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Production of Microbial Surfactants

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1 INTRODUCTION

Pollution treatment, abatement and environmental maintenance are among the most important challenges to any nation One biotechnological approach to this problem is the development of new surface active substances, biosurfactants, whose application to various environmental problems is likely to assume increasing significance We forsee the potential for relatively inexpensive applications in a variety of areas such as sludge liquification and bioremediation, heavy metal sorption and concentration, enhancement of bioremediation of hydrophobic pollutants such as pesticides, xenobiotics, solvents, explosives, cleaning etc In this regard it should be noted that 1 There is no universal surfactant formulation which can be used in every application, 2 Only relatively few organisms have been screened for biosurfactant production, 3 Biosurfactants compete favorably with non-toxic chemical surfactnats which are expensive and frequently require formulation with other more toxic material, 4 Non-toxic biosurfactant formulations might compete favorably with other materials used in a variety of industrial applications such as cosmetics, health care and food industries

The ultimate goal of this project is to develop a technology for the isolation, production, and characterization of specific microbial biosurfactants for application in pollution control and environmental maintenance Specific objectives are

1 To isolate, characterize and compare microbial biosurfactants from existing strains and newly isolated microorganisms

2 To develop a screening program for the isolation of surfactant-producing organisms which biosurfactants and surfaceactive biopolymers with novel properties

3 To investigate the physiological characteristics of growth and biosurfactant production with selected strains chosen on the basis of results obtained from Objectives 1 and 2 above

In addition, during the course of this work, we developed additional approaches for the production of novel surfactant formulations based on the interaction of specific proteins and various polysaccharides This work which is currently in progress, will also be presented in this report Finally, we have received a report directly from the laboratory of our colleague Dr Manasbaeva, who has submitted a report on her findings This work will also be included

2 NEW BIOEMULSIFIER-PRODUCING STRAINS.

Preliminary results of a screening program for new biosurfactant producing strains were described in our first progress report A series of strains able to grow on crude oil were isolated by enrichment culture from a soil sample and screened for bioemulsification properties The results are shown in Table 1 As expected, all of the strains produced some emulsifying activity although they did exhibit different properties For example, strains DG20, DG44, DG121, DG122 all produce emulsifying activity which is associated with the cell surface Interestingly, this activity frequently depends on the growth substrate For example, DG20 produced cell-associated bioemulsifier when grown either on glucose or on hexadecane Similar results were found for DG44 These two strains produced little extracellular emulsifier In contrast, however, DG121, produced copius amounts of cell-free emulsifying activity, yet also produced significant quantities of a cell-bound polymer Interestingly, DG121 produced both cell-bound and cell-free emulsifying activities only when grown on hexadecane Similarly, strain DG39 and DG122 produced emsulsifying activity in the cell-free broth only when grown on hexadecane

As shown in Table 2, the cells of strains producing the new biosurfactants varied in their adherence to hydrophobic surfaces Strains DG39, DG44, DG120 and DG121 resembled RAG-1 in their avid adhesion properties to hydrophobic surfaces Interestingly, strains DG 21, DG47, DG119 and DG123 strongly adhered to hexadecane when grown on the hydrocarbon substrate, but exhibited severely reduced adherence properties when grown on the water soluble carbon source This most likely reflects a carbon source dependent production of the hydrophobic fimbrae which generally mediate the adhesion of microbial cells to hydrophobic surfaces

One of the interesting features of the biopolymer emulsan is its ability not only to emulsify hydrocarbons, but also its ability to prevent the adhesion of other organisms to hydrophobic surfaces It was of interest therefore to compare the emulsan anti-adhesion activity with the those of the new strains (Table 3) Since DG44 was the most adhesive of the new strains, we tested the ability of the various emulsifier preparations to prevent its adhesion to hexadecane As can be seen in Table 3, DG39, DG120 and DG121 all produced potent anti-adherence activity In these cases, the anti-adherence activity was even more effective than emulsan itself In contrast, neither the cells of DG44, nor the extracellular products DG47 or DG119 were particularly effective in preventing the adhesion of DG44 The other emulsifiers showed intermediate activities in adherence inhibition These studies, along with others designed to purify and determine the structures of the various emulsifiers, are currently in progress

Table1 Emulsifying activity of oil-degrading microorganisms

Strain	Carbon Source	Biomass (KU)	Emulsifying Activity	
			Cells (U/g dcw***)	Supernatant (U/ml)
RAG-1	Glucose*	--	--	--
	Hex	1150	12	193
DG20	Glucose	437	95	42
	Hex	967	158	23
DG21	Glucose	856	<5	228
	Hex	1297	13	19
DG39	Glucose	289	29	79
	Hex	446	19	545
DG44	Glucose	529	226	55
	Hex	1097	163	87
DG47	Glucose	466	38	42
	Hex**	--	--	--
DG119	Glucose*	--	--	--
	Hex	774	65	87
DG120	Glucose	459	<10	322
	Hex	924	20	30
DG121	Glucose*	--	--	--

	Hex	1396	155	440
DG122	Glucose	598	<10	<10
	Hex	1119	<10	180

* These cells cannot use glucose as sole carbon source They can grow on acetate and on ethanol

** These cell cannot use pure hexadecane as sole carbon source They can grow on crude oils

*** dry cell weight

Table 2 Adherence of hydrocarbon-degrading microorganisms to hydrophobic surfaces

Strain	Growth Substrate	Adherence to Hexadecane %
RAG-1	Ethanol	78
	Hexadecane	98
DG20	Glucose	33
	Hexadecane	52
DG21	Glucose	42
	Hexadecane	87
DG39	Glucose	66
	Hexadecane	83
DG44	Glucose	100
	Hexadecane	100
DG47	Ethanol	23
	Crude oil	88
DG119	Ethanol	2
	Hexadecane	94
DG120	Glucose	62
	Hexadecane	85
DG121	Ethanol	96
	Hexadecane	89
DG123	Glucose	27
	Hexadecane	100

Adherence to hexadecane was monitored in a standard adherence assay in which 0 2ml hexadecane was vigorously mixed on a vortex mixture for 3 minutes with 1 ml of overnight washed culture (500 KU) of each strain grown on the indicated carbon source and allowed to settle After separation of the phases, the turbidity was measured Percent adherence was calculated as $(1-T_f/500) \times 100$

Table 3 Effect of bioemulsifiers on adhesion of strain DG44 to hydrocarbons

Source of emulsifier	Percent adherence
None	100
Emulsan	15
DG20*	35
DG21	32
DG39	3
DG44*	56
DG47	89
DG119	68
DG120	2
DG121*	23

DG121**	0
DG122	44

*- The source of the emulsifier consisted of washed cells

**- The source of the emulsifier was the dialyzed cell-free supernatant

3 THE DEVELOPMENT OF NEW BIOPOLYMER FORMULATIONS FOR ENVIRONMENTAL APPLICATIONS INCLUDING CATION BINDING

The purpose of this part of the project was to expand the spectrum of amphipathic biopolymers to include new formulations for bioemulsification and cation binding In this portion of the work, we used two basic approaches 1 Reconstitution of emulsifying activity using protein polysaccharide formulations, and 2 Genetic engineering of microbial polysaccharide to generate new biopolymers which could then be included in the program to test novel formulations

3a Reconstitution of activities using protein polysaccharide formulations.

In the first approach we exploited the fact that hydrophobicity of emulsan is enhanced when it is non-covalently complexed with crude protein preparations In the current project, protein was used to reconstitute the emulsifying activity of apoemulsan towards very hydrophobic substrates such as hexadecane This reconstitution was achieved with a cloned overexpressed, his-tagged esterase which was purified by affinity chromoatography on a nickel column using the T7 expression system Previous results had indicated that the esterase was a cell-surface protein involved in emulsan release from the cell surface Moreover, the amphipathic biopolymer could be partially deacetylated by either the RAG-1 enzyme or the recombinant enzyme Cloning of the enzyme in *E coli* was based on the ability of the recombinant protein to confer on *E coli* the ability to utilize simple triglycerides as sole sources of carbon and energy In addition to the wild-type esterase, we employed standard techniques to isolate site-directed mutants blocked either in the catalytic triad of the esterase, or in several other sites aparently involed in protein folding of the RAG-1 enzyme In addition to affinity chromatography, the esterase was occasionally purified on a monoQ column using a Pharmacia FPLC instrument

Use of esterase in reconstitution of hydrocarbon emulsifying activity

For reconstitution experiments, various concentrations of the recombinant esterase (either wild-type or mutants) were mixed with different biopolymers (at varying concentrations) including apoemulsan, the capsular polysaccharide from *Acinetobacter calcoaceticus* BD4, colanic acid from *E coli*, the capsular polysaccharide from *Erwinia stewartii* (termed stewartan), and the capsular polysaccharide from *Erwinia amylovora* (amylovoran) The protein polysaccharide mixtures were assayed for emulsification in a standard emulsification assay using hexadecane 2-methyl naphthalene (2 1) as the hydrocarbon substrate (as described previously) In addition, emulsification of hexadecane alone was also monitored These emulsification assays were also repeated using several of the esterase mutants in place of the wild-type recombinant enzyme

Use of esterase in cadmium binding

In addition to emulsion formation, "polymerasols" were generated according to the procedures described for preparing apoemulsanosols except that the pure biopolymer was replaced by the protein- polysaccharide formulation After centrifugation, the presence of the esterase in the polymerasol was verified using Western blot analysis with a polyclonal antibody prepared against the overexpressed, his-tagged recombinant esterase produced in *E coli* K12 Cd^{++} binding to the polymerosols was carried out as described above

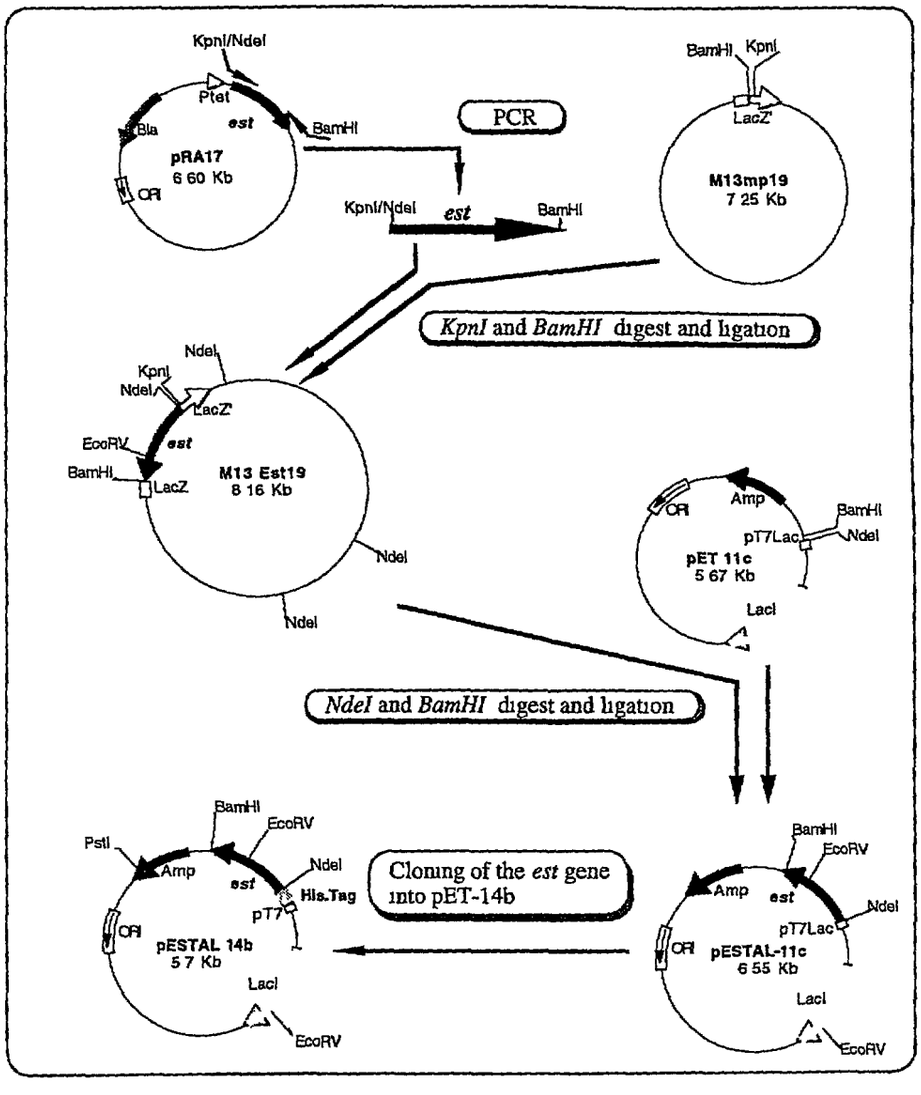
Previous results demonstrated that apoemulsan exhibits hydrocarbon substrate specificity Thus, for optimum emulsification, a mixture of aliphatic and aromatic hydrocarbons is required Pure aliphatic hydrocarbons such as hexadecane are not emulsified by apoemulsan Reconstitution of emulsification of hexadecane alone was achieved when emulsan associated protein was added back to the apoemulsan

preparation Since esterase was found to be associated with the normal suite of proteins associated with emulsan, it was of interest to see whether the recombinant esterase could restore emulsifying activity towards pure aliphatic hydrocarbon substrates A number of figures are included illustrating the cloning strategy and purification of the overexpressed esterase The recombinant protein was present in *E coli* on the cell surface, although about 75% of the enzyme was found in inclusion bodies The fraction associated with the cell surface was purified and shown to exhibit activities similar to those of the wild-type enzyme In addition to the wild-type esterase, a series of site directed mutants were isolated (see sequence and sites of mutations) Mutant glycine or alanine substitutions were made for each position None of the mutants exhibited esterase activity In addition, mutants made in the his-ser-glu catalytic triad, still exhibited relatively large amounts of appropriately folded protein, while the other mutants produced over 90% of the protein in the form of inclusion bodies

As shown in Figs 1-4, both wild-type and mutant esterases were able to reconstitute emulsifying activity to apoemulsan Activities using either mixtures of 2-mehtyl naphthalene + hexadecane, or hexadecane alone were obtained The fact that mutant esterases were also active in this reconstitution demonstrates that the protein need not be active catalytically in order to restore activity However, not all proteins work in this reconstitution system For example, a series of enzymes which interact with polysaccharides such as cellulase, lysozyme, as well as a series of other lipases, proteases and esterases were all found to be inactive in restoring activity (not shown) The specificity of interaction is currently under investigation One approach we are currently taking is to attempt to crystallize the protein with a view towards understanding its structure and function (for example, see Fig 7) Alternatively, we

are currently engineering various fragments from the *est* gene in order to map that region of the protein which may be involved in the reconstitution These studies are currently in progress We hope to be able to exploit the results in order to be able to define small peptides which could be produced synthetically in order to generate novel formulations In addition to the reconstitution of emulsifying activity, we have used the formulations described above and have shown that the recombinant esterases (mutant and wild-type) were able to form apoemulsanosols and were able to bind cadmium in the assay described above These studies are also currently in progress

Scheme of cloning of *est* gene into overexpression vectors pET-11c and pET-14b.



