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Production of Biosurfactant from Hexadecane by Crude Oil Utilizing Pseudomonas aeruginosa Strain K-3*

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ABSTRACT

Microbial growth on hydrocarbons accompanies the production of surface active agents which help in the dispersion of water immiscible substrate and its transfer into the cells. These biosurfactants having broad range of functional properties have great potential for emulsification, phase separation and viscosity reduction of heavy crude oils suggesting their probable use in enhanced oil recovery. Chemical nature of these biosurfactants varies with the substrate as well as the bacteria applied. Using one of the local isolate *Pseudomonas aeruginosa* strain K-3, capable of emulsifying crude oil from Khaskheli oil field within 2-3 days, and n-hexadecane as substrate, such surface active compounds were produced. Time course for the growth of this bacterium, pH changes and metabolite production were studied. Extracellular proteins, sugars, intracellular proteins and crude lipids were found to be growth associated. Neutral lipids increased during late growth whereas glycolipids were found to be growth associated. Phospholipids comprised only a minor portion and remained almost constant.

INTRODUCTION

In many hydrocarbon fermentations, hydrocarbons are emulsified by a phenomenon other than simple mechanical agitation. Emulsification results in the dispersion of small oil drops in the aqueous phase generating increased surface area, thus enhancing the microbial growth and metabolite production (Hisatsuka et al., 1971). Microorganisms while growing on hydrocarbons produce surface active agents or biosurfactants which emulsify the substrate, enabling its transfer into the cell (Goma et al., 1973).

The microbial oxidation of sparingly water soluble substrates is almost always associated with the production of biosurfactants (Cooper, 1986). Microbial biosurfactants are usually lipid in nature and can be grouped into different categories based upon their chemical composition (Parkinson, 1985). Glycolipids with different sugar moities comprise a major portion of the microbial biosurfactants in different bacterial species (Rapp et al., 1979; Hisatsuka et al., 1971; Itoh and Suzuki, 1974).

The potential application of microbial surface active compounds (biosurfactants) is based upon their broad range of functional properties such as emulsification and de-emulsification, phase separation, wetting, foaming, surface activity and viscosity reduction of heavy crude oils (Finnerty and Singer, 1984a). These properties suggest their probable use in enhanced oil recovery processes (Kosaric et al., 1983; Finnerty and Singer, 1984b). In the previous studies, *Pseudomonas aeruginosa* strain K-3 was found to be a bacterium of choice for biosurfactant production (Kokub et al., 1990). The present study was carried out for determining the time course for the growth, pH changes and production of different components of biosurfactant during growth of a *Pseudomonas aeruginosa* strain K-3, on a known hydrocarbon: n- hexadecane. These metabolites are being reported as extracellular proteins, sugars, intracellular proteins and crude lipids. Crude lipids were further fractionated into neutral-, glyco- and phospholipids and their growth relation was also studied.

MATERIALS AND METHODS

Microorganism

Pseudomonas aeruginosa strain K-3, isolated previously from oil contaminated soil samples (Kokub et al., 1990) was used for the production of biosurfactants.

Inoculum

A single colony of K-3 grown on nutrient agar plate, was transferred to nutrient broth and incubated on a rotary action shaker (100 rpm) at 30°C. After 48 hours of incubation the cells were separated by centrifugation at 12,000 rpm for 15 minutes. The cells were suspended in n-saline and an optical density (OD) of 0.62 was set against blank (n-saline) at 590 nm, and was added at the rate of 1% of the mineral medium in each flask as inoculum.

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Shake Flask Experiment

Bacterial growth and metabolite production especially extracellular lipids were studied in 1 litre Erlenmayer flasks containing 250 ml of BH medium (Bushnell and Haas, 1941) and 0.5% (v/v) n-hexadecane. A set of two flasks for three incubation periods i.e., 7, 14 and 21 days were reserved for the extraction of crude lipids. Whereas, another set of control and inoculated flasks were labelled as the sampling flasks and incubated at 30°C with 100rpm shaking for 22 days. Growth in terms of Colony Forming Units per millilitre (CFU/ml) was determined by spread plate method (Sharpley, 1966) and changes in the pH of the culture broth were also monitored by a pH meter (Corning-130) after every 48 hours of incubation upto 22 days.

