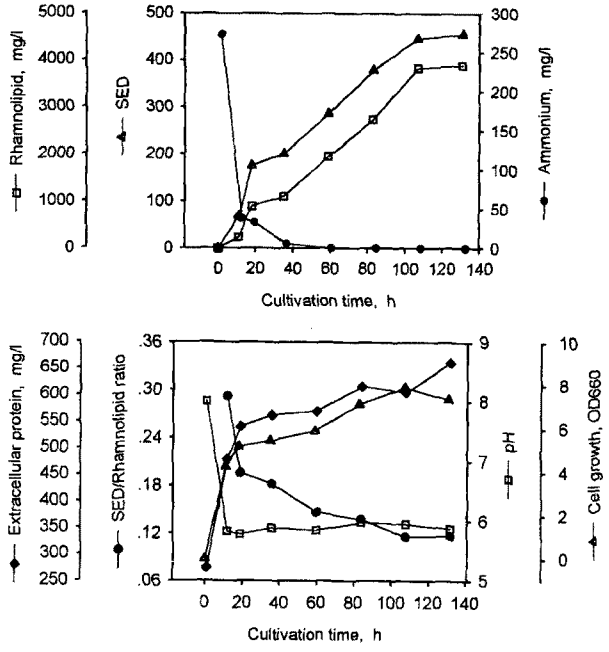

Fig. 3. Time course of bioemulsifier and rhamnolipid production.

effective measure of the bioemulsifiers concentration than CMC^{-1}.

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