Amino-acid sequence of RAG-1 esterase

Figure 1

```
MKFGTVWKYYFTESLLKATIRTPSQLNLAPNALRPVLDQLCRL
                                      79
FPQNPTVQIRPIRLAGVRGEEIKAQASATQLIFHIHGGAFFLG
                          110
SLNTHRALMTDLASRTQMQVIHVDYPLAPEHPYPEAIDAIFDV
                     149
YQALLVQGIKPKDIIISGDSCGANLALALSLRLKQQPELMPSG
                          196
LILMSPYLDLTLTSESLRFNQKHDALLSIEALQAGIKHYLTDD
                                 244
IQPGDPRVSPLFDDLDGLPPTLVQVGSKEILLDDSKRFREKAE
                274
QADVKVHFKLYTGMWHNFQMFNAWFPEAKQALADIAEFATSLD

LD*
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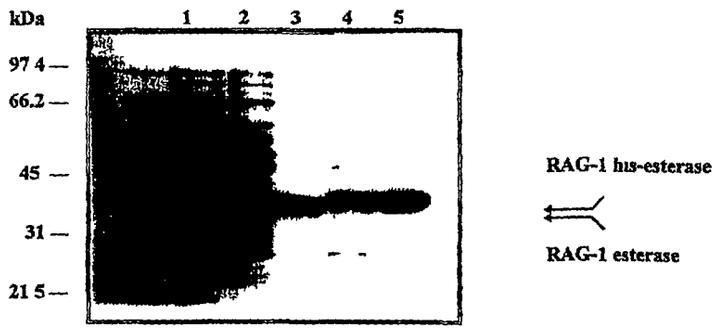
- Bold characters represent potential amino acids in the catalytic triad Ser-Asp(Glu)-His Their positions are indicated above them

- Amino-acids His^{79}, Asp^{110}, Ser^{149}, Asp^{196}, Glu^{244} and His^{274} were changed to glycine and alanine using site-directed mutagenesis

Overexpression of RAG-1 esterase in *E coli*

Figure 2



Esterase activity units/mg of protein 10 2300 15 2400 12

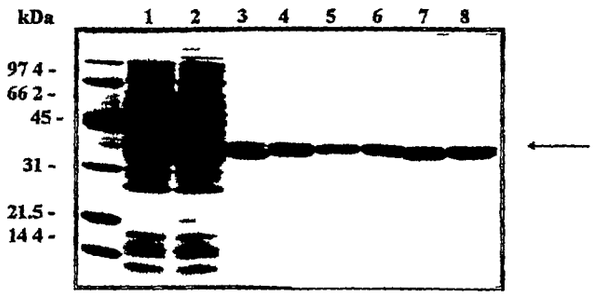
- Aliquots of cell-free extracts as well as insoluble fractions from each of the following strains were separated on a 12 % SDS-gel Lanes 1, cell free extract of uninduced BL-21(pESTAL-11), 2, cell-free extract of induced BL-21 (pESTAL-11c), 3, insoluble fraction of induced BL-21(pESTAL-11c), 4, cell-free extract of induced BL-21(pESTAL-14b), 5, insoluble fraction of induced BL-21(pESTAL-14b)

- The molecular weight of overexpressed RAG-1 wild type esterase and his-esterase were found to be 32 5 and 34 5 kDa respectively This difference in molecular weight corresponds to His-tag sequence and to the thrombin cleavage site which were fused to the wild type esterase

- The recombinant RAG-1 esterase and his-esterase were recovered in cell-free extracts as well as in inclusion bodies

Inclusion body formation of wild type and mutant esterases

Figure 3



- Inclusion body formation in induced strains carrying the alanine mutant proteins of RAG-1 esterase Lane 1-uninduced BL-21(pESTAL-c)(W t esterase), lane 2 - cell-free extract of induced BL-21(pESTAL-11c)(W t esterase) Lanes 3 - 8 represent washed inclusion bodies of strains lane 3 - BL-21(pESTAL-11c)(W t esterase), lane 4- BL-21(pYB-18)(H79A), Lane 5 - BL-21(pYB-19)(S149A), Lane 6- BL-21(pYB-20)(D196A), Lane 7 - BL-21(pYB-21)(E244A), and lane 8 - BL-21(pYB-22)(H274A) The arrow indicates position of the esterase bands

Overexpressed glycine mutants of the RAG-1 esterase in cell-free extracts

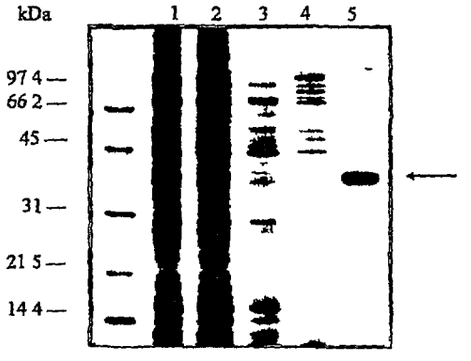
Figure 4

Esterase activity units/mg of protein	12	2200	14	550	9	10	8	10

- SDS-PAGE showing presence or absence of the mutant esterase proteins in sonicated cell extracts of *E coli* BL21 Lane 1) Control - BL21(pESTAL-11c) (W t esterase) without IPTG, Induction with IPTG 2) BL-21 (pESTAL-11c) (W t esterase), 3)BL-21 (pYB-1)(His^{79}Gly), BL21(pYB-2)(Asp^{110}Gly), 5)BL21(pYB-3)(Ser^{149}Gly), 6)BL21(pYB-4)(Asp^{196}Gly), 7) BL21(pYB-5) (Glu^{244}Gly), 8) BL21(pYB-6) (His^{274}Gly) The arrow indicates position of the esterase bands
- The wild type esterase as well as Asp^{110}Gly, Ser^{149}Gly and Glu^{244}Gly mutant proteins were detected in cell-free extracts
- The His^{79}Gly, Asp^{196}Gly and His^{274}Gly mutant proteins were not observed in soluble form, but were detected only in inclusion bodies
- Various efforts to decrease the inclusion body formation were unsuccessful
- Only Asp^{110}Gly mutant showed cell-bound or cell-free esterase activity of about 20% in comparison with wild type esterase

Purification of the wild type esterase by metal-chelation affinity chromatography

Figure 6

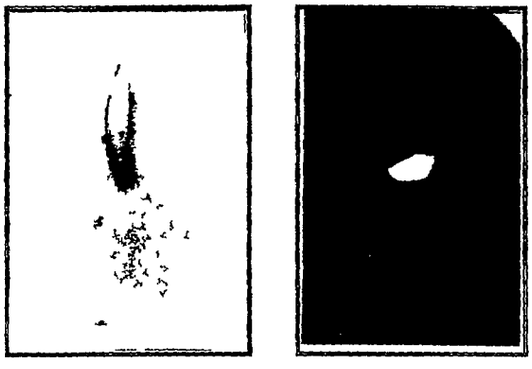


Esterase activity units/mg of protein 2340 331 100 310 50200

- The soluble RAG-1 esterase prepared from induced strain BL-21(pESTAL-14) was purified using Novagen His-Bind Resin Aliquots of column fractions were separated on a 12% SDS-gel and stained by Coomassie blue Lanes 1, start material, 2, column flow-through, 3, washing with 5 mM Imidazole, 4, washing with 80 mM Imidazole, 5, elution with 400 mM Imidazole The arrow indicates the position of the purified esterase protein The molecular mass markers are indicated to the left

Attempts at crystallization of RAG-1 his-esterase

Figure 7



Microcrystals of RAG-1 his-esterase

- The RAG-1 his-esterase was purified using metal-chelation affinity chromatography (Ni-column)
- The best microcrystals were obtained at a pH 8 1 in the presence of 0 2M ammonium sulfate, 10% polyethylene glycol and in the presence of Nonidet-40 detergent at protein concentration higher than 1 5 mg/ml

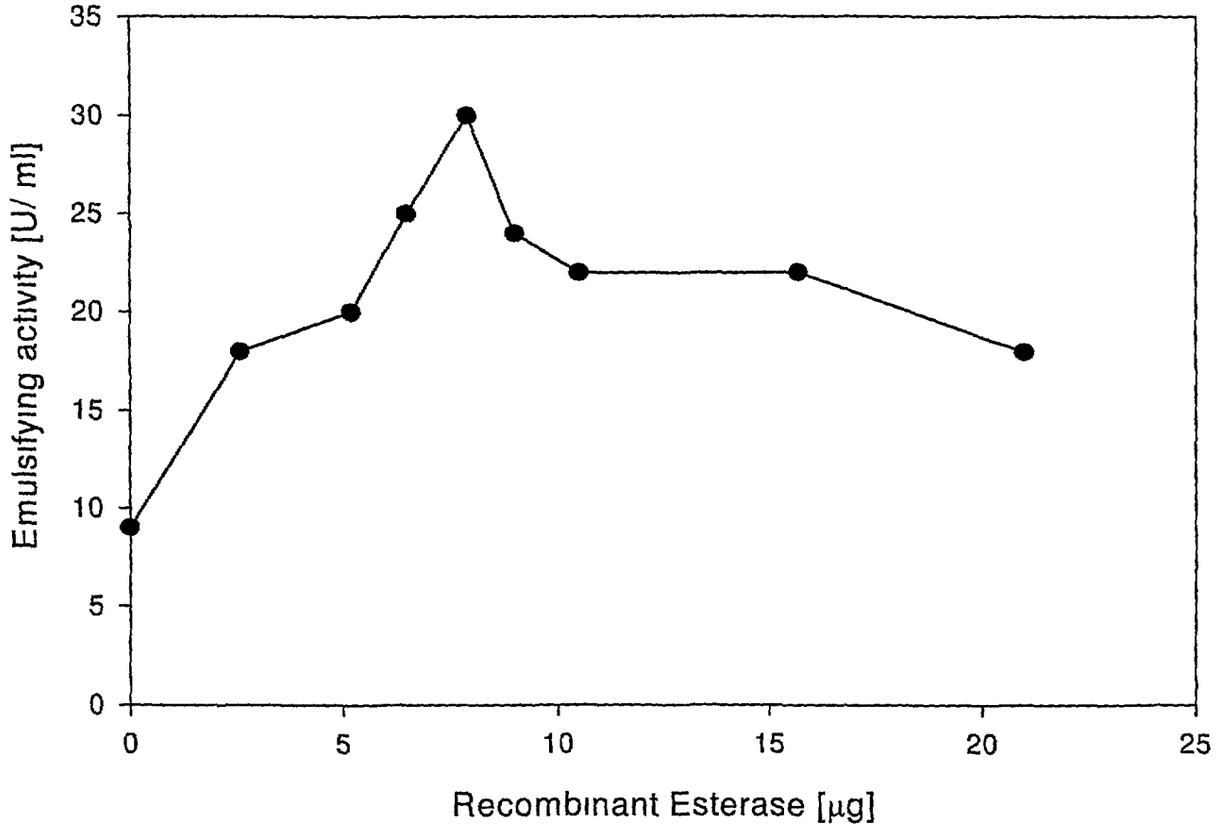


Figure 8 Emulsifying activity enhancement using recombinant RAG-1 esterase (wild-type) in presence of hexadecane 2 Methyl Naphthalene Apoemulsan at a fixed concentration of 60 μg/ml was used as emulsifier

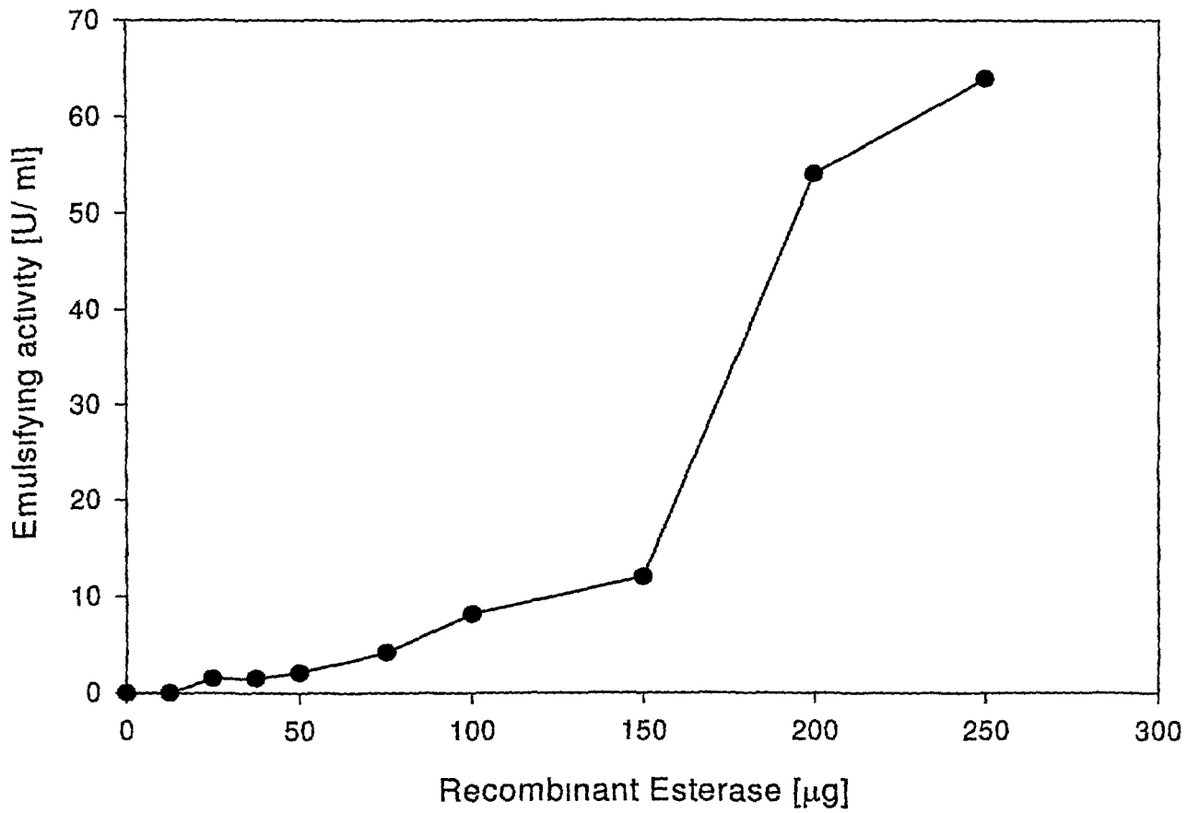


Figure 9 Emulsifying activity enhancement using recombinant RAG-1 esterase (wild-type) in presence of hexadecane Apoemulsan at a fixed concentration of 60 μg/ml was used as emulsifier

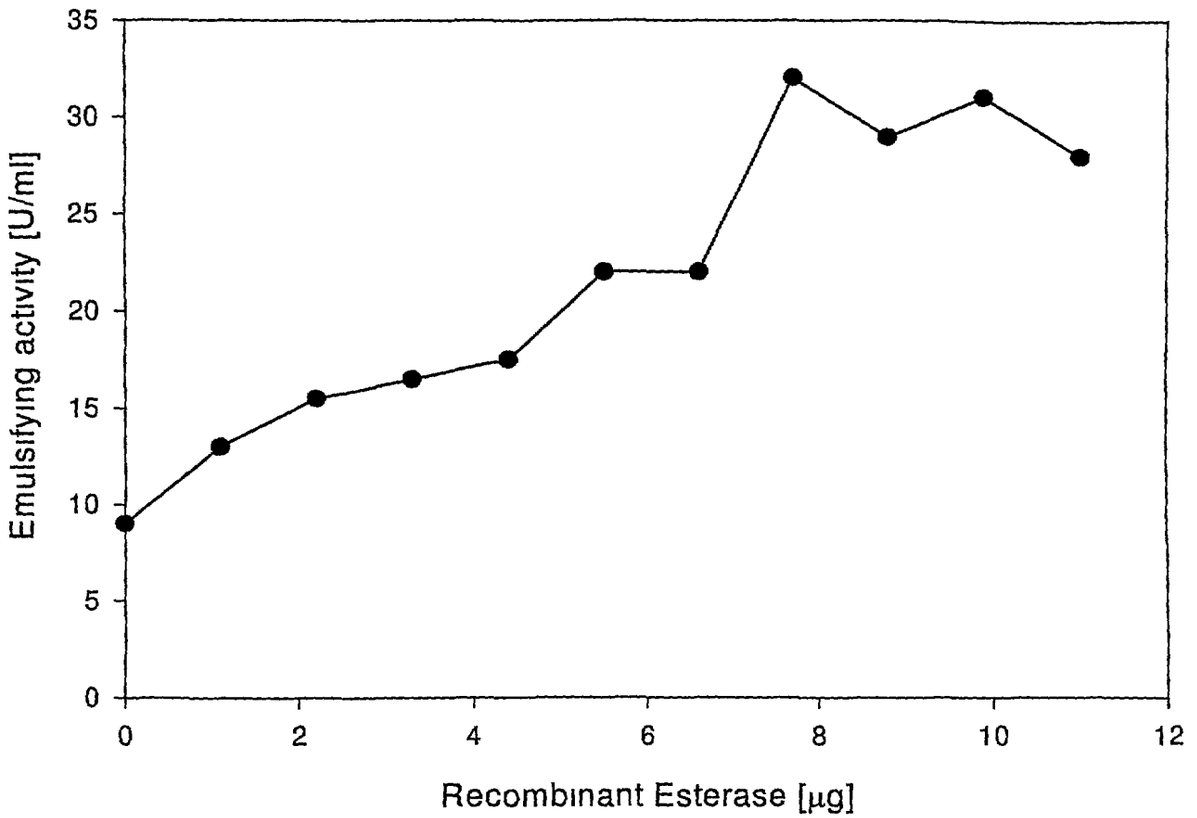


Figure 10. Emulsifying activity enhancement using recombinant RAG-1 negative esterase from $His^{79}Gly$ mutant in presence of hexadecane 2 methyl naphtalene Apoemulsan at a fixed concentration of 60 μg/ml was used as emulsifier

Finally, it was of interest to examine the specificity of reconstitution towards other polysaccharides These results are also currently in progress, although some of our preliminary observations are presented in the following table

Table 4 Cadmium binding to engineered polysaccharides [1]

Polysaccharides [2]	**Cd^{++} binding (μ equivalents/mg biopolymer)**
Apoemulsan	3 9
Colanic acid - Wild-type	1 3
Mutant 1	2 4
Mutant 2	0 5
Mutant 3	4 9
Stewartan - Wild-type	2 9
Mutant 1	0 [3]
Mutant 2	1 1
Mutant 3	1 8

[1] Biopolymerosols were prepared as described and cadmium binding was assayed by atomic absorption spectroscopy

[2] Mutant polysaccharides were produced in amounts only about 1/5 to 1/10 of the amount produced by the wild-type cells

[3] The biopolymerosol formed was too unstable to carry out the binding assay

Table 5. Esterase interaction with water soluble polysaccharides and their conversion to amphipathic bioemulsifiers.[1]

Polysaccharide	**Activity (u/ml)**
Capsular polysaccharide from *Acinetobacter calcoaceticus* BD4	75
Colanic acid	87
Stewartan (capsule from *Erwinia stewartii)*	122
Amylovoran (capsule from *Erwinia amylovora)*	133
Xanthan	39
Gellan	65
Alginic acid	22

[1] Esterase was added at concentration of 150 μg/ml Polysaccharides were added at concentrations of 60 μg/ml Hexadecane was the substrate for emulsification. The polysaccharides were completely inactive in the absence of esterase

Use of protein stabilized polymerosols in adhesion studies

The esterase-stabilized polymerosols described above were used in standard adhesion and anti-adhesion assays as described previously For these experiments, the test organism, RAG-1 was mixed vigorously with a standard volume of hexadecane, octane and xylene on a vortex mixer After standing the adherence of the cells to the hydrophobic surfaces was estimated from the lose of turbidity (due to adhesion of the cells to the hydrocarbons which floated to the surface after phase separation) The assays were repeated in the presence of the various biopolymer formulations in order to estimate the ability of the formulations to prevent adherence of microbes to hydrophobic surfaces

3b Engineering of novel polysaccharides for use in environmental applications.

