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PRODUCTION OF EMULSAN BY FREE AND IMMOBILIZED ACINETOBACTER CALCOACETICUS (RAG-1) USING ETHANOL OR HEXADECANE AS A SOLE CARBON AND ENERGY SOURCE

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ABSTRACT

Production of the bioemulsifier, emulsan, by Acinetobacter calcoaceticus RAG-1 (ATCC 31012) using ethanol or hexadecane as a sole carbon and energy source was performed. In free cell culture, ethanol was more favorable for growth than hexadecane. Most of the emulsification activity was found in the foam collected from the culture. Cells of A. calcoaceticus (RAG-1) were immobilized in Celite R-635, particles of calcined diatomaceous earth. In some experiments Celite particles were coated with alginate layer. In ethanol culture, coating the Celite particles resulted in reduction of the emulsification activity by 20% compared with culture containing the uncoated particles. In hexadecane culture no detectable effect of coating was observed. Immobilization of RAG-1 cells onto Celite particles greatly increased the total emulsification activity of either ethanol or hexadecane culture compared with their free cell cultures.

INTRODUCTION

Much attention has been focused on the potential of biological surfactants to replace conventional surfactants in textiles, pharmaceutical, cosmetics and food industries (1). They have been also used for enhanced oil recovery and oil spill clean-up (2,3). Biosurfactants are produced by a wide variety of microorganisms; Bacillus subtilis (4), Corynebacterium lepus (5), Acinetobacter calcoaceticus (6) and Pseudomonas (7), are reported as biosurfactant producers.

Acinetobacter calcoaceticus (RAG-1), a gram-negative bacterium, is capable of utilizing a variety of insoluble and soluble sources of carbon to produce the highly efficient bioemulsifier emulsan(8,9).

Immobilization of cells or enzymes in a variety of matrices such as alginate (10,11), sand particles (12), activated carbon (13), and porous glass (14,15) provides advantages over the use of free cells or enzymes in various biotechnological applications. For example, immobilization may increase the cell loading capacity (16) and/or increase the rates of production of microbial products (17) in bioreactors. In recent years, there has been an increased interest in the use of diatomaceous earth (Celite particles) as support matrices in biological process such as biodegradation (18) and biotransformation(15).

The specific aim of the present work is to assess the practical and economical feasibility of using immobilized A. calcoaceticus (RAG-1) cells to produce emulsan in culture media containing ethanol or hexadecane as sole carbon and energy source.

MATERIALS AND METHODS

Microorganism :

Acinetobacter calcoaceticus (RAG-1) ATCC 31012 was used.

Culture Media :

Nutrient agar; nutrient broth; tryptic soy-broth (TSB) and a chemically defined minimal salt medium, MSB-1 were used. The MSB-1 medium was prepared by dissolving the following salts in one liter of demineralized water : K_2HPO_4, 7.0g; KH_2PO_4, 3.0g; $MgSO_4.7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0g; $NaNO_3$, 1.0g; nitriloatriacetic acid, 1.0g. The pH was adjusted to 6.7.

Preconditioning of A. calcoaceticus (RAG-1) cells :

Preconditioned cells of RAG-1 were prepared in TSB, containing 2.5% (v/v) of the corresponding carbon source (ethanol or hexadecane). The cultures were incubated at 30°C in a gyrating water bath (150 rpm) for 24 hours. The grown cells were centrifuged, washed twice with sterile water and resuspended in sterile medium. The cell concentration was then adjusted to obtain OD_{546} = 0.1 for further investigation.

Production of emulsan by free cells :

Fermentation conditions. A volume of 2.5 ml of preconditioned cell suspension was added to 250 ml of freshly prepared MSB-1 medium containing 2.5% (v/v) of the carbon source (ethanol or hexadecane) in a 500 ml Erylenmeyer flasks. Sterile, humidified air (1 vol/vol/min.) was sparged directly through the culture broth. A foam trap was connected to each culture. Samples from culture broth were withdrawn for analysis every 24 hours and at the end of fermentation. The liquid collected in the foam trap was also subjected for analysis.

Because microbial cells were found adsorbed to the droplets of hexadecane (according to the microscopic examination), the optical density and the protein concentrations were determined only after extracting hexadecane with an equal volume of hexane. After hexane treatment, centrifugation and separation of the organic phase, cells were resuspended in the aqueous phase and the optical density was spectrophotometrically determined at 546 nm. Produced emulsan was recovered by ammonium sulfate precipitation as previously described (8).

