# PRODUCTION OF SURFACE ACTIVE LIPIDS BY SACCHAROMYCES UVARUM GROWN ON DECANE

SUBHI J. HAMZA*
ALA S. ABBAS*
AMAL A. HALOB*

*SUMMARY: A yeast capable of growth on n-decane as the sole source of carbon with production of appreciable quantities of biosurfactant has been isolated from soil and identified as Saccharomyces uvarum. The surface activity of the culture medium was attributed to lipids which were extracted and fractionated using column and thin-layer chromatograph. The lipid content of each fraction was characterized as phospholipids, triglyceride, and a small amount of palmitic acid using infrared spectrophotometry and gas chromatography. The amount of surfactant produced correlated with cell number, pH of cultures liquid and decane concentration.*

*Key Words : Saccharomyces uvarum, n-decane, biosurfactant.*

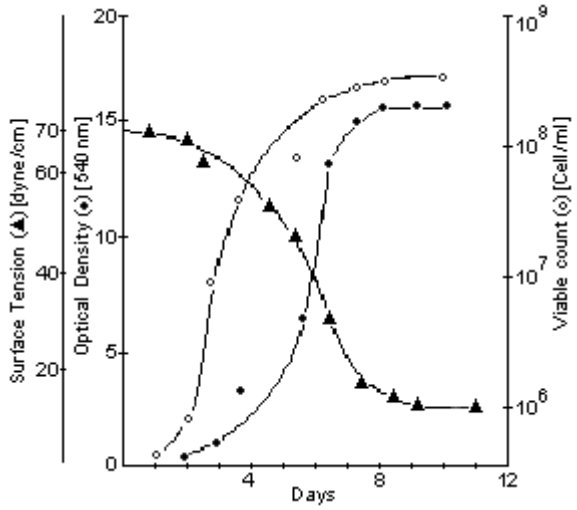
## INTRODUCTION

Genera of yeast include, *Candida, Rhodosporidium, Rhodotorula, Saccharomyces, Sporobolomyces,* and *Trichosporon* are reported as hydrocarbon degraders (1). The general route of biodegradation of aliphatic hydrocarbons is through the oxidation of alcohol and aldehyde, yielding... fatty acids (2). For instance, n-decane undergoes microbial oxidation by *Pseudomonas* sp. to produce ten fatty acids including stearic acid, maleic acid and palmitic acid (3).

The growth of microorganisms on hydrocarbons occur with the synthesis of surface active molecules which aid either in emulsification or attachment of the cell to an insoluble substrate (4). These molecules have been characterized as glycolipids, phospholipids, lipopeptides, or neutral lipids (5,6,7). It was found that *Torulopsis maynoliae* produce two major types of glycolipids, which both containing the disaccharide sophorose and a hydroxy fatty acid. However, Histasuka (6) characterized, rhaminolipids during the growth of P. aeruginosa on various alkanes. Rhaminolipid accumulation also occurred with the utilization of olive oil as carbon source (8). However, others found that suppression isocitrate dehydrogenase activity (9), addition of metal cation and continuous product removal (10) induced large yield of surfactant by *Bacillus subtilis.* However, enhancement of glycolipid production by *Torulopsis bombicola* was correlated with carbohydrate and vegetable oil as carbon sources. Addition of vegetable oil after exponential growth phase cause a huge biosurfactant production (11).

* Biological Research Center, Department of Applied Microbiology, P.O. Box 765, Baghdad, Iraq.

Figure 1: Changes in cell number, decane dispersion and emulsifier production by *Saccharomyces uvarum*. During growth on 10% (v/v) decane.




In this paper, we report the isolation and identification of yeast capable of assimilating n-decane with production of surface active lipids. The conditions for growth and emulsifier production have been elucidated in details.

## MATERIALS AND METHODS

Analytical grade n-decane was purchased from Fluka, Buchs, Switzerland. Soil samples were collected from area previously contaminated with motor and cutting oil. Standard lipids and Triglycerides were obtained from Applied Science Laboratories, Inc., state college, Pennsylvania, U.S.A.