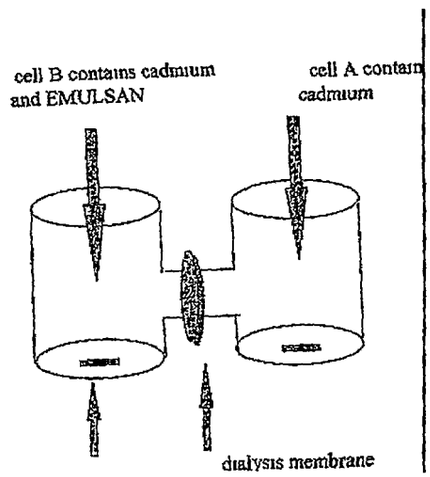
This is an approach which we began before the collaborative project was initiated, but which was continued during both the second and third years of the project In this approach we exploited the fact that certain biosynthetic pathways for polysaccharide production are already known and quite similar The basis of the approach is to use mutants blocked in polysaccharide synthesis in specific steps, and then attempt to complement the defect using cloned genes from a heterolgous organism to complement the defect in biosynthesis If the defect is in a particular transglyosidation reaction which adds an activated sugar onto a growing oligosaccharide chain, the hope was that a heterologous transglycosidase could be introduced which replaced the wild type sugar component with a new sugar in the subunit backbone of the polymer The general approach involved the complementation of a series of biosynthetic mutants defective in the production of the capsular polysaccharide stewartan produced by the corn pathogen *Erwinia stewartii* The mutants were complemented with homologous genes from *E amylovora* as well as with a library from *E coli* The resulting polysaccharide was reocovered from the supernatant fluid of glucose grown cultures, and recovered by precipitation in the presence of 80% ethanol This material was dried and spun in an ultracentrifuge at 100,000 X G for 24h to remove possible residues of lipopolysaccharide, and treated with protease K to remove residual protein Final purification involved two additional cycles of ethanol precipitation The putatively engineered polysaccharides were used in the emulsification, adherence and cation binding assays as described above In addition, they were hydrolyzed and the sugar monomers analyzed in order to determine the changes in polymer composition

3c Characterization of metal binding to the biopolymer emulsan

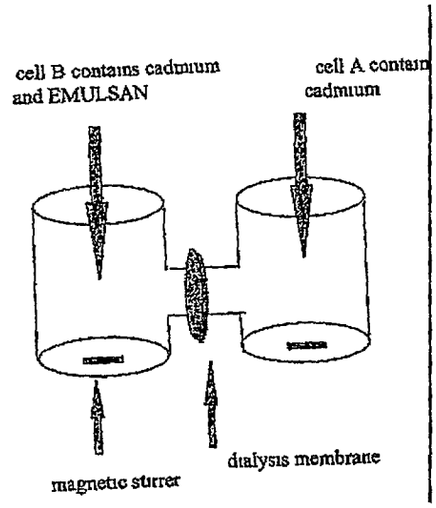
In this work we studied the binding of cadmium and other metal ions to the protein-free biopolymer, apoemulsan which was produced by an ethanol fermentation as previously described The emulsan polymer was first purified by ammonium sulfate precipitation an dialysis followed by treatment with protease K to remove the 10% protein which was associated with emulsan The protein-free material, termed apoemulsan, was still capable of stabilizing oil/water emulsions Previous results had shown that the binding of both organic and inorganic cations to apoemulsan was significantly enhanced when the biopolymer was present in an emulsion at the oil/

when the latter was either free in solution or bound at an apoemulsan-stabilized hexadecane/water emulsion (termed *apoemulsanosol*) The biopolymer-stabilized emulsion was first formed by sonic oscillation of a 3% hexadecane/water suspension in a solution of apoemulsan This emulsion was then concentrated by centrifugation to yield a thick, cream consisting of a hexadecane/water (70% hexadecane) emulsion, the apoemulsanosol At the concentrations used to form the emulsions, virtually all of the added apoemulsan could be recovered within the apoemulsanosol Apoemulsanosols are stable at room temperature for periods of several months before phase separation occurs

Binding measurements In order to carry out the binding experiments we designed a



simple double-cell chamber in which the two cells were separated by a dialysis membrane



Equal concentrations of Cd^{++} were added to each cell (A and B), while various concentrations of either apoemulsan or apemulsanosol were added to Cell B After steady state was reached, the concentration of residual Cd^{++} was monitored in Cell A (in the absence of biopolymer) Binding was determined from the decrease of Cd^{++} cell A Cd^{++} was measured using two methods 1 electrochemically using the Differential Pulse Anodic Stripping Voltometry (DPSAV) technique, and 2 atomic absorption spectroscopy Results from the two techniques were found to be within 15% of each other, although the atomic absorption spectroscopy was somewhat less cumbersome and more routine In all cases the results were determined from an average of three independent measurements for each concentration

Using the chamber described above we developed cadmium binding to apoemulsan and apoemulsanosols was determined using both the DSPAV electrochemical technique as well as atomic absorption spectroscopy The results

presented here were based on the atomic absorption measurements for cadmium Apoemulsan, with a molecular mass of 10^3 kilodalton, has been shown to 1295 carboxylate anions in the form of uronic acid moieties It was found that at concentrations of apoemulsan free in solution as low as 0 4μg/ml, one molecule of the polymer bound up to 1375 equivalents of cadmium ion In agreement with previous results, this suggests that the binding is stoichiometric In contrast, when present in an apoemulsanosol, the same biopolymer was able to bind 2125 equivalents of cadmium This represents a 60% increase in binding over that observed with the biopolymer free in solution The results are in agreement with previous results obtained for binding of both uranyl ion as well as the organic cation rhodamine The results are consistent with the notion that the biopolymer undergoes a conformational change at the oil/water interface of a biopolymerosol which enables greater than stoichiometric binding Moreover, when the apoemulsanosol containing the bound cadmium was washed in solutions at different pH, the cadmium could be completely removed by lowering the pH below 3 05 (the pK_{app} of the uronic acid residues)

4 DEVELOPMENT OF A MODEL SYSTEM FOR METABOLIC ENGINEERING OF NEW PATHWAYS INTO ROBUST, ENVIRONMENTALLY USEFUL BACTERIAL STRAINS

The approach taken here was to develop models for metabolic engineering of the oil-degrading RAG-1 strain which we have found to be quite robust in various environmental situations The spectrum of hydrocarbons normally degraded by RAG-1 includes straight chain aliphatic hydrocarbons (C12-C20) The model system chosen was to introduce the genes for aromatic degradation from the *tol* plasmid of *Pseudomonas putida* into RAG-1 and select for strains which retained their ability to grow on crude oil, produce emulsan, and exhibit all of the properties of the original

strain coupled with the expression of new genetic potential for degrading aromatic hydrocarbons Two general approaches were used to engineer RAG-1 to grow on aromatics

4a Conjugation

In the first approach the entire pTOL was introduced by conjugation from the parent organism into RAG-1 selecting for kanamycin resistant ex-conjugants capable of utilizing toluene or xylene as sole sources of carbon and energy The conjugation was carried out in a triparental mating system in which *E coli* carrying pRK200 was used as a helper to promote the transfer of pTOL from *P putida* into RAG-1 selecting for kanamycin resistance on plates containing crude oil as a carbon source (to select against the donor and helper strains, respectively and kanamycin as a marker to select against the recipient Exconjugants were then screened for their ability to grow on plates supplemented with either toluene or xylene as carbon sources in the vapor phase

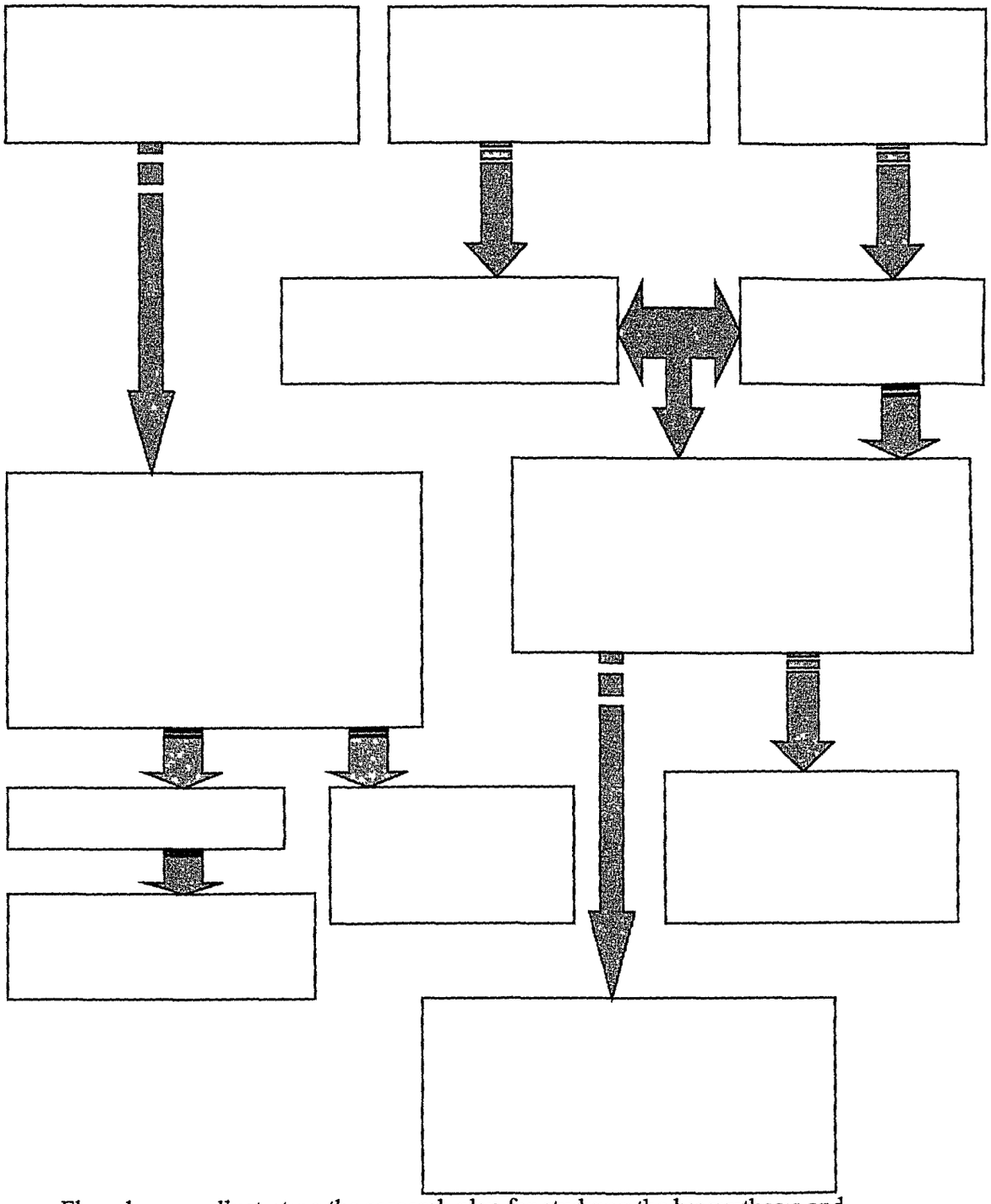
4b Recombination into the chromosome