Production of emulsan by immobilized cells :

1- **Immobilization technique :** Celite R-635 was used for immobilization of RAG-1 cells as described before (19). The sterilized particles were agitated at 150 rpm for one hour in 150 ml of the preconditioned cells (OD_{546} of 0.38 and a total protein concentration of 14 µg/ml). After decanting the liquid, the beads were transferred to sterilized MSB-1 medium.

2- **Coating the immobilized cells with a thin layer of calcium alginate :** The method of coating was similar to the method described before (19). Immobilized RAG-1 cells on Celite particles were gently agitated for about 30 min in 4% sodium alginate solution. The sodium alginate-coated Celite was then filtered under vacuum then resuspended in 300 ml of 2% $CaCl_2$ solution to harden the alginate layer for one hour. The calcium alginate-coated Celite was then resuspended in 250 ml of fermentation medium.

3- **Fermentation conditions :** Immobilized cells (coated or non coated) were added to 250 ml sterile MSB-1 medium containing 2.5% (v/v) ethanol or hexadecane in aerated shacked flasks with a foam trap and incubated at 30°C on a shaker water bath (200 rpm). Sterile air was continuously sparged into the culture (1 vol/vol/min).

Analytical Procedures :

Determination of cell growth :

Cell growth was followed by monitoring the optical density of the culture

broth (i.e., OD_{546}). Total protein was determined using previously reported procedure [20].

Determination of ethanol and hexadecane by gas chromatographic analyses :

For Ethanol: Ethanol samples were diluted with demineralized water (1:100) and centrifuged for 5 minutes at 13,000 xg. Ethanol concentration in the clear supernatent was then analyzed. A Shimadzu Model GC-8A gas chromatography with a propapak Q-80/100, 6' x 1/8", stainles steel packed column with a flame ionization detector (FID) was used. Hydrogen and oxygen flowed through the FID at 90 ml/min and 200 ml/min, respectively. Nitrogen was used as the carrier gas which flowed through the column at 60 ml/min. The column temperature was held at 160°C while the injector and detector temperature were held at 180°C. The quantity of ethanol in the 10 µl sample was determined by comparing the area under the eluted ethanol peak against that obtained with standard samples of ethanol.

For Hexadecane: Hexadecane was extracted into hexane. An aliquot of 1 µl of hexane solution was gas-chromatographed. The resulting peak area was used to quantitate the recovery of hexadecane using a standard curve prepared by dissolving standard n-hexadecane in hexane. A Shimadzu model GC-8AX gas chromatograph equipped with FID and split/splitless injector SPL-G9, SPL-G9M were used. The sample was chromatographically separated by passing through 100 meters of fused silica capillary column with inner diameter 0.25 mm and film thickness 0.5 microns (Petrocol DH, Supelco). The sample was split 100:1 using nitrogen with a pressure of 3.6 kg/cm^2. Nitrogen carrier gas flowed through the column at 60 ml/min. The starting column temperature of 50°C was maintained for 10 minutes; the temperature was then increased 10°C/min. to a final temperature of 250°C. Once the column temperature reached 250°C, the temperature was held constantly for 30 min. Both injector and detector were held constantly at 280°C.

Determination of the Emulsification Activity : An emulsification activity assay was developed based on the method of Pendrey [21]. It based on the surfactant's ability to stabilize an emulsion. A finely dispersed emulsion of Jet-A fuel and water was prepared by mechanically mixing 25 µl of jet-A fuel in 2 ml of 1 M Tris base for 1 minute. A 100 µl aliquot of the emulsion was added to 2 ml of a 0.1 M Tris buffer containing different concentrations of test solutions. Sodium dodecyl sulfate (SDS) was used as a standard. The emulsion was mixed gently and then centrifuged at 700 xg for 10 minutes at room temperature (23°C). The optical density of the emulsion, following centrifugation was then determined spectrophotometrically at 530 nm. The stability of the emulsion, after centrifugation, was determined by light dispersion at OD_{530}. A standard curve was prepared using SDS. The OD_{530} of the emulsion is plotted vs. the final concentration of SDS in the emulsion solution.

Emulsification Unit : One emulsification unit (EU) is defined as the amount of SDS (or other surfactants) that stabilizes a preformed emulsion made and centrifuged as described above to give a net absorbance of 0.1 OD_{530} units.