### Medium and culture conditions

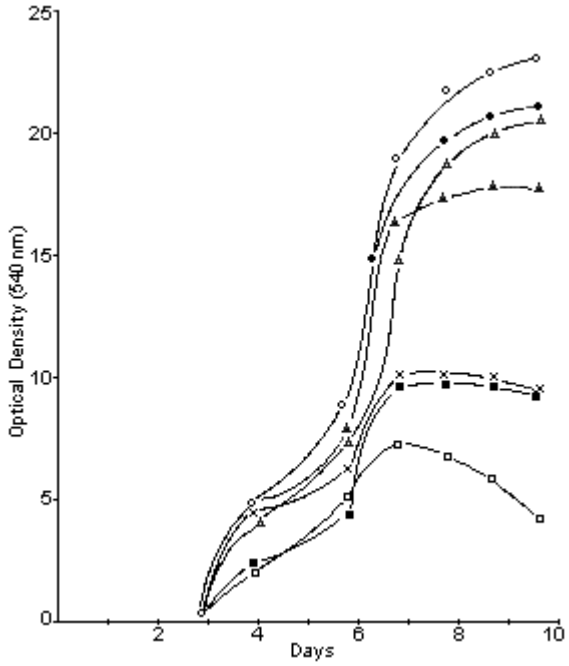
Yeast was maintained aerobically at 30°C on basal salts medium of the following composition (gram per liter) : $KH_2PO_4$, 3.0; $NH_4NO_3$, 5.0; $MgSO_4$, 0.2; $FeSO_4$, 0.002; chloramphenicol, 0.03. Decane was added after sterilization. The pH of the medium was adjusted to 4.5. Decane-degrading yeast was isolated by selective enrichment from soil previously contaminated with motor oil and culting oil. A soil sample (lg) was added to 100 ml of basal salts medium containing 10 ml of decane. "The Flasks were then incubated at 30°C for 15 days. Subcultures were transferred every 10 days for five passages into fresh medium containing decane as the sole source of carbon. After each transfer, the cultures were checked for turbidity and substrate disappearance. The cultures were then plated onto Davis yeast agar and nutrient agar. The methods and Key for taxonomic classification of the isolated yeast were done according to Lodder (12) and Beech *et al.* (13).

### Extraction and fractionation of lipids

Culture medium (2 liters) were left over night leading to the separation of emulsion from aqueous phase. Lipids were extracted from the emulsion following the method of Kates (9). The emulsion was shaked three times with chloroform-methanol-water (2:1:0.8 ml per g of emulsion) for 6 h. The organic phase was pooled and washed twice with water then evaporated to dryness. Extraction of the aqueous phase with the solvent system indicated that 99% of the lipid had been removed by this procedure. Lipids were fractionated by chromatography on to column (1 by 10 cm) containing 10 g of activated silicic acid which was prepared in chloroform. Lipid samples were applied to the column in chloroform. Methanol or gradient of chloroform-methanol were used to elute phospholipids or individual lipids, respectively. Mono-, di-, and triglycerides were separated onto silicic acid column packed in hexane. The following solvent systems were used to elute Mono-, di- and triglyceride: Ether, 10 ml; Hexane-30% ether, 10 ml; Hexane-15% ether, 20 ml; respectively.

Lipids were examined for purity using thin layer chro-

Figure 2: Time course of emulsifier accumulation by *Saccharomyces uvarum* in relation to pH of culture medium. Symbols: pH 2.5, □; 3.0,■; 3.5,X; 4.0, ▲; 4.5,△; 5.0,❍ and 5.5,●.




matography (TLC). Merck G silica gel plates (20 by 20 cm) were spotted with lipid fractions and developed in the following solvent system : chloroform-methanol-water (65:25:4); and chloroform - acetone - methanol - acetic acid - water (7:8:2:2:1). After developing the spots, the following standard spray reagent (14) were used Zinzadaze reagents, Ninhydrin, rhodamine 6G. Identification were made on the basis of comparisons with published data (14,15) and available phospholipids; including phosphotidyl glycerol, phosphotidyl choline and phosphatidic acid.

**Analytical methods**

Lipid phosphate was assayed by the method of Bartlett (16). Free fatty acids were determined by gas chromatography. The silyated derivatives of the dried material were prepared using pyridine based solutions of N-methyl-N-trimethylsilyle trifluoroacetamide (Pierce Chemical Co.). The derivative was resolved over 230°C in Shimadzu-6AG gas chromatograph equipped with 5% diethylene glycol succinate (DEGS) column and has detected with a flameionization detector. Infrared spectra were obtained on Beckman IR-20 spectrometer.