Results from the conjugation experiments demonstrated that RAG-1 was able to grow on benzoic acid via the ortho- pathway It, therefore seemed reasonable to try to engineer RAG-1 to grow on aromatics, by introducing the so-called "upper" *xyl* operon into the chromosome In the second approach, the *xyl* "upper" operon together with *xyl* R was introduced as a 13 kb insert into the *est* gene and the entire construct flanked on both sides with the 500 b p of the *est* was cut out of the construct using Sph I and subsequently extracted from an agarose gel This linear fragment was then introduced into RAG-1 via electroporation and recombinants were selected which had the ability to grow on toluene Recombination into chromosome occurred via homologous recombination between the *est* flanking sequences, and the *est* in the chromosome Recombinants were then screened for emulsifier production, growth on crude oil, adherence to hydrocarbon, and esterase activity Genomic DNA of the recombinants was extracted, cleaved with Sal I to yield an 8 kb fragment which was probed with either *est* or *xyl* R in Southern hybridization experiments In addition, PCR analysis was used to amplify both *xyl* R and *est* from the chromosome in order to demonstrate the presence of specific sequences following recombination

5 STUDIES ON THE BIOSYNTHESIS OF THE CATION BINDING BIOPOLYMER EMULSAN DISCOVERY OF A NEW GENE POTENTIALLY INVOLVED IN A SIGNAL TRANSDUCTION PATHWAY CONTROLING BIOEMULSIFIER SYNTHESIS

Thus far, little is known concerning neither the organization nor the regulation of the genes required for emulsan production We have therefore initiated a study of these aspects of emulsan production and present here our preliminary results The overall scheme for this part of the research is outlined in the flow diagram illustrated

below A genetically modified derivative of Tn10, mini-Tn10PttKm (developed by our cooperative partner in this project, V de Lorenzo) was used for isolating emulsan-defective mutants of *A lwoffii* RAG-1 The plasmid was transferred in plate matings from *E coli* S17[(λpir)(pLOFPttKm)] to *A lwoffii* RAG-1, and selecting for kanamycin resistance Emulsan deficient transconjugants were isolated on the basis of their characteristic translucent colony morphology The transconjugants were then screened for resistance to phage ap3 which utilizes cell-bound emulsan as receptor In addition, the mutants were screened for extracellular emulsifying activity using the a standard assay The mini-Tn10Km^r was labeled using the DIG system and used as probe in Southern hybridization experiments with EcoR1-digested genomic DNA of the insertion mutants Thus far 26 mutants were screened The approach includes cloning specific fragments into into pUC18 and subsequent sequencing with the help of the automatic sequencing unit provided by the Faculty of Life Sciences Thus far, the entire *ptk* (protein tyrosine kinase) gene from RAG-1 along with the *ptp* (phosphotyrosine phosphatase) gene were completely sequenced Experiments are currently in progress to characterize the biosynthetic intermediates which may accumulate in the mutants In addition, phosphorylation studies and the purification of PTK and PTP are currently underway Finally, the entire regulon has been cloned and sequencing studies are currently underway



Flow diagram illustrating the research plan for studying the biosynthesis and regulation of emulsan production

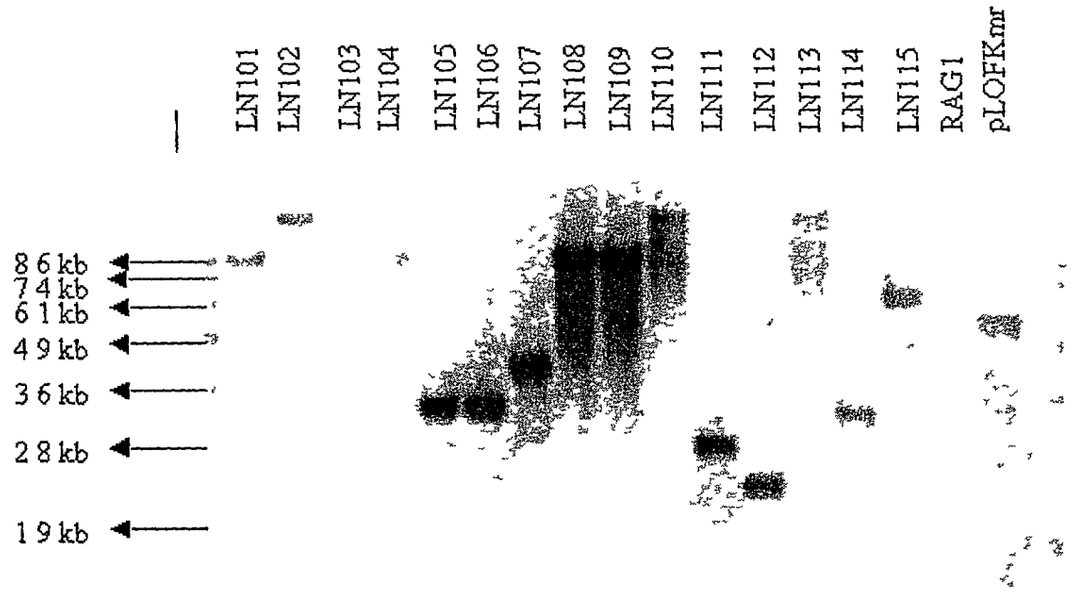


Figure 12 The probe mini-Tn10 was DIG labeled and hybridized to EcoR1-digested genomic DNA of 15 *A lwoffii* RAG-1 transconjugants with a translucent morphology The negative control was *A lwoffii* RAG-1 and the positive control was EcoRI digested pLOFKm^r

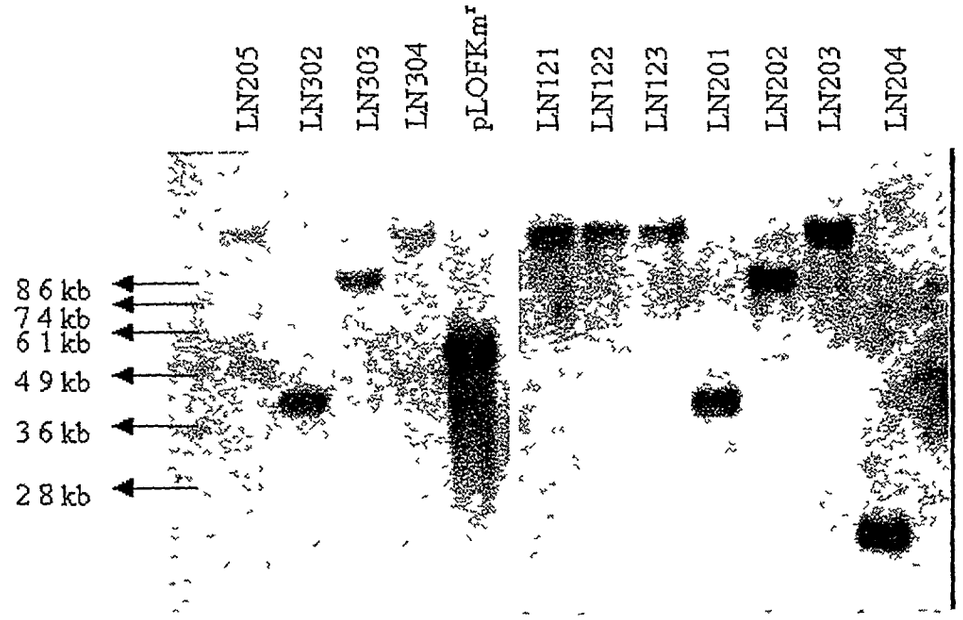


Figure 13 The probe mini-Tn10 was DIG labeled and hybridized to EcoR1-digested genomic DNA of 11 *A lwoffii* RAG-1 transconjugants with a translucent morphology The positive control was EcoRI digested pLOFKm^r

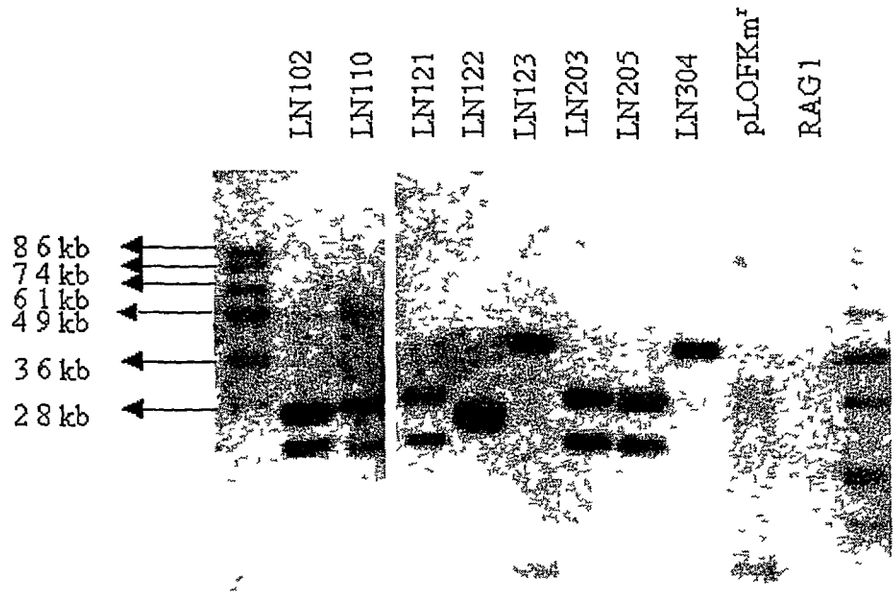


Figure 14 The probe mini-Tn10 was DIG labeled and hybridized to HindIII-digested genomic DNA of 8 *A lwoffii* RAG-1 transconjugants with a 10 5 kb EcoRI fragment consisting the transposon The positive control was HindIII digested pLOFKm^r

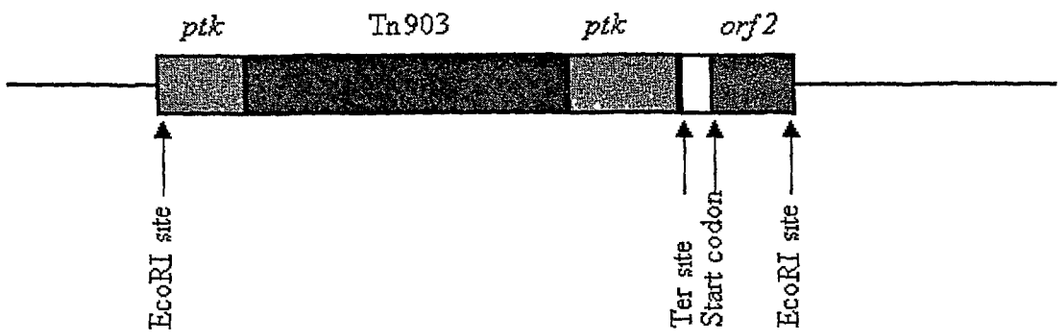


Figure 15 The genetic map of the cloned fragment of mutant *A lwoffii* LN201

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                      421    430       440       450       460       470       480       490
                      |--------+---------+---------+---------+---------+---------+---------|
orf1_A.lwoffiiRAG-1   TQLNSTLKQLPDIQRQYLQLFREVEVKTQLYTALLNSYQQLRIAKAGEIGNVRIVDTAVEPVEPIKPKKL
    ptk_A.johnsonii   GELNATLKELPDLQRRYLQLYREVEVKQQLYTALLNSYQQLRIAKAGEIGNVRIVDTAVEPIEPIAPKKL
  orf6_K.pneumoniae   ATM-------PETQQEILRLSRDVESGRAVYMQLLNRQQELNIAKSSAIGNVRIIDSAVTQHKPVKPKKI
   amsA_E.amylovora   SQM-------PQTQQEIVRLTRDVQAGQEIYMQLLNRQQELNISKASTVGDVRIIDHAETAAKPVAPKSI
epsB_P.solanacearum   NSMTRRLKSLPNIEQDTVRLMRDVQVDNELYVSLLNDMQQLKLYKAGKVGNVRLVDGAAVPEEPVKPKKL
          Consensus     m   lk lP iqq  lrL RdVev   lY  LLN  QqL iaKag iGnVRivD Av p ePvkPKkl

                      491    500       510       520       530       540       550       560
                      |--------+---------+---------+---------+---------+---------+---------|
orf1_A.lwoffiiRAG-1   QILILSIFLGGFLGTLLALLRNMLRTGVKDSTQIEREFDLPVYATVPRSPVQETRMSI--LKK------K
    ptk_A.johnsonii   QILILSIFLGGFLGTLLALLRNMMRSGIKDSTQIENELDLPVYATVPRSPVQESRINI--LKK------K
  orf6_K.pneumoniae   IVVLAGLFIGLVISVSLVLVRILLRKGIETPEQLEEL-GINVYASIPVSESNPKNVIAKRLNKRDDSRPK
   amsA_E.amylovora   LIVAGSLILGLVVSVGLVLMKALFHHGIDNPEQLEEL-GLNVYASVPLSEWQRKKDQETLLKRKLDARTD
epsB_P.solanacearum   TVTPLAGVLGVVLGVMAAFVRNALFGGITDPQDIEEHTGLSVYATVPLSDTQVD------LSGQLTTR-K
          Consensus    i  ls flG vlgv lal rn lr Gi dp qiEe  gl VYAtvP S  q        Lkk    r k

                      561    570       580       590       600       610       620       630
                      |--------+---------+---------+---------+---------+---------+---------|
orf1_A.lwoffiiRAG-1   KSIPILAVKHSDDIAIESLRSIRTAIHFSLASAKNNIIMIAGPAPEVGKSFISTNLSTIFAQSDKKVLLI
    ptk_A.johnsonii   KNIPILAVKNSDDIAIESLRSMRTAIHFALSSARNNLITISGPAPEVGKSFISTNLATILAQSDKRVLII
  orf6_K.pneumoniae   V---LLATENPADLAIEAIRGLRTSLHFAMLEARNNLLMISGASPNAGKTFVSSNLSSVISQTGKKVIFI
   amsA_E.amylovora   PHNRLLALGNPTDLSIEAIRSLRTSLHFAMMDAQNNILMITGASPGIGKTFVCANLATLVAKTGEKVLFI
epsB_P.solanacearum   RGQYLLARRVPDDPSIEALRSLRTALQFAMQDAGNNLVVLTGPTPGVGKSFVSANLAAVIATGGKRVLLI
          Consensus      lLA  npdD aIEalRslRTalhFam  A NNl mi Gp P vGKsFvs NLat  aq gkkVllI

                      631    640       650       660       670       680       690       700
                      |--------+---------+---------+---------+---------+---------+---------|
orf1_A lwoffiiRAG-1   DADMRRGYMHKYFDVDIKPGLSEFLSNQAELSQVIHQTEVNGLNVITRGKSPTNPSEMLGSQKFKALLDE
    ptk_A.johnsonii   DADLRRGYLHKYFNLDTQPGLTELLNGQQSLETVIRHTEVPGLSVISRGKSPANPSELLSSNQFKNLLEQ
  orf6_K.pneumoniae   DADLRKGYTHKLFNIKNTNGLSDYLSGRVALDKIINNLQTEGFDYISRGSVPPNPAELLMHNRLAELLEW
   amsA_E.amylovora   DGDMRRGYTHELLGAESKTGLSDILSGKLPFNTDLVQRGDYGFDFIARGQVPPNPSELLMNSRMKELVHW
epsB_P solanacearum   DADMRKGYLHQYFGKDRKPGLLDLLAGNRSIEQVVHREVVPGLDFIATGLFPHNPSELLLNPRMVELMDT
          Consensus   DaDmRrGYlHkyf  d kpGLsdlLsg  sleqvih   vpGld I rG  P NPsElL   r keLld

                      701    710       720       730       740       750       760       770
                      |--------+---------+---------+---------+---------+---------+---------|
orf1_A.lwoffiiRAG-1   LSQQYDHIIIDTPPILAVTDGIIISQYAGVNLLIARYSKSQMKELELSLNRFEQAGVKVNGFILNDIQRT
    ptk_A.johnsonii   MSEKFDHVIIDTPPVLAVTDGIIISQYTGVNLVIARYAKTQMKELELTLNRFEQAGVKVNGFILNDIQRS
  orf6_K.pneumoniae   ANKSYDIVILDTPPILAVADAAIIGNYVGTTLLVARFEENTPKEIDISVKRFQNSGVNIKGCILNGVVKK
   amsA_E.amylovora   ASQNYDLVLIDTPPILAVTDASIIGKLAGTSLMVARFETNTVKEVEISYKRFIQNGIDIKGIILNAVVRK