RESULTS

Production of emulsan by free A. calcoaceticus (RAG-1) cells in presence of ethanol or hexadecane as a sole carbon and energy source :

Optical density (OD) and total protein concentration of the broths and foams from ethanol and hexadecane cultures are shown in Table 1. Changes in the OD and protein concentration in the culture broths are nearly parallel to changes in OD and to protein concentrations in the foams. After addition of either of the carbon sources at 96 hours fermentation (2.5%), an increase in the protein content and OD of the culture broth was observed. In hexadecane

Table (1): Optical density and protein concentrations of the culture broth and foam of cultures of A. calcoaceticus (RAG-1) with ethanol or hexadecane

Time[1] (hrs)	Cultures with ethanol [2]		Cultures with hexadecane[2]	
	OD_{546}		OD_{546}	
	Broth	Foam	Broth	Foam
0[2]	0.005	NF[3]	0.01	NF[3]
24	0.083	41.0	1.21	18.3
48	0.137	50.0	0.34	13.0
96	0.083	42.0	0.51	23.4
120	0.221	24.0	10.04	27.6
144	0.441	16.0	3.56	21.5
168	0.393	11.5	0.67	19.5
Time[1] (hrs)	**Protein mg/L**		**Protein mg/L**	
	Broth	**Foam**	**Broth**	**Foam**
0	0.01	NF[3]	0.02	NF[3]
24	5	1800	60	650
48	9	2060	40	410
96	9	1920	50	1090
120	12	1670	260	1510
144	15	1630	110	650
168	16	1760	60	670

Legend :

(1) The hydrocarbon substrate (ethanol or hexadecane) was added to the fermentation broth at zero time and after 96 hours of fermentation.

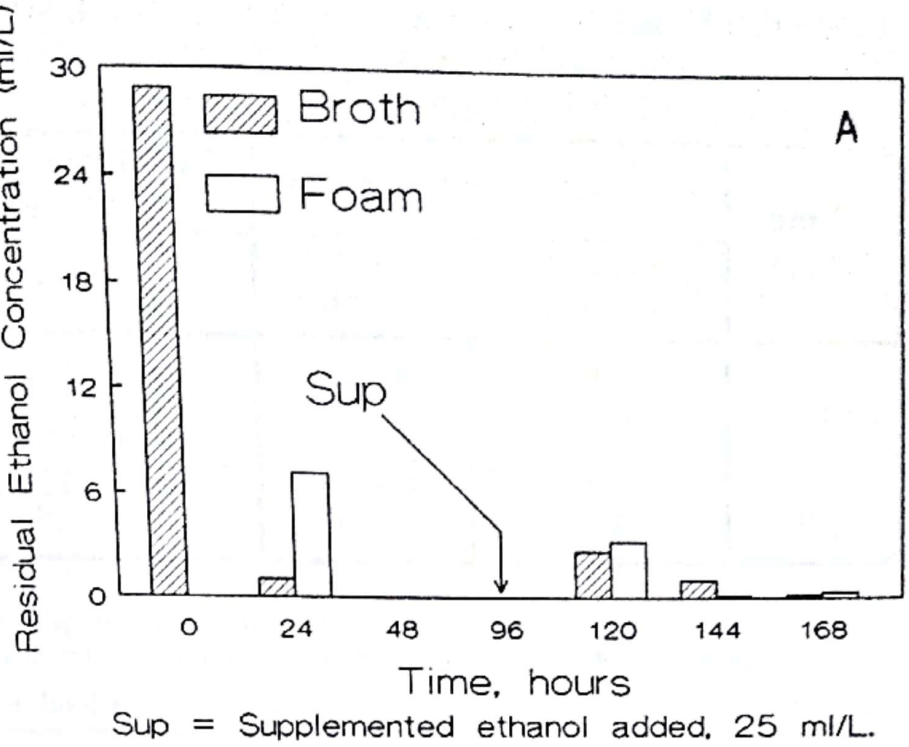
(2) Highly turbid samples were diluted with demineralized water prior to measuring optical density. Data reported in table was corrected for dilution.

(3) NF = no foam.

culture, the OD increased about 20-fold and the protein concentration increased nearly 5-fold. However, the increases in ethanol cultures were not as great as those seen in cultures supplemented with hexadecane. The pH of the culture broth of both ethanol and hexadecane decreased in the first 48 hours (6.9 to 6.5 for ethanol and 6.7 to 6.4 for hexadecane) and then remained relatively steady during the remaining time of fermentation.