Extracellular Metabolic Products

Ten millilitre of the culture broth was taken from the sampling flasks after every 48 hours during 22 days of incubation and was analyzed for different metabolic products. Cell free extract (supernatant from the centrifuged sample) was used for analyses of extracellular proteins (Lowry et al., 1951) and sugars (Spiro et al., 1966). The cell pellet was washed with sterile water, centrifuged and then resuspended in distilled water. A known portion of this suspension was used for biomass estimation (Guerra-Santos et al., 1984) and other portion after hydrolyzing with NaOH, was used for intracellular proteins (Lowry et al., 1951).

Isolation of Crude Lipids

A set of two flasks was removed from the shaker at the end of each incubation period i.e., 7, 14 and 21 days and the residual hydrocarbon was removed by solidifying the spent culture medium at 4°C for separating the solid hydrocarbons by filtration. The spent medium was then centrifuged at 12,000 rpm for 15 minutes to remove the cells and extracted with two volumes of chloroform: Methanol (2:1) mixture. The organic solvent was evaporated by using rotary evaporator at 30°C under vacuum and further dried to constant weight by flushing with a stream of nitrogen. This organic crude extract was referred as the crude lipids (biosurfactants).

Silicic Acid Column Chromatography

The crude lipids were fractionated into different lipid classes by silicic acid column chromatography (Finnerty and Singer, 1984a). Twelve grams of activated silicic acid (325 mesh) in 30- 40 ml chloroform was poured into 1x24cm glass column (Kates, 1972). Crude lipid extract re-dissolved in 2 ml chloroform was layered on the top of the column and eluted sequentially with chloroform, acetone, and methanol, to collect the neutral lipids, glycolipids and phospholipids respectively. These three fractions were concentrated on a rotary evaporator and dried to constant weight by flushing with a stream of nitrogen.

RESULTS AND DISCUSSION

Bacterial Growth (CFU/ml) on Khaskheli Crude Oil

Pseudomonas aeruginosa strain K-3, in pure culture emulsified 0.5% (v/v) Khaskheli crude oil in BH-medium under shaking (100 rpm) at 30°C. The emulsification of crude oil after 3 days of incubation in comparison to control is shown in (Figure 1), which reveals the possible use of this strain for biosurfactant production. Strain K-3 showed no lag phase in utilizing Khaskheli crude oil (Figure 2) probably because it was isolated from oil contaminated soil sample and may have adapted to utilize crude oil as carbon source. The absence of lag phase might be due to the catabolism of oil by direct enzyme induction (Van Eyk and Bartels, 1968).

Bacterial Growth (CFU/ml) on n-Hexadecane

The bacterial strain K-3, when grown on n-hexadecane, exhibited an increase in viable cells with time as shown in (Figure 2). But it remained in lag phase for six days, after which a logrithmic increase in viable cells was observed (log phase). This prolonged lag phase might be due to the fact that during initial stages of growth the cells remained attached to the hydrocarbon phase and since viable counts were determined from the aqueous phase, the increase in viable counts could not be observed. With the passage of time, more of the hydrocarbon is emulsified into aqueous phase, more number of cells come in contact with oil drops, therefore, further increase in viable cells was observed. This observation has further been quantified by determining the ratio of the cell bound to the hydrocarbon phase to that not bound (Panchal and Zajic, 1978). It has been observed that this ratio dropped from 0.99 at the beginning of the fermentation to around 0.66 after 70 hours of batch growth. On the other hand, just after two days of inoculation, the pH of the medium started decreasing, it dropped from 7 to 5.9 in the late exponential phase (Figure 2). This gradual drop in pH is associated with the production of fatty acids in the growth medium (Gray et al., 1984).