epsB_P.solanacearum   FRSQYDLVLVDTPPVLAVADTAILAARAGLVLLVTRFERSTLGEIRETIKQLQHANVDVRGVVFNALDPN
          Consensus    s qyD viiDTPPiLAVtD  Ii  yaG  LlvaRfe st kE e s krf qagv v G ilN   r

                      771    780       790       800 804
                      |--------+---------+---------+---|
orf1_A.lwoffiiRAG-1
    ptk_A.johnsonii   SAGYGYGYGYNYA------YAYKANK-----ESD
  orf6_K pneumoniae   AT-NKYGYGYNYYDYSYSD-KK
   amsA_E.amylovora   SA-NNYGYGYDYYDYSYQQ-GEKS
epsB_P.solanacearum   -T-YRYGYGSRYGRYRYVQYGYTSNSKPPEAESA
          Consensus     y ygygy y  y y    y
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                    |--------+--------+--------+--------+--------+--------+--------|
orf1_A.lwoffiiRAG-1 TQLNSTLKQLPDIQRQYLQLFREVEVKTQLYTALLNSYQQLRIAKAGEIGNVRIVDTAVEPVEPIKPKKL
    ptk_A.johnsonii GELNATLKELPDLQRRYLQLYREVEVKQQLYTALLNSYQQLRIAKAGEIGNVRIVDTAVEPIEPIAPKKL
  orf6_K.pneumoniae ATM-------PETQQEILRLSRDVESGRAVYMQLLNRQQELNIAKSSAIGNVRIIDSAVTQHKPVKPKKI
   amsA_E.amylovora SQM-------PQTQQEIVRLTRDVQAGQEIYMQLLNRQQELNISKASTVGDVRIIDHAETAAKPVAPKSI
epsB_P.solanacearum NSMTRRLKSLPNIEQDTVRLMRDVQVDNELYVSLLNDMQQLKLVKAGKVGNVRLVDGAAVPEEPVKPKKL
          Consensus  n   lk IP iqq  IrL RdVev   IY  LLN  QqL iaKag iGnVRivD Av p ePvkPKkl

                    491    500       510       520       530       540       550       560
                    |--------+--------+--------+--------+--------+--------+--------|
orf1_A.lwoffiiRAG-1 QILILSIFLGGFLGTLLALLRNMLRTGVKDSTQIEREFDLPVYATVPRSPVQETRMSI--LKK------K
    ptk_A.johnsonii QILILSIFLGGFLGTLLALLRNMMRSGIKDSTQIENELDLPVYATVPRSPVQESRINI--LKK------K
  orf6_K.pneumoniae IVVLAGLFIGLVISVSLVLVRILLRKGIETPEQLEEL-GINVYASIPVSESNPKNVIAKRLNKRDDSRPK
   amsA_E.amylovora LIVAGSLILGLVVSVGLVLMKALFHHGIDNPEQLEEL-GLNVYASVPLSEWQRKKDQETLLKRKLDARTD
epsB_P.solanacearum TYTPLAGVLGVVLGVMAAFVRNALFGGITDPQDIEEHTGLSVYATVPLSDTQVD------LSGQLTTR-K
          Consensus  i  ls flG vlgv lal rn lr Gi dp qiEe  gl VYAtvP S  q       Lkk    r k

                    561    570       580       590       600       610       620       630
                    |--------+--------+--------+--------+--------+--------+--------|
orf1_A.lwoffiiRAG-1 KSIPILAVKHSDDIAIESLRSIRTAIHFSLASAKNNIIMIAGPAPEVGKSFISTNLSTIFAQSDKKVLLI
    ptk_A.johnsonii KNIPILAVKNSDDIAIESLRSMRTAIHFALSSARNNLITISGPAPEVGKSFISTNLATILAQSDKRVLII
  orf6_K.pneumoniae V---LLATENPADLAIEAIRGLRTSLHFAMLEARNNLLMISGASPNAGKTFVSSNLSSYISQTGKKVIFI
   amsA_E.amylovora PNNRLLALGNPTDLSIEAIRSLRTSLHFAMMDAQNNILMITGASPGIGKTFVCANLATLVAKTGEKVLFI
epsB_P.solanacearum RGQYLLARRVPDDPSIEALRSLRTALQFAMQDAGNNLVVLTGPTPGVGKSFVSANLAAVIATGGKRVLLI
          Consensus     lLA  npdD aIEalRslRTalhFam  A NNl mi Gp P vGKsFvs NLat  aq gkkVlLI

                    631    640       650       660       670       680       690       700
                    |--------+--------+--------+--------+--------+--------+--------|
orf1_A.lwoffiiRAG-1 DADMRRGYMHKYFDVDIKPGLSEFLSNQAELSQVIHQTEVNGLNYITRGKSPTNPSEMLGSQKFKALLDE
    ptk_A.johnsonii DADLRRGYLHKYFNLDTQPGLTELLNGQQSLETVIRHTEVPGLSYISRGKSPANPSELLSSNQFKNLLEQ
  orf6_K.pneumoniae DADLRKGYTHKLFNIKNTNGLSDYLSGRVALDKIINNLQTEGFDYISRGSVPPNPAELLMNNRLAELLEW
   amsA_E.amylovora DGDMRRGYTHELLGAESKTGLSDILSGKLPFNTDLVQRGDYGFDFIARGQVPPNPSELLMNSRMKELYHW
epsB_P.solanacearum DADMRKGYLHQYFGKDRKPGLLDLLAGNRSIEQVVHREVYPGLDFIATGLFPHNPSELLLNPRMYELMDT
          Consensus DaDmRrGYlHkyf  d kpGLsdlLsg  sleqvih   vpGld I rG  P NPsElL   r keLld

                    701    710       720       730       740       750       760       770
                    |--------+--------+--------+--------+--------+--------+--------|
orf1_A.lwoffiiRAG-1 LSQQYDHIIIDTPPILAVTDGIIISQYAGVNLLIARYSKSQMKELELSLNRFEQAGVKVNGFILNDIQRT
    ptk_A.johnsonii MSEKFDHVIIDTPPVLAVTDGIIISQYTGVNLVIARYAKTQMKELELTLNRFEQAGVKVNGFILNDIQRS
  orf6_K.pneumoniae ANKSYDIVILDTPPILAVADAAIIGNYVGTTLLVARFEENTPKEIDISVKRFQNSGVNIKGCILNGVVKK
   amsA_E.amylovora ASQNYDLVLIDTPPILAVTDASIIGKLAGTSLMVARFETNTVKEYEISYKRFIQNGIDIKGIILNAVVRK
epsB_P.solanacearum FRSQYDLVLVDTPPVLAVADTAILAARAGLVLLVTRFERSTLGEIRETIKQLQHANVDVRGVVFNALDPN
          Consensus  s qyD viiDTPPiLAVtD  Ii  yaG  LlvaRfe st kE e s krf qagv v G ilN   r

                    771    780       790       800 804
                    |--------+--------+--------+---|
orf1_A.lwoffiiRAG-1
    ptk_A.johnsonii SAGYGYGYGYNYA------YAYKANK-----ESD
  orf6_K.pneumoniae AT-NKYGYGYNYYDYSYSD-KK
   amsA_E.amylovora SA-NNYGYGYDYYDYSYQQ-GEKS
epsB_P.solanacearum -T-YRYGYGSRYGRYRYVQYGYTSNSKPPEAESA
          Consensus    y ygygy y  y y    y
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CDR Grant No TA-MOU-94-CA14-043

Production of Microbial Biosurfactant for Environmental Applications

by

Dr Aliya Baryevna Manasbaeva, Institute of Microbiology, Ministry of Science - Academy of Sciences, Republic of Kazakhstan

REPORT

1 Introduction and background

During the period 1996-1998 studies were conducted to investigate the various microbial strains in order to characterize their ability to produce bioemulsifying agents There are major sources of pollution in a variety of industries in Kazakstan and we hope to investigate the potential exploitation of microbial biodegradation and biosurfactant production as approaches to clean-up technology of this pollution The increasing the availability of hydrocarbons to microorganisms is useful method to accelerate the rate of biodegradation at contaminated sites There are also many another applications of microbial surfactants because the study of them role in different processes which are connected with there ability to mediate the adsorption to or detachment from an interface is extremely useful with the point of view of the utilitarianism approach

2 Objectives

The ultimate goal of this project is to develop a technology for the isolation, production and characterization of specific microbial biosurfactants for aplication in pollution control and environmental mainenance Specific objecives are

1 To develop a screening program for the isolation of surfactant-producing organisms which produce biosurfactants and surface active biopolymers with novel properties

2 To isolate, characterize and compare microbial biosufactants from existing strains and newly isolated organisms

3 To investigate the physiological characteristics of growth and biosurfactant production with selected strains chosen on the basis of results obtained from Objectives 1 and 2 above

3 Materials and Methods

Microorganism strains We have concentrated on two bacterial and two yeast strains 1 *Pseudomonas alcaligenes*, 2 *Acetobacter aceti* and 3 *Rhodotorula glutinis/var glutinis*, 4 *Candida krusei* (collection of Institute of Microbiology and Virology, Ministry of Science - Academy of Science, Republic of Kazakstan) This cultures produce bioemulsifying agents under growth in mineral liquid medium with a single source of carbon which is ethanol We have previously shown that all of these strains produce biosurfactants

In addition, we have also focused some attention on a new bioemulsifier produced by the iron-oxidizing organism *Thiobacillus ferrooxidans* *T ferrooxidans* is a gram-negative obligate chemolithotroph active in the bioleaching of minerals The basis for working with this strain was the fact that bacterial adhesion which is the initial step in bacterial bioleaching changes the surface properties of pyrite in crushed fine coal from hydrophobic to hydrophilic We hypothesized that such a change may be related to the the production of new microbial biosurfactants

Medium and growth conditions

A single colony from plates with nutrient agar was incubated into tubes with nutrient agar fron 1 to 9 days These cultures served as inoculums for growth experiments All strains