The concentration of ethanol in broth and foam was shown in Figure 1A. Most of the ethanol added to the culture medium at zero time and after 96 hours of culturing disappeared within the first 24 hours of addition. Figure 1B shows the concentration of hexadecane in broth and in the collected foam. The hexadecane is utilized less rapidly by RAG-1 cells than ethanol. Furthermore, hexadecane was consistently found in higher concentrations in the foam than in the culture broth.

Table 2 shows that the emulsification activity of the ethanol culture broth and foam increased with time up to 48



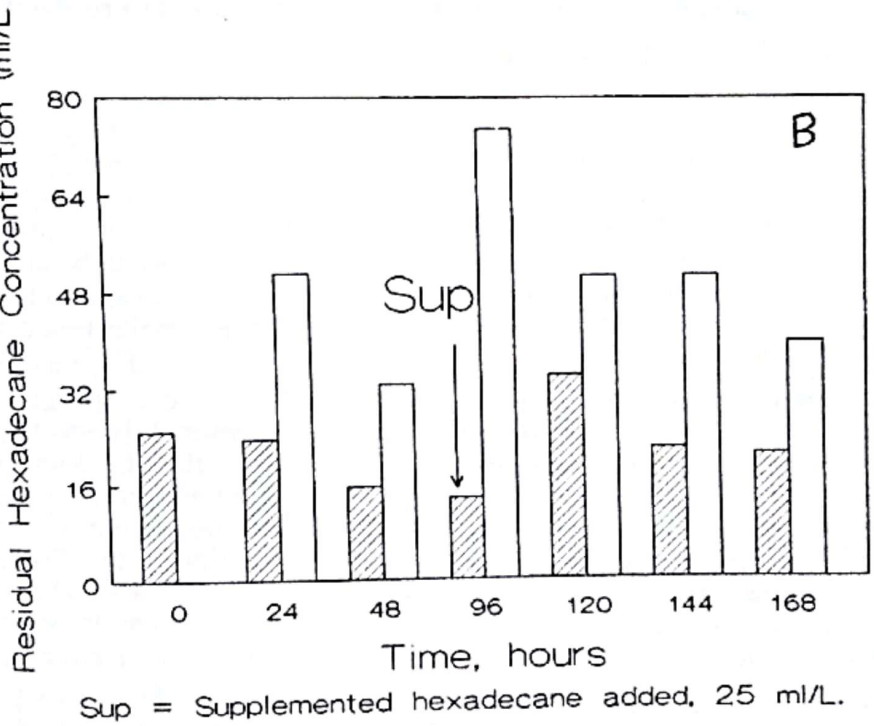


Fig. (1): Residual ethanol (A) and hexadecane (B) concentrations in the bulk volume and in the collected foam of the free cell culture of A. calcoaceticus (RAG-1).

Table (2): Emulsification activity of culture broth and foam produced by a A. calcoaceticus (RAG-1) culture containing ethanol[1]

Time (hrs)	Emulsification Activity		Total Emulsification Activity[2]
	Broth[3] (EU/ml)[4]	Foam (EU/ml)[4]	(Broth + foam) (Eu)
24	3.6	43.2	1029.6
48	5.8	800.0	2650.0
96	4.2	678.0	1728.0
120	2.4	176.0	776.0
168	3.0	755.0	2260.4

(1) The hydrocarbon substrate (ethanol or hexadecane) was added to the fermentation broth at time zero and after 96 hours of fermentation.

(2) Emulsification activity = [(250 ml) x (Broth Activity)] + [(vol. Foam) x (Foam Activity)]

(3) The total volume of culture broth during fermentation equalled approximately 250 ml. Samples of broth assayed were about 5 ml.

(4) EU = Emulsification units.

hours where it reached maximum. After that time a decrease in emulsification activity was detected. The emulsification activity of the foam is nearly greater (approximately 7-fold) than that found in the foam collected from the hexadecane culture (Table 3).

Table 3 shows the emulsification activity of hexadecane culture broth and foam. No emulsification activity in the broth was observed; this may be attributed in part, to the procedure for sampling of only aqueous phase. In contrast, the collected foam possessed both aqueous and hydrocarbon phases, so possessed the most emulsification activity.