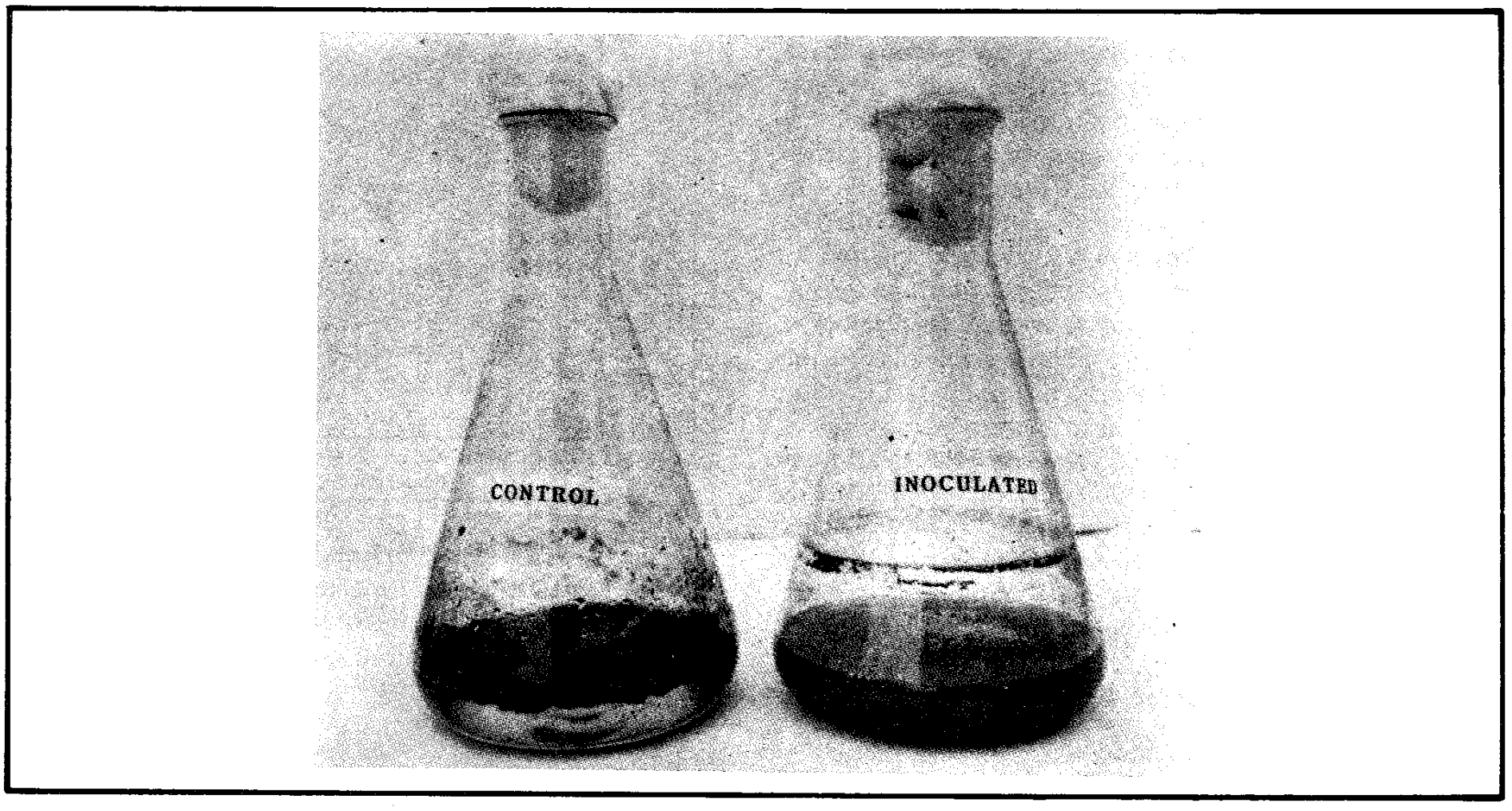

Figure 1— Emulsification of 0.5% (v/v) Khaskheli crude oil by *Pseudomonas aeruginosa* strain K-3 after 72 hours of incubation at 30°C, under shaking condition (100rpm).