were cultivated in a minimal ethanol salts medium (8E medium) containing (per liter of water) 1,5 g of $(NH_4)_2HPO_4$, 1 g of KH_2PO_4, 0,5 g of NaCl, 0,8 g of $MgSO_4$, and 5,10,or 20 ml absolute ethanol provided as sole source of carbon and energy Volume of medium in Erlenmejer flask was 125 ml Cultures were grown on shakerunit with 180 rpm, at 30°C and growth was monitored by absorbence at 540 nm with a photocolorimeter FEC-56M starting with initial concentration 20-30 mg/ml (dry cell weight)

Minimal agar media was liquid 8E media solidified with 2% agar On this media growth was determined viablity

Cell surface hydrophobisity index (H%) The microorganisms were grown at 30°C with shaking in nutrient broth or in 8E medium, harvested at different growth phases and resuspended in 8E medium to an initial absorbance at 400 nm to approx 0,3 To test tubes (10 mm diameter) containing 1,8 ml cell suspension were added 0,2 ml n-hexadecane Following 10 min the mixtures were agitated uniformly for 2 min After allowing 10 min for the hydrocarbon phase to rise completely, the aqueous phase was care fully removed with a Pasteur pipette and transferred to a cuvette Light absorbance was determined at 400 nm, using photocolorimeter FEC-56M Data are presented as H (%)

$$H (\%) = 100 - (D_{exp} \times 100)/D_{contr},$$

where H (% hydrophobicity index,

D_{exp} 400nm),

D_{contr} - optical density of suspension (400nm)

Emulsification assay For determination of emulsifying activity in the cell-free culture broth we used a standard emulsification assay wich is based on emulsification of a mixture of 0,2 ml of hexcadecane 2-methylnaphthalene (1 1 [v/v]) in 2 ml broth After the mixture was shaken for 2 min at 25° C it was allowed to stand unshaken for an additional 10 min after which

the turbidity of the stable hydrocarbon-in- water emulsion was measured at 600 nm in photocolorimeter FEC-56M

Surface tension The surface tension of the biosurfactant solutions was measured by the method of Wilgelmi using the tensiometer of Dyu-Nui

Growth on different hydrocarbons In order to determine the capacity of strain to grow on different hydrocarbons, microorganisms were cultivated either on minimal (8E) solid agar medium or in liquid media (8E) supplemented with the following hydrophobic substrates as single source of carbon gasoline, kerosene, diesel fuel, vaseline oil, sunflower oil, hexadecane, and crude oil (2% [vol/vol])

Hydrocarbon dispersion assay The emulsification of a variety of pure hydrocarbons and mixtures of aromatic and aliphatic hydrocarbons was tested in order to determine the biosurfactant hydrocarbon substrate specificity The range of oils and greases included gasoline, kerosene, diesel fuel, vaseline oil, margarine, sunflower oil, hexadecan and crude oils 1,2,3, respectively Crude oil 1 contains paraffins, crude oil 2

crude oil 3 For screening we used standard assay wich is based on measuring the increase in turbidity resulting from the emulsification of some hydrocarbons following reciprocal shaking (180rpm) for 2 h at 30°

Crude preparations were obtained by centrifugation (at 10,000 x g for 10 min) of cultures which were grown in minimal liquid medium The cell-free supernatant fluids stored as a lyophilized powders

Cetyl trimethyl ammonium bromide (CTAB)-tolerance assay The CTAB-tolerance assay is based on exposure of cells to various CTAB concentrations in defined minimal media (8-E) or drinking water with series of culture tubes containing 1,9 ml of medium (or water) and increasing CTAB concentrations up to 30 mg/l at intervals of 2 mg/l The cultures were shaken at 30°C for 24 h At the end of the incubation the cultures were examined for growth CTAB

tolerance (expressed in milligram of CTAB per milliliter) was determined as the highest concentration of the detergent in the presence of which growth occurred

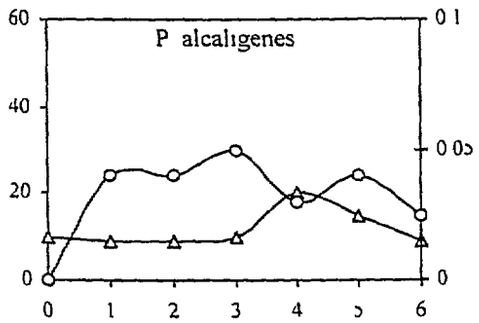
4 RESULTS

Our results showed that CTAB tolerance of yeast *Rhodotorula glutinis var glutinis* is 22 mg/l, CTAB tolerance of yeast *Candida krusei* and bacteria *Pseudomonas alcaligenes* are 28 mg/l When we used water with CTAB instead 8-E media yeast *Candida krusei* did not demonstrate growth on the solid media after incubation with toxic cationic surfactant

To investigate aggregation of bacteria Pseudomonas lcaligenes Acetobacter aceti and yeast Rhodotorula glutinis var glutinis, Candida the cell surface hydrophobicity index (H%) as flocculation factor was defined In some phases of growth negative H% were obtained for P lcaligenes, A aceti, R glutinis in the first day of cultivation on nutrient broth and for C krusei - in the third day of growth on mineral liquid medium (Fig 1)

The negative H% was not described earlier It was possible when optical density in test variant is more then in control In this situation cells had capacity to disperse the hydrophobic substrate, to adsorb on droplets and to keep its in water As result the optical density increased Emulsifying activity of cultural liquid was found when H% was negative We suggest, that the cell-bound biosurfactants are able to keep the hydrophobic droplets in water and cell itself carries a function of surface active molecular This property we called as "hyperhydrophobicity", and the phenomena as "effect of hyperhydrophobicity"

1


P alcaligenes
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20
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0 1
0 05
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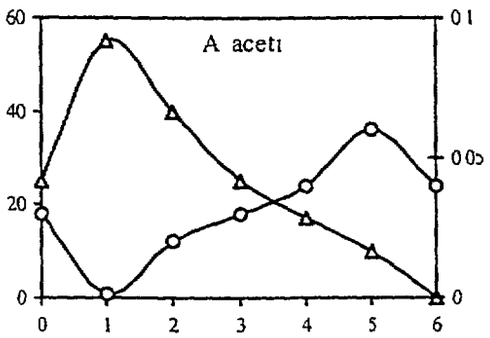
2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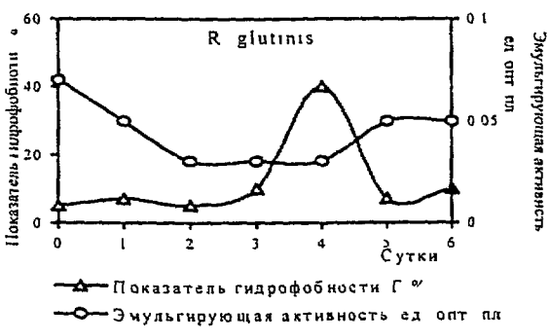
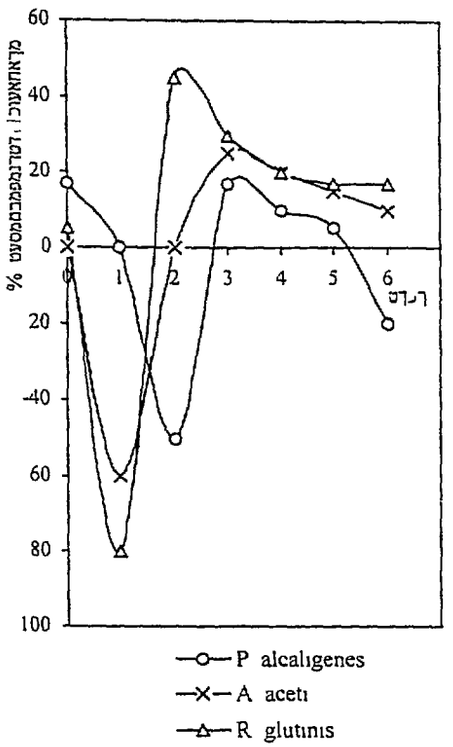
3


R glutinis
Показатель гидрофобности %
Эмульгирующая активность ед опт пл
Сутки
Показатель гидрофобности Г %
Эмульгирующая активность ед опт пл

4


% выделения углеводородного слоя
Сутки
P alcaligenes
A aceti
R glutinis

5

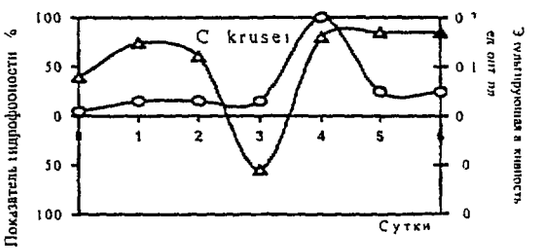
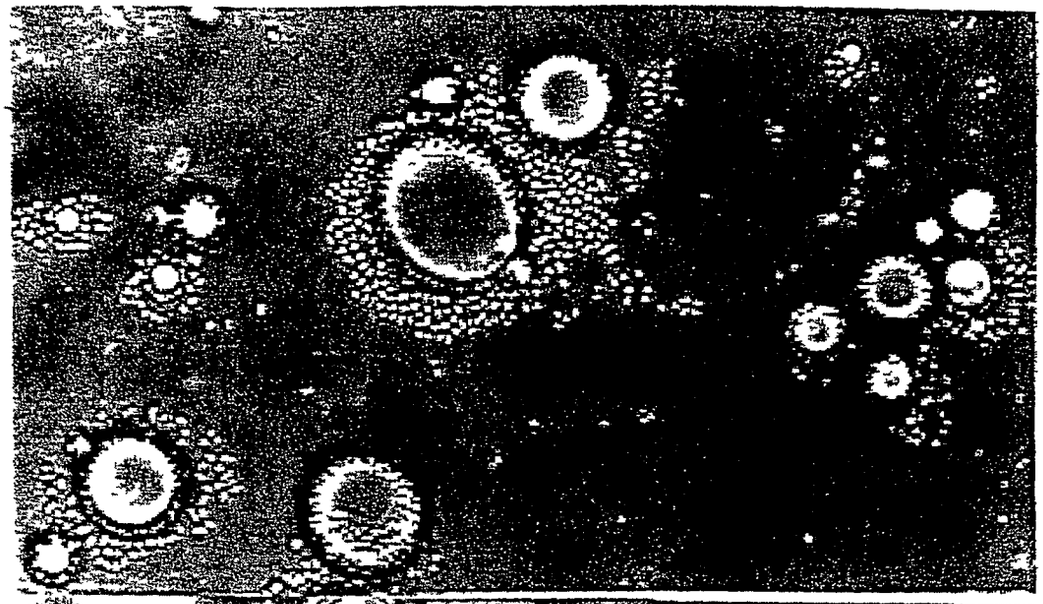

C kruse
Показатель гидрофобности %
Эмульгирующая активность ед опт пл
Сутки

Fig 1 Cell surface hydrophobicity index (H%) and emulsifying activity culture medium under bacteria Pseudomonas lcaligenes Acetobacter aceti and yeast Rhodotorula glutinis var glutinis, Candida krusei grouth in a minimal ethanol salt medium (1,2,3,5) and in nutrient broth (4)

1



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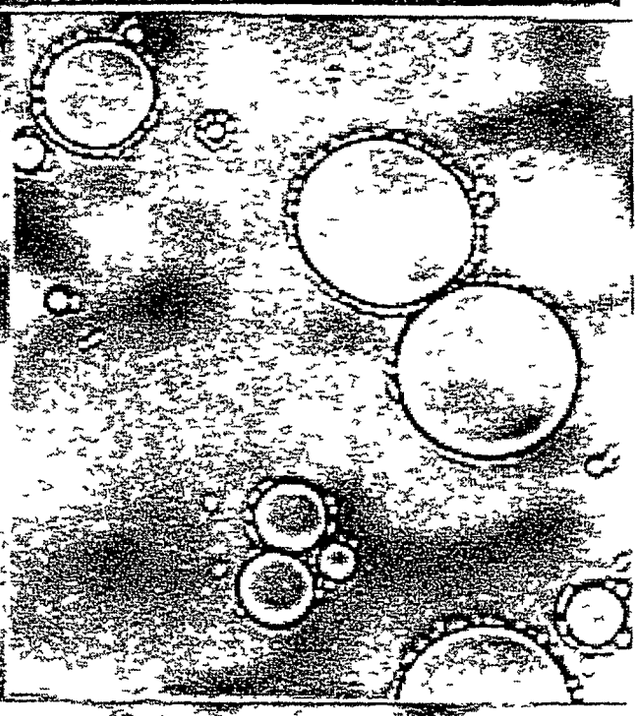

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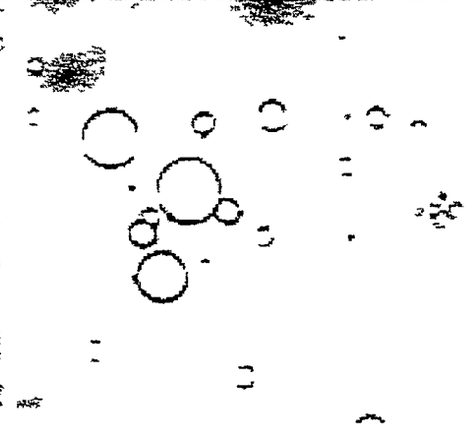


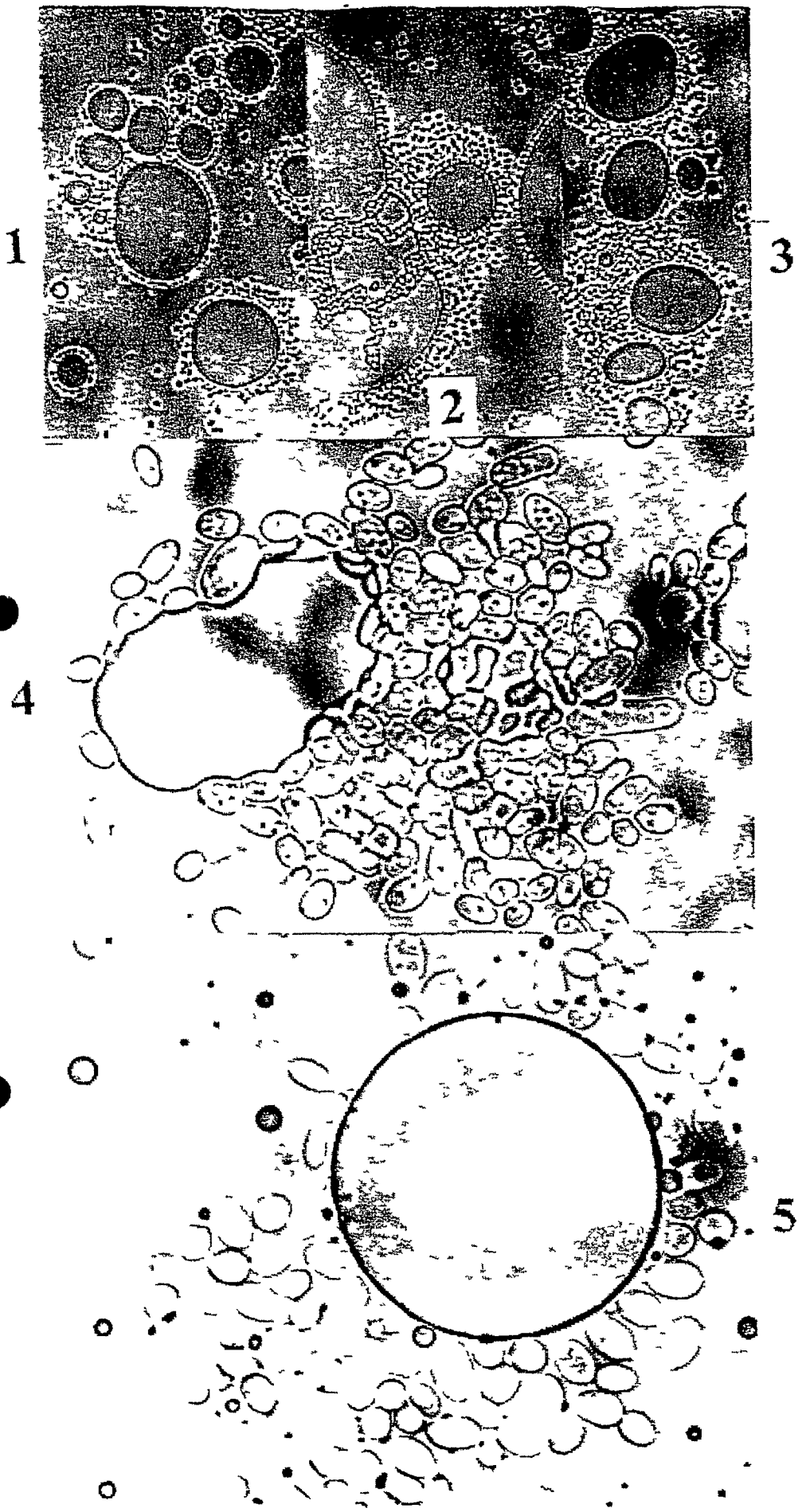

1
2
3
4
5

Fig 2 Light micrograf showing cells Pseudomonas alcaligenes (1 2,3), Candida krusei (4) and Rhodotorula glutinis var glutinis (5) adhering to diesel fuel droplet folowing mixing (800*)

Table 1 Hydrocarbon utilization

Medium	Culture	Hydrocarbon substrate								
		Ben-zine	Kero-sene	Diesel fuel	Vazeli-ne oil	Sunflo-wer oil	Hexa-decan	Crude oil*		
								1	2	3
Solid minimal medium	P alcaligenes									
	A aceti	-	+	+ *	-	+	+	-	-	-
	R glutinis									
Liquid minimal medium	P alcaligenes									
	A aceti									
	R glutinis									

Solid minimal medium	P alcaligenes									
	A aceti	-	+	+**	-	+	+	-	-	-
	R glutinis									
Liquid minimal medium	P alcaligenes									
	A aceti									
	R glutinis									

Crude preparations of P lcaligenes, C krusei, R glutinis biosurfactants were obtained on synthetic medium without growth factors and with ethanol as a sole carbon source (10 g/l) To demonstrate surface-active properties decrease in water surface tension when different concentrations of dry preparations were added, dependence of the decrease in the surface tension on pH of solutions (Fig 3) The preparations (1%) showed themselves as iongenic surface-active agents which decreased the water surface tension (72,75 mH/m) P lcaligenes - 42,4 mH/m, R glutinis - 47,4 mH/m, C krusei - 48,5 mH/m