Emulsan production by A. calcoaceticus (RAG-1) cells immobilized on Celite :

Ethanol containing cultures :

Optical density (OD_{546}) and total protein content of the foam and broth samples of the two fermentation cycles are shown in Table 4. Results show that both the OD and protein content of the uncoated immobilized cell culture is greater than that obtained from the culture with alginate-coated immobilized cells. This can be attributed to decreasing the cell leakage to the surrounding medium. A similar result was obtained previously (15). The total emulsification activity of the broth and foam samples was shown in Table 5. The uncoated immobilized cell culture produced total emulsification activity, on average, 25% greater than the culture with coated immobilized cells during the first fermentation cycle. During the second cycle of fermentation, very little emulsification activity was detected in either the broth or foam of either culture.

The concentrations of residual ethanol are shown in Figure 2A for the coated and Figure 2B for the uncoated immobilized cell cultures. It is evident that at zero time the concentration of ethanol in the culture broth is 1.7 and 1.9% for the coated and uncoated immobilized Celite cultures, respectively, rather than the expected 2.5%. This loss of ethanol may be attributed to the sorption of ethanol by

Table (3): Emulsification activity of culture broth and foam produced by A. calcoaceticus (RAG-1) culture containing hexadecane(1)

Time (hrs)	Emulsification Activity		Total Emulsification Activity(2)
	Broth(3) (EU/ml)(4)	Foam (EU/ml)(4)	(Broth + foam) (Eu)
24	None	89.0	178.0
48	None	107.6	215.2
96	None	91.6	91.6
120	None	100.8	201.6
168	None	110.8	443.2

See legend of table 2

Table (4): Optical density and total protein concentrations of immobilized A. calcoaceticus (RAG-1) cells using ethanol as sole carbon source

Time (hrs)		$OD_{(546)}$(1)				Total protein concentration (µg/ml)			
		Uncoated		Coated		Uncoated		Coated	
		Broth	Foam	Broth	Foam	Broth	Foam	Broth	Foam
Cycle 1	0	0.024		0.03		9		8	
	24	7.3	114.7	5.7	82.9	710	6000	600	5300
	48	14.1	136.8	6.0	91.2	1200	9000	800	6100
	72	31.5	98.2	4.3	52.8	1700	6000	1100	4600
	96	42.6	None	9.5	None	1700	None	500	None
	120	33.6	None	24.3	None	800	None	400	None
Cycle 2	24	12.123	109.0	5.1	109.8	400	4100	400	5280
	48	14.7	141.2	5.2	None	500	6100	600	None
	72	20.0	-	5.3	None	710	None	800	None

(1) For highly turbid samples, a 1:100 dilution with demineralized water was performed before measurement. The obtained adsorption was multiplied by 100 to calculate the OD in the original sample.

Table (5): Total emulsification activity of immobilized A. calcoaceticus (RAG-1) using ethanol as sole carbon source

Time (hrs)		Total emulsification units (1,2)					
		Uncoated			Coated		
		Broth	Foam	Broth & Foam	Broth	Foam	Broth & Foam
Cycle 1	24	2150	1800	3950	4820	225	5045
	48	750	2500	3250	1435	1075	2510
	72	3025	1980	5005	1284	1825	3110
	96	3450	NA	3450	NA	1925	1925
	120	3650	NA	3650	NA	1750	1750
Cycle 2	24	400	392	792	105	0.0	105
	48	0.0	612	612	NA	0.0	0.0
	72	0.0	NA	0.0	NA	0.0	0.0

Legend :
(1) EU = Emulsification unit
(2) The total EU was determined by multiplying the EU/ml times the broth volume or times the volume of foam.

Celite or dilution of the medium constituent by presence of wet Celite. For this reason culture was supplemented with 6.25 ml (2.5%, v/v) of ethanol every 24 hours of the fermentation time.