## RESULTS AND DISCUSSION

The results reported in this investigation show the development from soil, previously contaminated with motor oil, and cutting oil, apure culture of yeast capable of decane utilization using selective adaptation and enrichment technique. After several transfers of the five enrichment cultures in decane containing medium, turbidity measurement and GC analysis showed that decane was being metabolized. Dilutions were plated onto Davis yeast agar and nutrient agar. On the basis of colonial morphology, one isolate able to degrade decane efficiently and produce surface active agent was obtained. The isolate was subjected to different taxonomic tests according to the key described by Lodder (12). Figure 1 summarizes four parameters which accompanied decane degradation. The microscopic count increased thousand folds during 7 days, a visible turbidity of culture liquid with extensive production of emulsifying agent was observed and indicated by drop down of surface tension reading from 72

Figure 3: Time course of emulsifier accumulation by *Saccharomyces uvarum* in relation to decane concentration. Symbols: percent (v/v) decane 1,X ; 3,■; 5,❍; 7,□; 10,●; 13,△ and 17,▲.

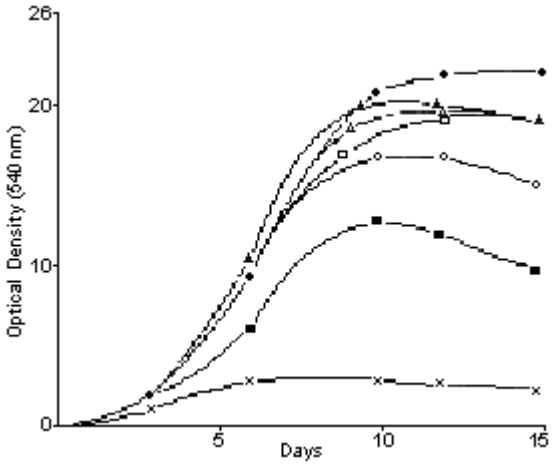
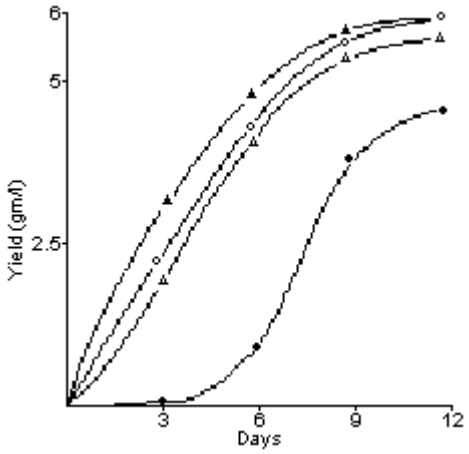
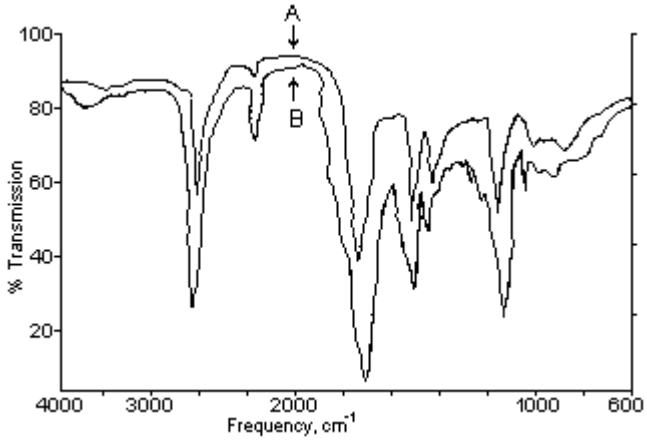



Figure 4: Time course of emulsifier production by *Saccharomyces uvarum* in relation to decane concentration. Symbols: percent (v/v) decane 5,●; 10,▲; 15,△ and 20,❍.




dyne/cm to 20 dyne/cm, and decane loss off from culture medium was evident by GC analysis of extracted culture liquid. Figure 2 shows the kinetics of cell growth in relation to pH of culture medium. The data clearly indicate that decane utilization occur in all pH ranges assigned for good yeast growth. However, best decane utilization observed at pH 5.0. In all cases, the pH of culture liquid drop down to 2.5 and then remained constant for the duration of the experiment. Most of the drop occur during the exponential phase of growth, a time when there was extensive production of emulsifying agent. Reisfeld *et. al.* (17) also observed a decrease in pH of culture medium of *Arthrobacter* sp. from 7.5 to 5.0 during a time when there was no net increase in number of viable cells. However, the PH rose slowly back to about 7.0 accompanied with oil dis-

Figure 5: Infrared spectra of triglyceride. (A) standard triglyceride; (B) triglyceride purified from emulsifier of *Saccharomyces uvarum* grown on decane.