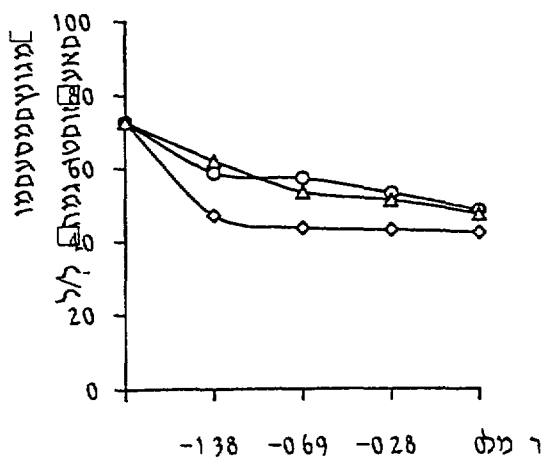


Fig 3 Surface tension of P alcaligenes, C krusei and R glutinis crude biosurfactant preparate solutions (lyophilized cell-free supernatants)

X P alcaligenes, O C krusei, Δ R glutinis

The ability of biosurfactants to emulsify a hidrophobic phase (various types of oil and -in- hydrophobic components The preparation of R glutinis dispersed maximum the oil at pH=9, and P lcaligenes - at pH=5 Both preparations emulsified the margarine very well at pH=7 However, C krusei preparation did not interact with oil and margarine (Tab 2, 3)

Table 2 Special emulsifying activity of crude biosurfactants under formation oil in water emulsion, u/mg d w biosurfactant

Strain	Hydrophobic substrate	pH		
		5	7	9
P alcaligenes	oil 1	183,5	145,0	71,0
	oil 2	15,0	52,5	63,5
	oil 3	122,5	14,5	91,0
C krusei	oil 1	8,0	2,5	4,5
	oil 2	9,5	2,0	33,5
	oil 3	8,0	1,0	48,5
R glutinis	oil 1	72,5	70,5	570,5
	oil 2	96,0	18,5	137,5
	oil 3	78,0	111,0	312 5

Table 3 Special emulsifying activity of crude biosurfactants under formation margarine in water emulsion, u/mg d w biosurfactant

Strain	pH		
	5	7	9
P alcaligenes	129,0	140,0	110,0
C krusei	23,5	6,0	26,0
R glutinis	252,0	348,0	180,5

Table 4 Special emulsifying activity of *R glutinis* crude biosurfactant under formation hexadecan in water emulsion, u/mg d w biosurfactant

Biosurfactant concentration, %	0,25	0,50	0,75	1,00	1,25
Special emulsifying activity, u/mg d w biosurfactant	106,6	213,3	210,0	209,7	200,8

Pseudomonas alcaligenes The results of study of Pseudomonas alcaligenes optimal age and quantity of inoculum for maximum synthesis of extracellular emulsifying agent are given

At first bacteria of P alcaligenes were grown in bacterial agar, next - in mineral liquid medium 8-E with a single source of carbon which was ethanol (10g/l) Influence the inoculum age and quantity on the synthesis of emulsifying agent was evalueted by 2 stages The first was an observation of P alcaligenes which was grown in medium 8-E from one-day and four-days inoculums These inoculums were cultivated on bacterial agar After the growth

one-day bacterial culture in 8-E medium it became an inoculum for the second passage in 8-E medium On this second stage the inoculum in different volumes - 3, 5, 10 ml , on dry weight accordingly-1,5, 3,0, 4,5 mg/l, was put into flasks

Daily P alcaligenes culture obtained from one-day and four-days inoculum had no differences on biomass, emulsifying activity (EA) of cultural liquid and cell productivity (table 5,6)

Table 5 Pseudomonas alcaligenes cultivation conditions used under passage 1

Inoculum age, day	Medium of cultivtion	V of medium, ml	Concentration of ethanol, g/l	Dry weight of inoculum, g
1	8 - E	125	10	0,015
4	8 - E	125	10	0,012

Table 6 Pseudomonas alcaligenes cultivation parameters obtained under passage 1

Daily culture of P alcaligenes in 8 - E medium							
Inocu-lum age, day	Opt density (540nm) of culture medium	Emulsifying activity,unit of opt density, A_{600}	Em activi-ty, u/ml	Special em activity, u/mg d w of emulsifier	Yield of biomass g dry weight/L	Yield of emulsifier g dry weight/L	Cell productivity g d w of em / g d w of biomass
1	1,9	1 8	900	15,5	1,3	5,8	4 5
4	2,0	1,7	800	13,5	1,6	6,3	4 0

The P alcaligenes culture after the second passage in liquid medium 8-E was observed for 3 days The process of biomass and EA were more intensive in variants which were developed from the four-days inoculum Bacterial growth from 3ml and 5ml inoculums were identical Maximum biomass was observed on the first day and maximum of EA - on the second day The bacterial growth from 10ml inoculum was like the above-mentioned variants, but EA was two times below (table 7)

Table 7 Pseudomonas alcaligenes cultivation parameters obtained under passage 2

Inoculum age under passage 1, day	Time of cultivation, day	V of inoculum, ml	Emuls activty, u/ml	Yield of biomass g d w /L	Yield of emusifier g d w /L	Cell productivity, g d w of emul /g d w of biomass	Special em act u/mg d w emulsifier
1	0	3 5 10	25,0 35,0 60,0				
1	1	3 5 10	460 385 400	0,5 0,92 0,8	3,85 4,16 4 1	7,7 4,5 5,1	11,9 9,3 9,8
1	2	3 5 10	230 275 275	0,43 0,66 0,5	3,75 3,95 4,7	8,7 5,9 9,4	6,1 6,9 5,9
1	3	3 5 10	330 300 230	0,73 0 53 0,75	3,4 4 0 3,9	4,2 7 6 5,2	9,7 7 5 ɔ 9
4	0	3 5	15 0 45,0				

		10	65,0				
4	1	3 5 10	475 420 550	1,2 1,5 1,63	6,0 6,5 5,4	5,0 4,3 3,3	7,9 6,5 10 2
4	2	3 5 10	1000 1050 550	1 0 1,18 0 96	5,4 5,3 5,0	5,4 4,5 5,2	18,5 19,8 11 0
4	3	3 5 10	725 850 520	1,28 1,35 1,03	4,4 5,9 5,6	3,4 4,4 5,4	16,5 14,4 9 3

Differences on biomass and EA among variants P alcaligenes culture grown from primary daily inoculum was not considerable These populations were less active than those from four-days inoculum In maximum points biomass and EA were 1,5-2,0 times below Availability of EA minimum points suggest that deemulsifying agent was present In order to check it we determined EA in cultural mixture with different primary EA For example, 2,1 and 0 6 unit of the optical density We obtained total EA equal to 1,95

Cell productivity and specific EA were determined for all variants As shown in table 2 the specific EA in maximum points of EA of population grown from four-days inoculum was 1,6 times higher than in the culture from one-day inoculum

Thus, to obtain active inoculum of P alcaligenes in mineral liquid medium 8-E it is better to use four-days culture grown on bacterial agar(Fig 4) The optimal quantity of inoculum is to be from 1,5 g/l up to 3,0 g/l

The emulsifier *Pseudomonas alcaligenes* was prepared from the cell-free supernatant of a culture of ethanol-grown cells Microbial surfactant produced by bacterium *Pseudomonas alcaligenes* is termoresistant (to 80°C), extracellular polysaccharide-protein complex (containing a 2 1 molar ratio of polysaccharide to protein) of mol wt within 3,5-6,0 kDa This emulsifying

complex in culture media was able effectively to form stable emulsion of sunflower oil in water A breakage of the emulsion on 50% occurs through eight days Concentrated in 3 times culture liquid interacted with the mixture n-hexadecane and 2-methylnaphthalene with forming an emulsion, 50% breakage of which approached in 24 hours, but full disinteraction - through 16-18 days

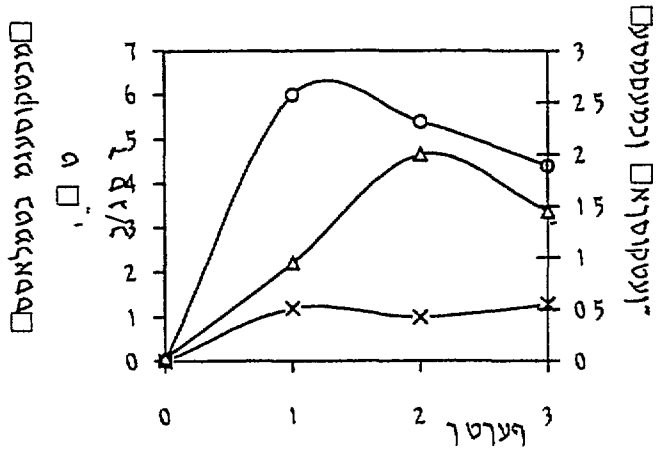


Fig 4 Pseudomonas alcaligenes biomass, emulsifying activity of cultural medium and cell productivity from four-days inoculum, passage 2

In order to study growth of the culture on diesel fuel strain *P alcaligenes* was grown on a minimal salts medium containing 0 5% (v/v) diesel fuel as carbon source During a period of 10 days the cultures were examined for biomass and substrate emulsification (Fig 5) Cell biomass was followed by measuring turbidity at 540 nm The culture showed relatively high turbidity following the first 24h of growth and thereafter However, this turbidity was complicated by the presence of both cells and emulsified oil droplets

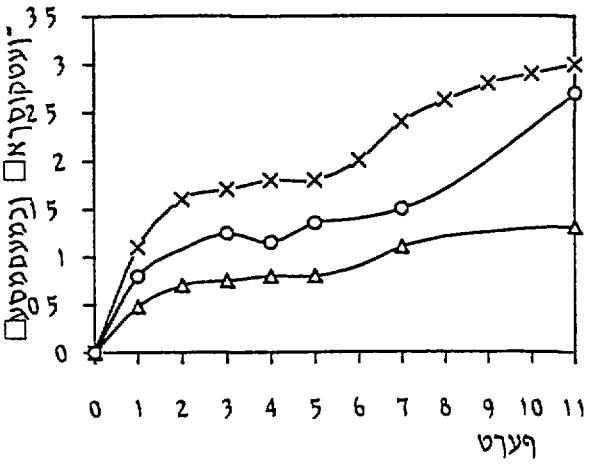


Fig 5 P alcaligenes grouth dinamic and emulsification of diesel fuel