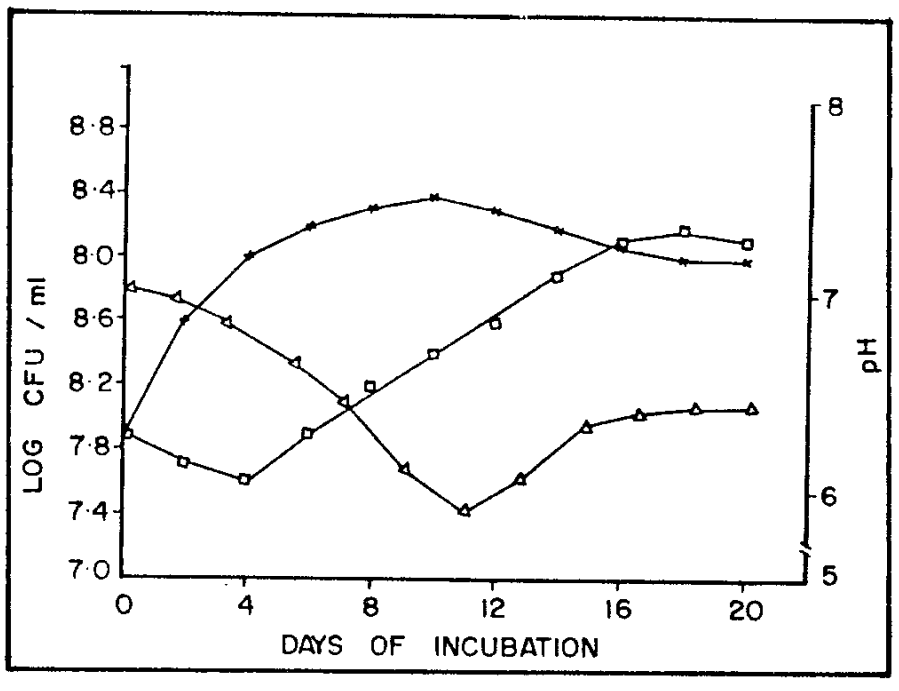

Figure 2— Growth comparison of *Pseudomonas aeruginosa* strain K-3 on 0.5% (v/v) n-hexadecane (□) and Khaskheli crude oil (×) in BH mineral medium and pH changes during growth on n-hexadecane (△).

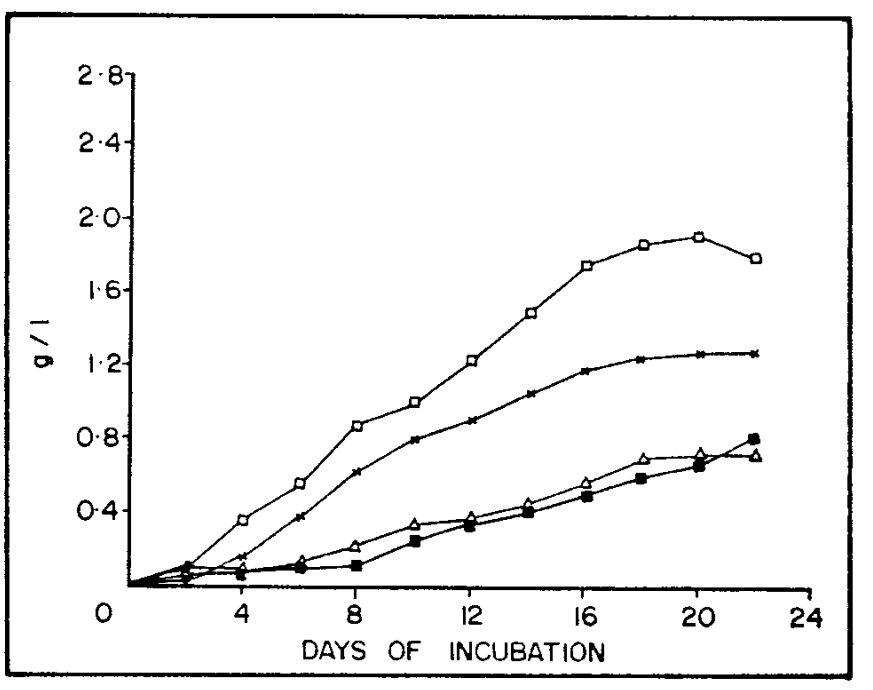

Figure 3— Time course of metabolite production by *Pseudomonas aeruginosa* strain K-3 grown on 0.5% (v/v) n-hexadecane, dry biomass (□), extracellular proteins (×), intracellular proteins (■)and extracellular sugars (△).

Extracellular Metabolic Products

During the growth of strain K-3 on n-hexadecane, proteins and sugars were excreted into the medium, while time dependent increase in cell biomass and intracellular proteins was also observed. Figure 3 shows that there was a lag phase of only two days in the production of extracellular proteins, while other metabolites were excreted into the medium right from the day of inoculation, inspite of the fact that there was no observable increase in cell number during this period (cells were probably present in the hydrocarbon phase). It has been observed that as long as the cells remain in hydrocarbon phase, they obtain

carbon for energy and growth by direct contact which enable them to continue the metabolic process and hence secrete various metabolites into the culture medium (Nakahara et al., 1977). With the passage of time, cell growth in the aqueous phase is enhanced resulting in higher emulsifier production. Extracellular emulsifier production alters the surface active properties, thus affecting the solubility of liquid hydrocarbon into the aqueous phase. The liquid hydrocarbon is emulsified in the form of fine oil droplets. These oil droplets accommodate themselves in the aqueous phase, thus improving the availability to the cells. Increased cell growth may be due to the fact that the cells growing in the aqueous phase, obtain hydrocarbon as "accommodated" oil drops (Chakravarty et al., 1975). As shown in Figure 3, the production of extracellular proteins during the first two days of cultivation was not observed but it sharply increased after the initial lag period. Similar increase was observed in other metabolic products. Such increase with growth was also observed earlier (Roy et al., 1979). These results clearly indicate that production of different metabolites by strain K-3 are growth dependent.