Hexadecane containing cultures :

The optical density and protein content of culture broth only (very little foam was formed) are shown in Table 6. There is no significant difference between OD and protein content of the culture broth in the two cases. The pH of the broth containing coated immobilized cells decreased from 6.2 to 5.2 during the first 24 hours of culturing then gradually increased reaching a steady range (pH 6.2 - 6.4). In contrast, only small changes in the pH were observed in the culture broth containing uncoated immobilized cells. The emulsification activity of the culture are shown in Table 7. Levels of emulsification activity of both cultures are quit similar. Furthermore, they remain relatively constant during the time of fermentation. According to results of free cells, hexadecane was slowly utilized by RAG-1 cells. So, each 250 ml culture was supplemented with 6.25 ml (2.5%) of hexadecane after 96 hours of fermentation. Residual hexadecane concentrations in both cultures are shown in Figure 3. At zero time the concentration of hexadecane in the broth of the culture containing uncoated, immobilized cells is close to the added level of 2.5% (v/v), whereas the hexadecane in culture of coated immobilized cells is markedly lower than added. During the first 96 hours of fermentation the concentration of hexadecane decreased steadily in the culture with coated immobilized cells. In contrast, hexadecane concentration, in the culture containing uncoated, immobilized cells, remains relatively constant. Addition of supplemental hexadecane (6.25 ml; 2.5%, v/v) resulted in increasing hexadecane level in the medium. The rapid decrease in the concentration of hexadecane in the broth of the uncoated immobilized cells is in contrast to the much slower decrease observed during the initial 96 hours of culturing. Interestingly, there is no corresponding change

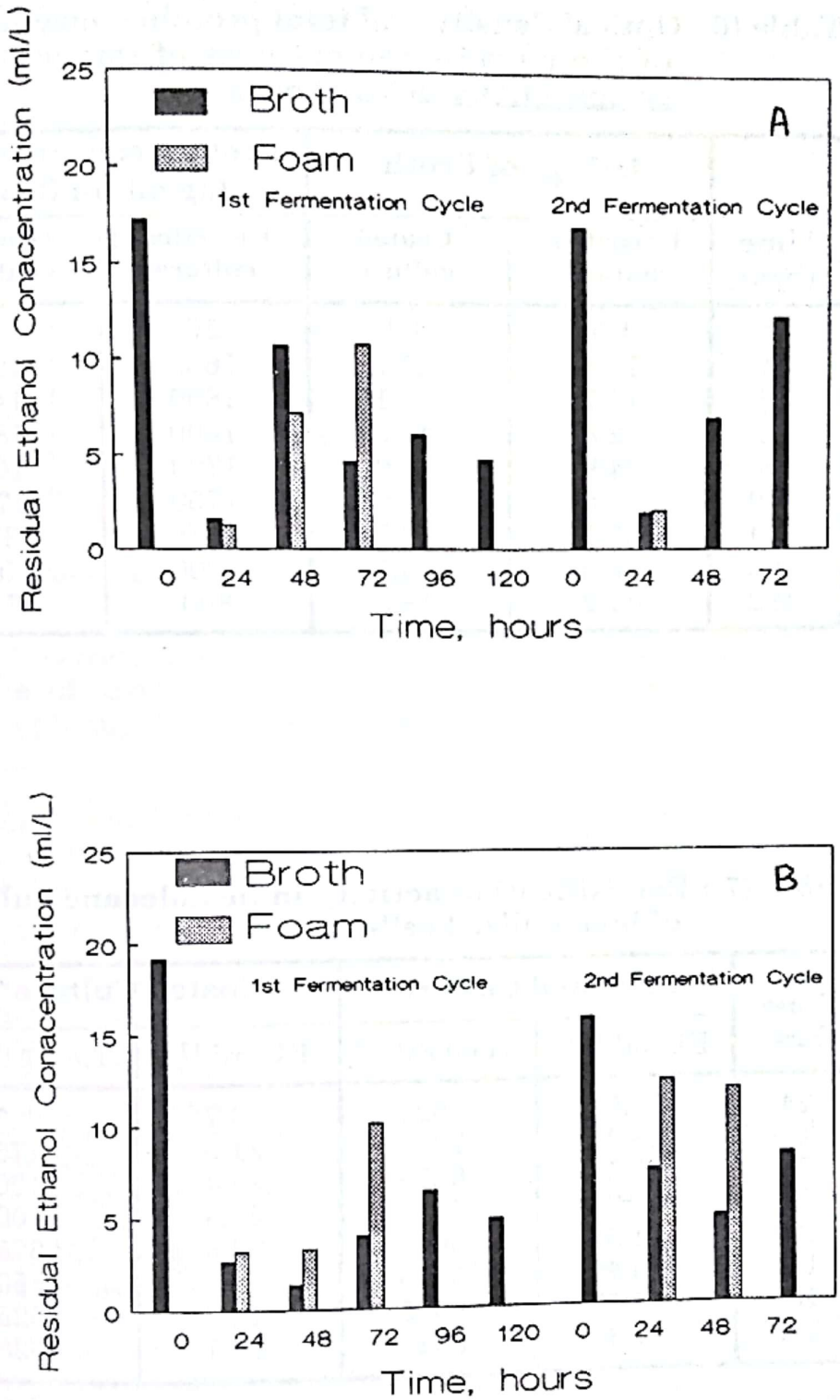


Fig. (2): Residual ethanol concentrations in the bulk volume and in the collected foam of the coated (A), and uncoated (B), immobilied cell cultures of A. calcoaceticus (RAG-1).