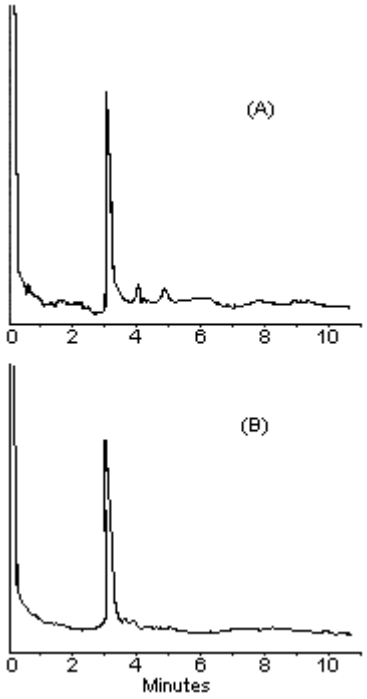

persion. The increase in pH attributed to the production of emulsifying agent during growth on hydrocarbons. Most of the emulsions were obtained from pH 5.0 to 9.0. The maximum production occurred between pH 5.0 and 6.0 (4).

Growth and production of emulsifying agent as a function of decane concentration is shown in Figure 3. Since turbidity attributed to yeast growth was negligible (only about 2.5 at late exponential phase), over the range 1 to 117 ml of decane per 100 ml, the amount of emulsifier increased with increasing substrate concentration up to 10 percent and the maximum amount produced was within 9 days of incubation (Figure 3). Like wise, maximum yield was observed when decane concentration increased up to 10 percent. No net increase in emulsifier concentration even with 20 percent decane (Figure 4). The time course of emulsifying agent production mimic that of growth. No emulsifier production was noticed after growth ceased. A dried sample of emulsion was analyzed for C-H-N- ratio and calculated to be 57:10:7. Rosenberg *et. al.* (4) also found an association between growth and emulsifier production; but the production continued during stationary phase. They also found a relationship between he rate of emulsion formation and gas oil concentration. Emulsifier concentration increased gradually with increasing substrate concentration up to 100 mg/ml.

The lipids of emulsifier were extracted and fractionated by silicic acid chromatography into fractions of polar lipids and lipoproteins. Further fractionation and purification were done using thin layer chromatography. It was found that phospholipids constituted the major fraction as indicated by TLC and a phosphorus content of 125 moles per gram dry weight of emulsifier. The result was confirmed by silylation and gas chromatography of the acid hydrolysate. The major components of phospholipids were phosphotidyl glycerol phosphatidic acid, and unidentified component presumably being phosphotidyl choline.

There are relatively few examples of appreciable amounts of phospholipids being isolated from cell free microbial broth. During growth of *Thiobacillus thiooxid* and several different phospholipids were found. However, the major component were phosphotidyl glycerol and phosphatidic acid (18). Similarly, the production of phospholipids by *Acine-tobacter* sp. could be enhanced by using Hexadecane and Heptadecane as a carbon source (19). Triglyceride was found in culture liquid as indicated by comparation of the infrared spectrum of purified triglyceride to that of tripalmitin (Figure 5). The

Figure 6: Gas chromatographic analysis of palmitic acid. (A) free fatty acid purified from emulsifier of *Saccharomyces uvarum* (B) standard palmitic acid.




spectrum shows identity and revealed the following groups : (OH, carboxyl, 3000 and 2700 cm; C=0 1710; C=C (aliphatic), 1650 cm; C-0, 1100, 1200). GC analysis indicate the presence of small amount of simple fatty acid identified as palmitic acid (Figure 6). Thorpe and Ratledge (20) analyzed lipids of number of yeast species grown on n-dodecane and found in each yeast about 80% of the lipid consisted of triglycerides. They also found an appreciable amount of dodecanoic acid in the triglyceride.

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Correspondence:
Subhi J. Hamza
Biological Research Center,
Department of Applied Microbiology,
P.O. Box 765, Baghdad,
IRAQ.