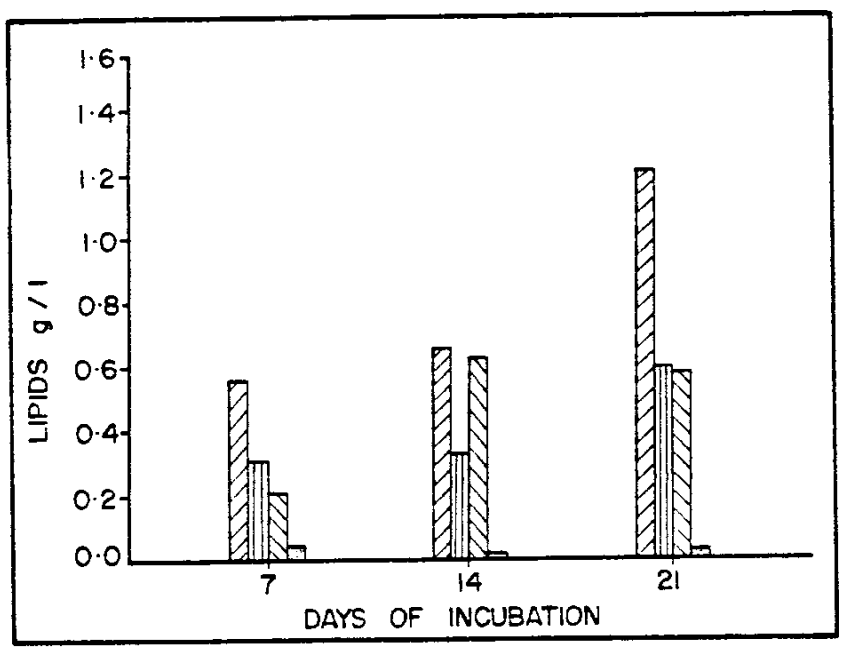

Figure 4— Crude biosurfactant (▨) production by *Pseudomonas aeruginosa* strain K-3 grown on n-hexadecane and fractionation of crude biosurfactant into neutral (▥), glycolipid (▧) and phospholipid (▩) by silicic acid column chromatography.

Fractionation of Crude Biosurfactant (Lipid)

The bacterial strain K-3 produces appreciable amount of crude biosurfactant into the culture medium, when grown on n- hexadecane. It was determined gravimetrically that crude biosurfactants are produced during the entire growth cycle of K- 3, with major amounts of extracellular production (1.22g/l) occurring in stationary growth phase. Similar observations were made by others (Wagner et al., 1983; Syldatk et al., 1985). The crude biosurfactants produced by strain K-3 after 7, 14 and 21 days of incubation were fractionated into neutral, glyco, and phospholipids by silicic acid column chromatography (Figure 4). It has been found that neutral lipids remained almost same at 7 and 14 days of incubation, but increased significantly on day 21 confirming that synthesis of neutral lipids is growth independent. Increase in crude lipids on day 14 was found to be mainly due to the glycolipids. More of the glycolipids are produced extracellularly in the beginning of the exponential phase and kept on increasing till the end of the experiment. The glycolipids appeared to be synthesized throughout the growth cycle while the major amounts being produced in the stationary phase. These observations are in agreement with the findings of others (Finnerty and Singer, 1984a).

During this study, it has been observed that the crude biosurfactants are produced over the entire growth cycle of K-3. The amount increased with increase in incubation time, confirming in part, that the extracellular production of crude biosurfactants are growth associated (Mekula et al., 1975).

CONCLUSION

This local isolate *Pseudomonas aeruginosa* strain K-3, produced extracellular biosurfactants which emulsified crude oil in 2-3 days of incubation. Extracellular proteins, sugars, intracellular proteins and crude lipids were found to be growth associated. Fractionation of crude lipids by silicic acid column chromatography revealed that only glycolipids were growth associated and found to be Rhamnolipid in nature. (Unpublished results).

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