Table (6): Optical density and total protein concentrations of the hexadecane cultures of immobilized A. calcoaceticus (RAG-1) cells

Time (hrs)	$OD_{(546)}$ of Broth		Protein concentration (μg/ml) in Broth	
	Uncoated culture	Coated culture	Uncoated culture	Coated culture
0	1.9	1.1	10	11
24	11.8	27.7	1600	1000
48	15.7	37.1	1800	1400
72	22.8	40.6	1400	1800
96	24.9	31.0	1700	1600
120	26.0	43.0	1700	1700
144	35.1	38.7	500	610
168	56.0	25.5	1200	700
192	41.2	18.8	800	710

Table (7): Emulsification activity in hexadecane culture of immobilized cells.

Time (hrs)	Uncoated Culture		Coated Culture	
	EU/ml (1)	Total EU (2)	EU/ml (1)	Total EU (2)
24	22.5	5625	23.7	5925
48	28.9	7225	21.9	5475
72	25.5	6375	28.4	7100
96	21.3	5325	26.8	6700
120	14.5	3625	22.3	5575
144	20.4	5100	14.6	3650
168	22.5	6625	11.7	2925
192	21.3	5325	18.1	4525

Legend :
(1) EU = Emulsification unit
(2) The total EU was determined by summation of EU/ml times the volume of broth (250 ml) and for collected foam.

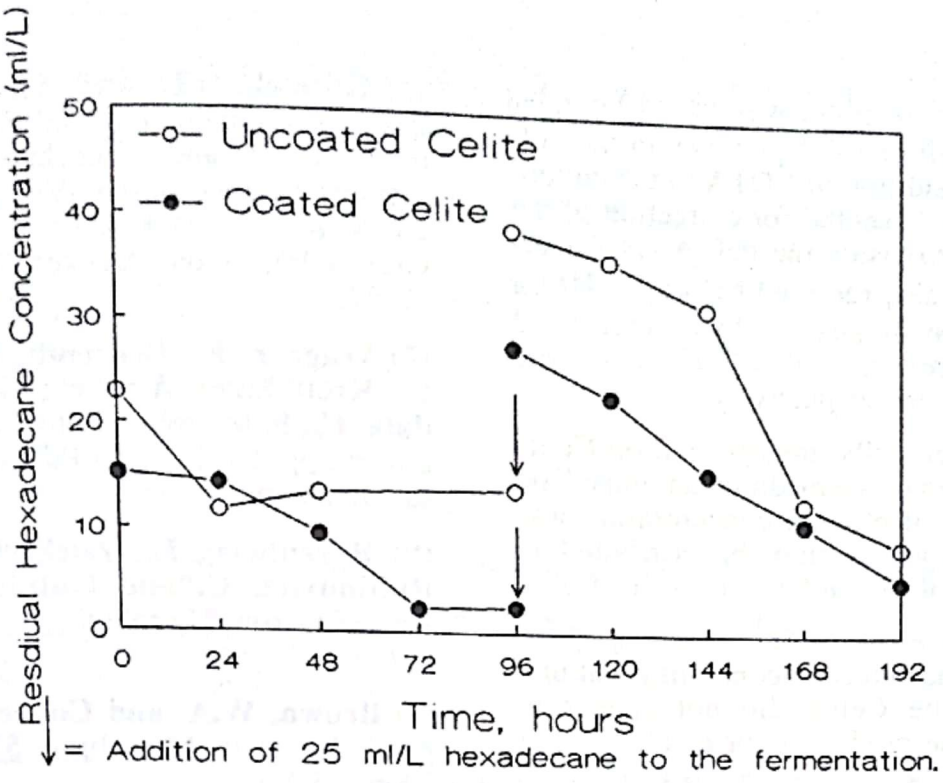


Fig. (3): Degradation of hexadecane in cultures containing coated and uncoated immobilized Celite particles with A. calcoaceticus (RAG-1) cells.

in pH, OD_{546}, protein level or emulsification activity after addition of supplemental hexadecane.

DISCUSSION

Ethanol as carbon/energy source was more favorable for growth than hexadecane. The emulsifying activity of the foam produced by free A. calcoaceticus (RAG-1) cell cultured on ethanol was about seven times greater than that produced with hexadecane. This may be related to the observations that hexadecane was utilized less rapidly than ethanol by the microogranism. Furthermore, it was observed that hexadecane was consistently found in higher concentration in the foam than in the culture broth so much of the hexadecane was not utilizable in the culture.

When RAG-1 cells immobilized on Celite R-635 and cultured with ethanol as the sole carbon source in MSB-1 medium, the total emulsification activity (an indicator for the emulsan production) was increased by about three time that recorded with free cells. Diatomaceous earths are the fossilized silica remains of diatom skeletons. They are characterized by having large surface areas and are reasonably strong (18). Therefore, Celite beads were found to adsorb considerably high bacterial cells from a solution and consequently resulted in increasing the production capacity.

In general, coating Celite particles with Ca-alginate layer markedly reduces the fragmentation of Celite caused by the agitation of the culture broth and it also tends to secure the immobilized cells to the solid support. But when the Celite beads, in this study, were coated (after immobilization) with calcium alginate, the total emulsification activity produced was reduced by about 20%. This may be attributed in part to the characters of the produced emulsan. The Acinetobacter RAG-1 emulsifier is a highly acidic polysaccharide with a molecular weight average close to 10^6 (22). As a result, release of this high molecular weight emulsifier to the culture medium through the alginate layer will be difficult. According to Wang and Wang

(1989)[23], emulsan was found bounded to cell wall or solid particles in the culture and addition of EDTA to the culture broth was essential for extraction of the emulsifier outside the gel. A similar effect was also reported before [19,24] for production of dextran by immobilized bacterial cells in coated Celite and porou stainless steel, respectively.

When cells immobilized on Celite were added to the medium, an immediate reduction of ethanol concentration was observed which may be attributed to sorption of ethanol by the solid Celite support.

In hexadecane-containing culture, coating the Celite did not appear to change the production of emulsification activity. The levels of emulsification activity remained fairly constant during the time of experiment (up to 192 hours), whereas they appeared to fall after similar periods of culture on ethanol.

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انتاج الإملسان بواسطة خلايا الأسينيتوباكتر كالكواسيتيكس الحرة والمثبطه باستخدام الكحول الإيثلى والهكساديكان كمصدر وحيد للكربون والطاقة

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فى هذه الدراسه تم استخدام خلايا الأسينيتوباكتر كالوأسيتيكس (ATCC 31012; RAG-1) فى انتاج الإملسان باستخدام الكحول الإثيلى والهكساديكان كمصدر وحيد للكربون والطاقه ووجد أن الكحول الإيثلى كان مفضلا عن الهكساديكان لنمو الخلايا الحره فى المستنبتات. كما وجد أن القدره على انتاج وسيط الاستحلاب (الإملسان) قد تركزت فى الرغاوى المتجمعه من المستنبت. فى هذه الدراسه تم ايضا تثبيط خلايا RAG-1 على حبيبات السيليت (Celite R-635 - تربه الدياتوم المتكلسة) حيث تم فى بعض التجارب تكسية هذه الحبيبات بعد تثبيط الخلايا عليها بواسطه طبقه رقيقه من اللجينات الكالسيوم. وقد نتج عن ذلك تخفيض القدره على انتاج الإملسان لهذا الميكروب (RAG-1) بنسبة ٢٠٪ مقارنه بما تم قياسه فى المستنبت المحتوى على الحبيبات الغير مكسيه فى وجود الكحول الإيثلى بينما لم تسجل هذه الطريقه أى تأثير فى المستنبت المحتوى على الهكساديكان. كما أثبتت هذه الدراسه أن تثبيط الـ RAG-1 على حبيبات السيليت قد أدى بصفه عامه إلى زيادة كبيرة فى قدرة الميكروب على انتاج الإملسان فى مستنبتات الكحول الإيثلى والهكساديكان مقارنه بما تم قياسه مع المستنبتات المحتوية على الخلايا الحرة.