As shown in Fig 6, the interraction bacteria with diesel fuel were examined under the light microscope The Pseudomonas strain formed highly aggregated flocs whose formation did not appear to be dependent on growth on the diesel oil Interestingly, the Pseudomonas strain produced smaller droplets in the order of 5 microns in diameter and the cells were easily separated from the hydrophobic sustrate by centrifugation SDS had no effect on the Psedomonas cultures, the highly aggregated cells remained as flocs The culture resulted in the formation of stable emulsions suggesting that it produced bioemulsifiers and/or biosurfactants as a result of growth on diesel fuel Experiments are currently in progress to isolate and characterize the emulsifier(s) produced by the cells on diesel oil and to compare them with the extracellular products produced by the cells grown on ethanol

Modification of a polymer carrier when it interacts with a microbial surfactant (P lcaligenes) which is used as a receptor support in immobilization of Lactobacillus lactis producing lactic acid on foam polyurethane was studied Foam polyurethane was kept in 2%

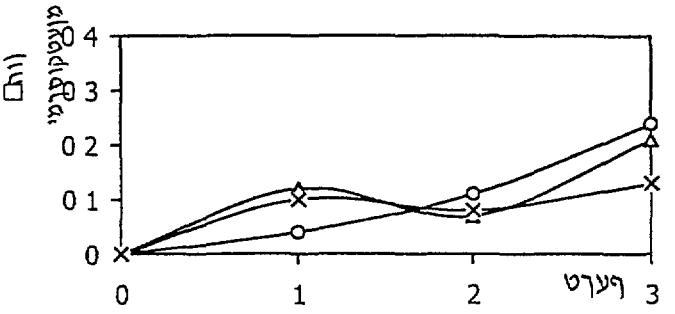
solution of the biosurfactant at 37° C for 48 hours and was immersed in suspension of twice washed cells of Lactobacillus lactis (0,018 g/ml) at 22° C for 24 hours with constant stirring Then the time of sorption equilibrium establishment, specific sorption, desorption and the yield of lactic acid were determined Such method of treatment was proved to bring about modification of the polymer carrier The time of sorption equilibrium establishment for untreated foam polyurethane was found to be about 16 min and for a modified one - 6 min The specific sorption was about $5,11 \times 10^{9}$ cells/g and $2,00 \times 10^{10}$ cells/g, respectively The desorption rate decreased by a factor of 4 when foam polyurethane was treated with the biosurfactant, and the yield of lactic acid increased by 1% The studies we have done showed that modification of polymer carriers using biosurfactant of P lcaligenes may be an effective technique for biotechnological processes in adhesion immobilization of microorganisms

Fig 6 Pseudomonas alcaligenes cell adhesion to diesel fuel droplets at the first (1), second (2,3) and third (4,5) days of cultivation (800*)

Table 8 Extracellular biosurfactants produced by Pseudomonas alcaligenes characteristics before and after partial purification

Polymer type	Pattern (sample) type	Emuls activty, opt density, (600nm)	Dry weight emusifier g /L
Extracellular biosurfactants	Cell-free supernatant	1,17	3,98 0,05
	Id after dialyze	1,35	0,40 0,05
	Id after freeze-drying	1,35	0,40 0,05

Rhodotorula gflutinis var glutinis



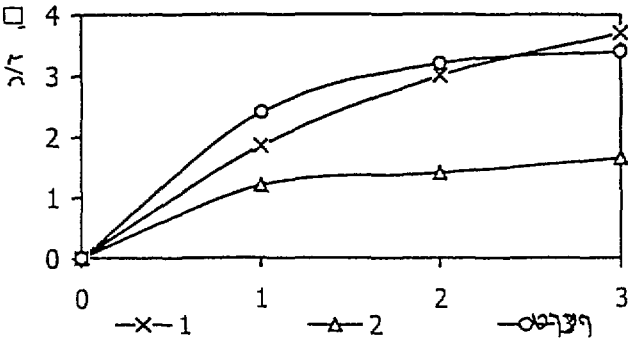


Fig R glutinis emulsifying activity and biomass on different mediums (1 8-E, 2 Rider, 3

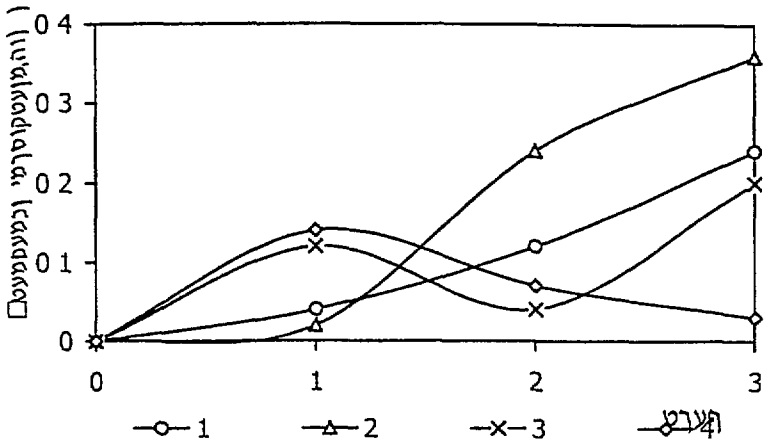


Fig R glutinis growth on different carbon sources 1 - ethanol (10 g/l), 2 - ethanol (10 g/l) + sunflower oil (0,25 g/l), 3 - ethanol (10 g/l) + kerosene, 4 - ethanol (10 g/l) +benzine

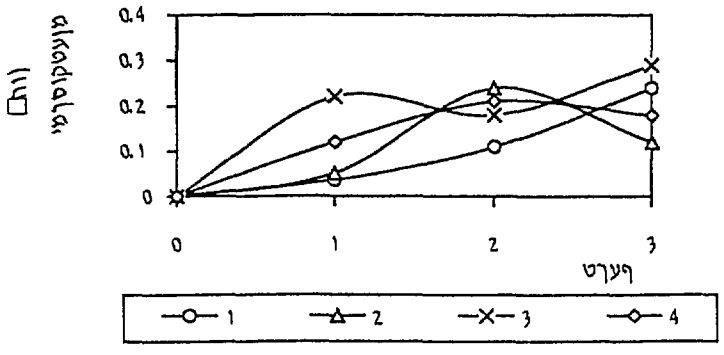


Fig R glutinis emulsifying activity under the usind inoculum of different ages 1- 3-days-inoculum, 2 - 5-days-inoculum, 3 7-days-inoculum, 5 9-day-inoculum

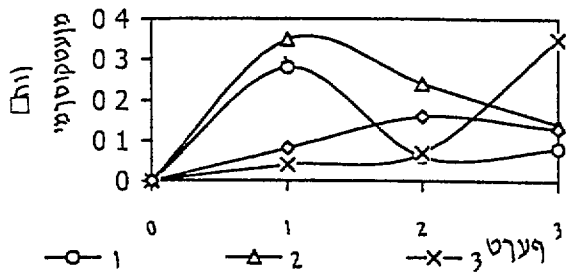


Fig R glutinis emulsifying activity under the usind synchronized inoculum of different ages 1- 3-day-inoculum, 2 - 5-day-inoculum, 3 7-day-inoculum, 5 9-day-inoculum

Table 9 R glutinis cultivation parameters obtained under optimization of grown conditions and with using 5-day-inoculum

Time of cultivation day	Emuls activty opt density, (600nm)	Yield of biomass, g d w /L	Yield of emusifier g d w /L	Cell productivity, g d w of emul /g d w of biomass	Special em act opt density (600 nm)/mg d w emulsifier
0	0,07	-	-	-	-
1	0 40	1,83	1,54	0,84	0,256

2	1,05	3,00	2,83	0,94	0,371
3	2,40	3,75	3,54	0,94	0 678

The potential of R glutinis extracellular biosurfactants and cell surface-bound polymers to complex platinum group metal (Os) was investigated

The amount of free and bound osmium was determined spectrophotometrically The initial osmium ion concentration in solution was 0,1 mg/ml

R glutinis extracellular biosurfactants were prepared from the cell-free supernatant of a culture of ethanol-groun cells Cell-free supernatant fluid was obtained by centrifugation of culture at 5 000 x g for 10 min Suprenatant was filtered across membrane filter for yeast cells, dialyzed agains distilled water, and freeze-dried

R glutinis cell surface-bound polymers were prepared from the cells of ethanol-groun culture Cells- were obtained by centrifugation of culture at 5 000 x g for 10 min The cell pellet was washed twice in 8-E media without ethanol and suspended to 2% EDTA solution (pH 8,0) The suspension was mixed on shaker at 30°C for 2 h and after centrifuging, cell-free supernatant was filtered across membrane and dialyzed agains distilled water Cell surface-bound polymers were precipitated by isopropanol with 4% of sodium chloride at 4°C and dried at room temperature

In sorption experiment dry powders of R glutinis extracellular biosurfactants and cell surface-bound polymers were added to Os containign solution to a final concentration of 1

mg/ml and mixed on shaker at room temperature for 30 min The polymers were then separated from solutions by ultracentrifugation In Os recovery from gas phase experiment the precipitates were suspended in water to a final concentration of 1 mg/ml and were used for complexation of osmium during 3 hours

Emulsion was prepared by shaking of a mixture of 1,5 ml vaseline oil and 8,5 ml of aqueus solution containing 10 mg R glutinis extracellular biosurfactants and used for complexation of osmium from gas phase during 3 hours Osmium binding to emulsion was determined after separating the aqueous phase from vaseline oil droplets by centrifugation at 6000 x g for 20 min Binding of osmium to vaseline oil in water emulsion(no biopolymers) served as control and was measured after breakage of system into phases

The data summarized in Table 8,9,10

Table 10 Extracellular biosurfactants, cell surface-bound polymers produced by R glutinis characteristics before and after partial purification

Polymer type	Pattern (sample) type	Emuls activty, opt density, (600nm)	Dry weight emusifier g /L
Cell surface-bound polymers	Washing solution	0,45	0,74 0,08
	Id after dlialyze	0,37	0,68 0,04
	Id after freeze-drying	0,32	0,68 0,02
Extracellular biosurfactants	Cell-free supernatant	2,4	3,54 0,05
	Id after dlialyze	2,2	3,05 0,05
	Id after freeze-drying	1,95	3,05 0,04

Table 9 Osmium complexation (biosorption) capacity of extracellular biosurfactants and cell surface-bound polymers produced by R glutinis

Polymer type	Cell productivity, g d w of crude polymers / g d w of biomass	Complexation capacity, %
Cell surface-bound polymers	0,3-0,5	4,25±0,25
Extracellular biosurfactants	0,8-1,0	7,70±0,25

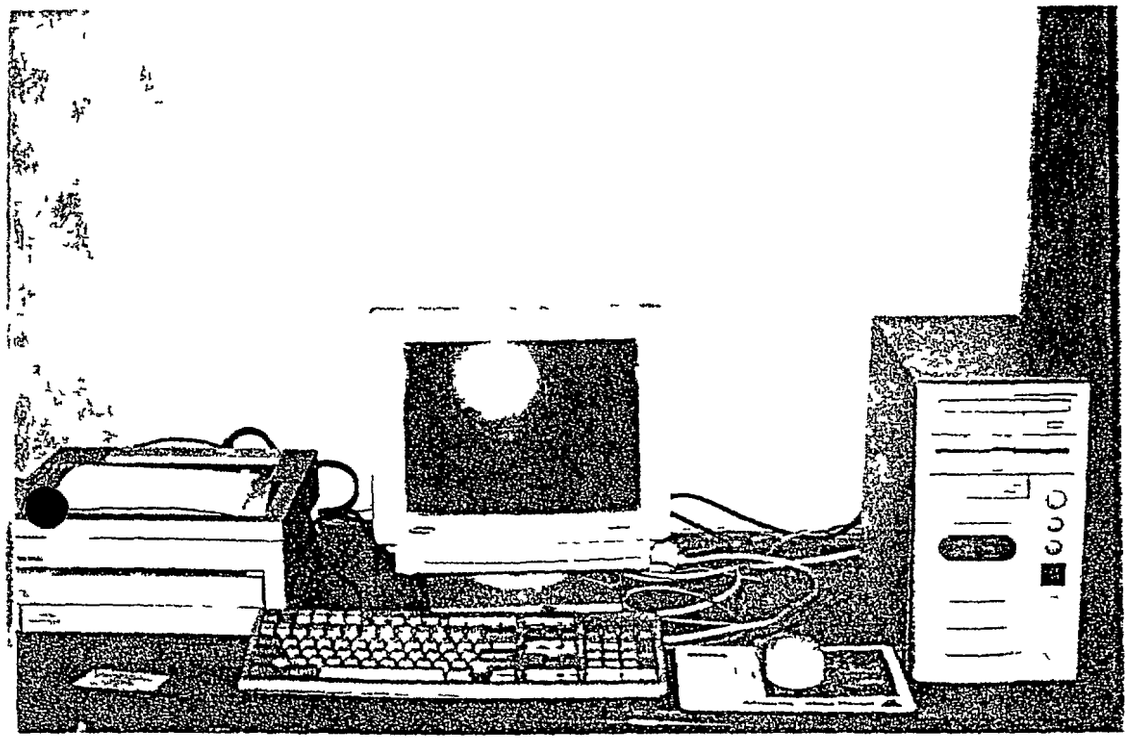
Table 11 Osmium complexation capacity of extracellular biosurfactants, cell surface-bound polymers produced by R -in- extacellular biosurfactants of R glutinis during 3-hours contact with Os containign gas phase

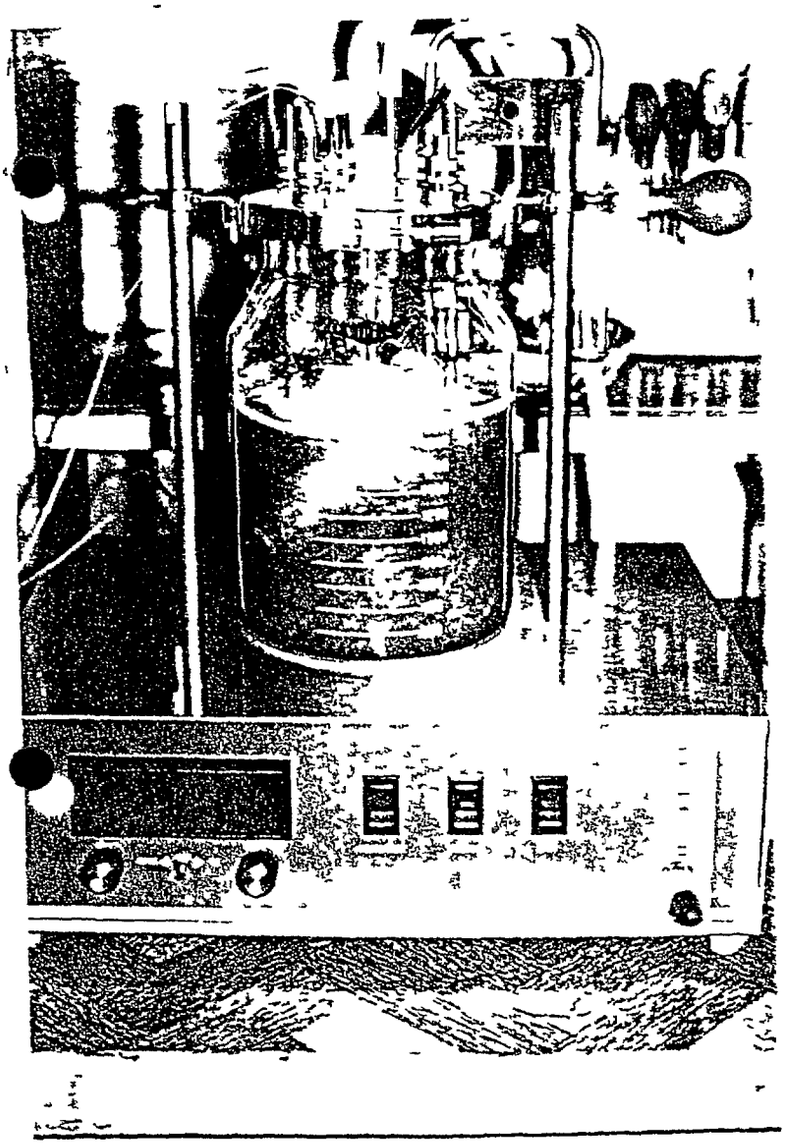
Polymer type and hydrophobic substrate	Pattern (sample) type	Complexation capacity, %
Extracellular biosurfactants	Solution	0,060
Extracellular biosurfactants	Freeze-dried solution	10,068
Cell surface-bound polymers	Solution	0,025
Cell surface-bound polymers	Dried solution, 20°C	4,350
Extracellular biosurfactants+vaseline oil	Emulsion	0,0834

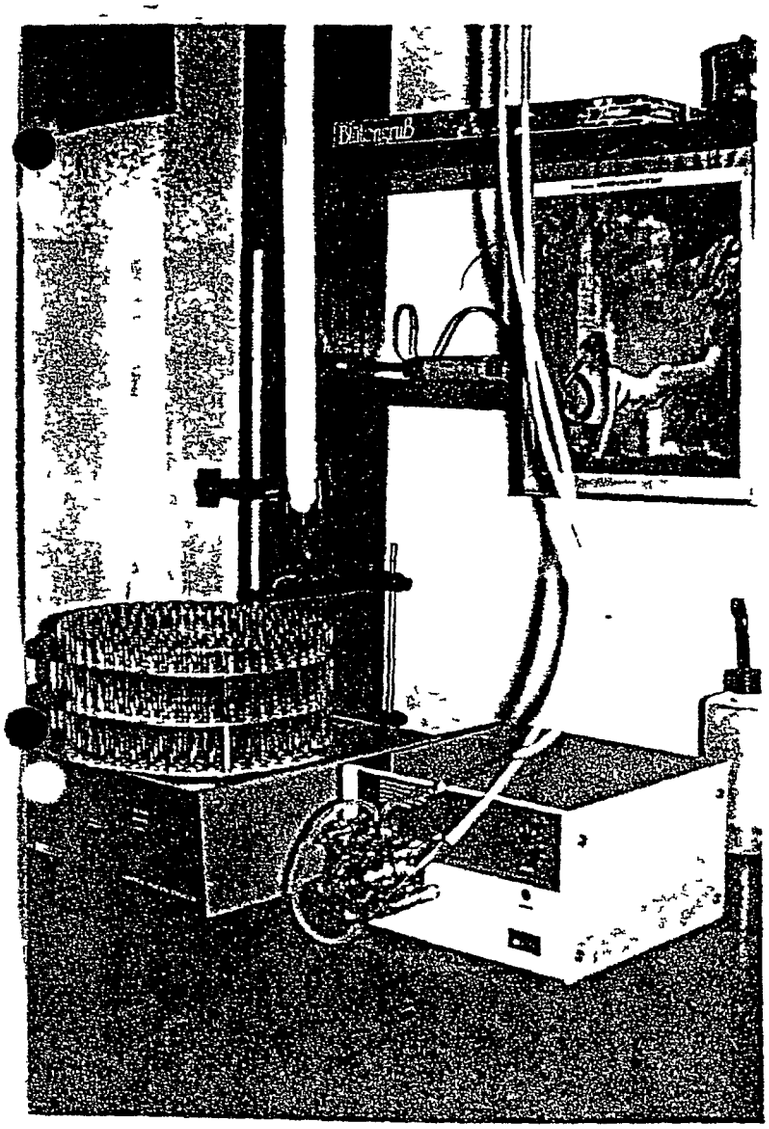
Extracellular biosurfactants+vaseline oil	Dried emulsion, 50°C	13,996
Vaseline oil in water emulsion	Vaseline oil	0,00

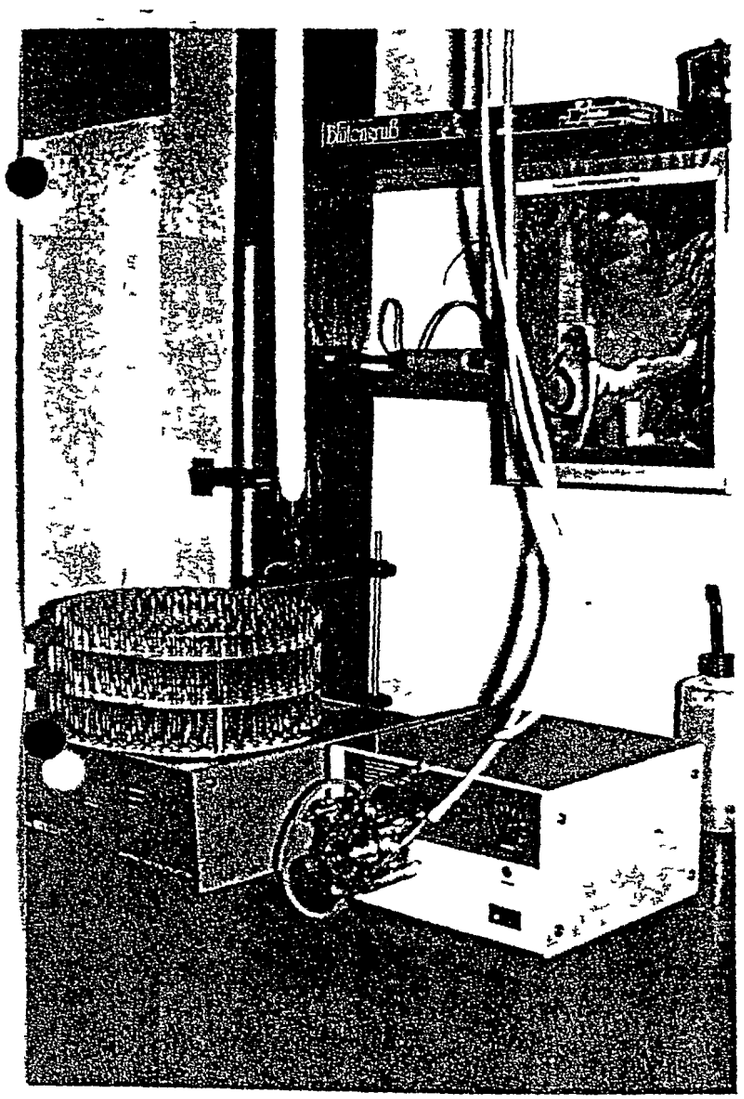
APPENDIX I

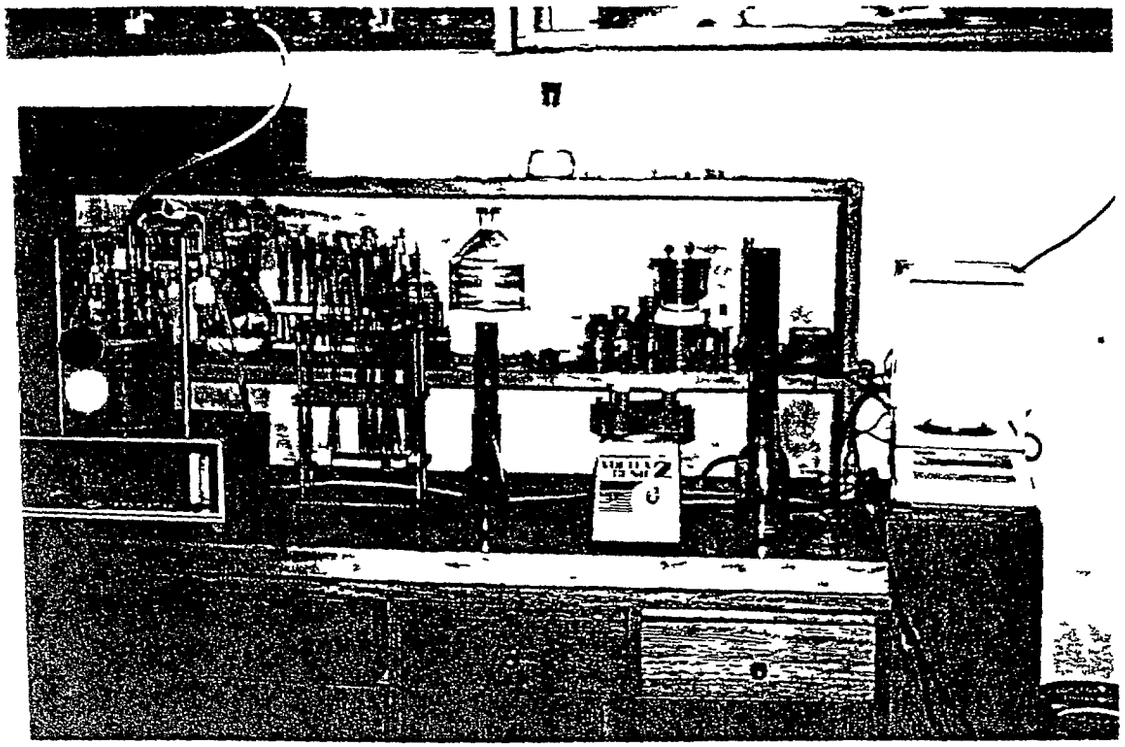
Photos of new laboratory in Almaty, Khazakstan

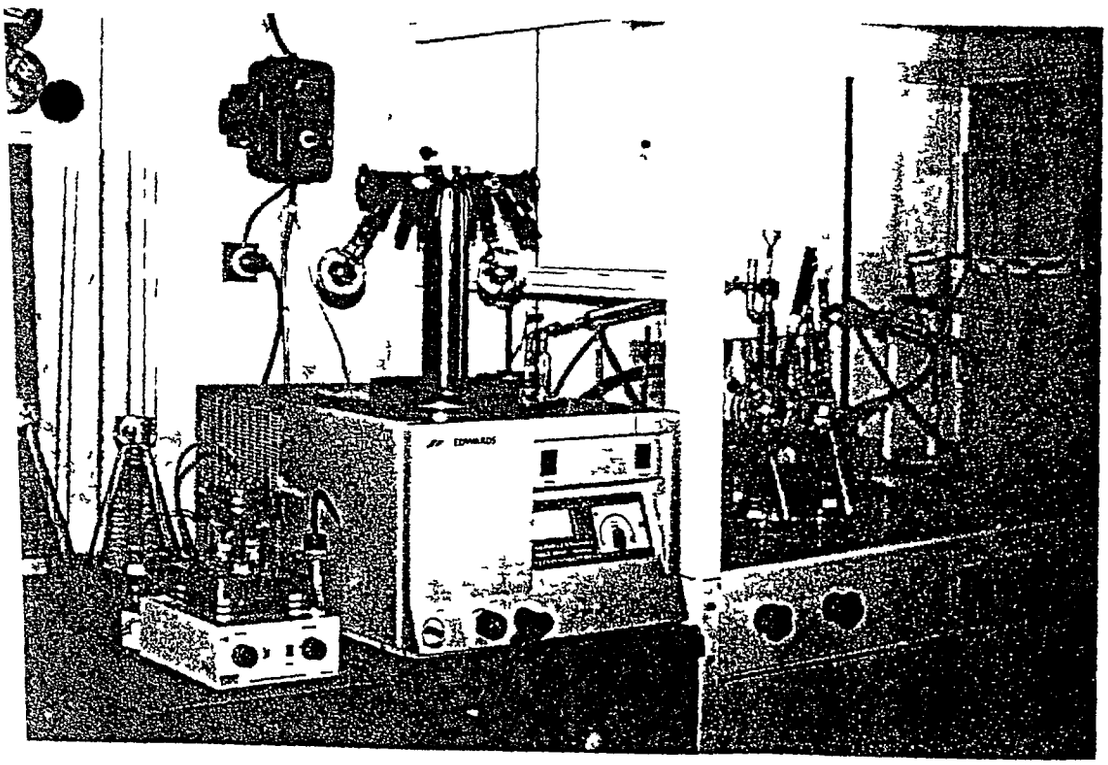








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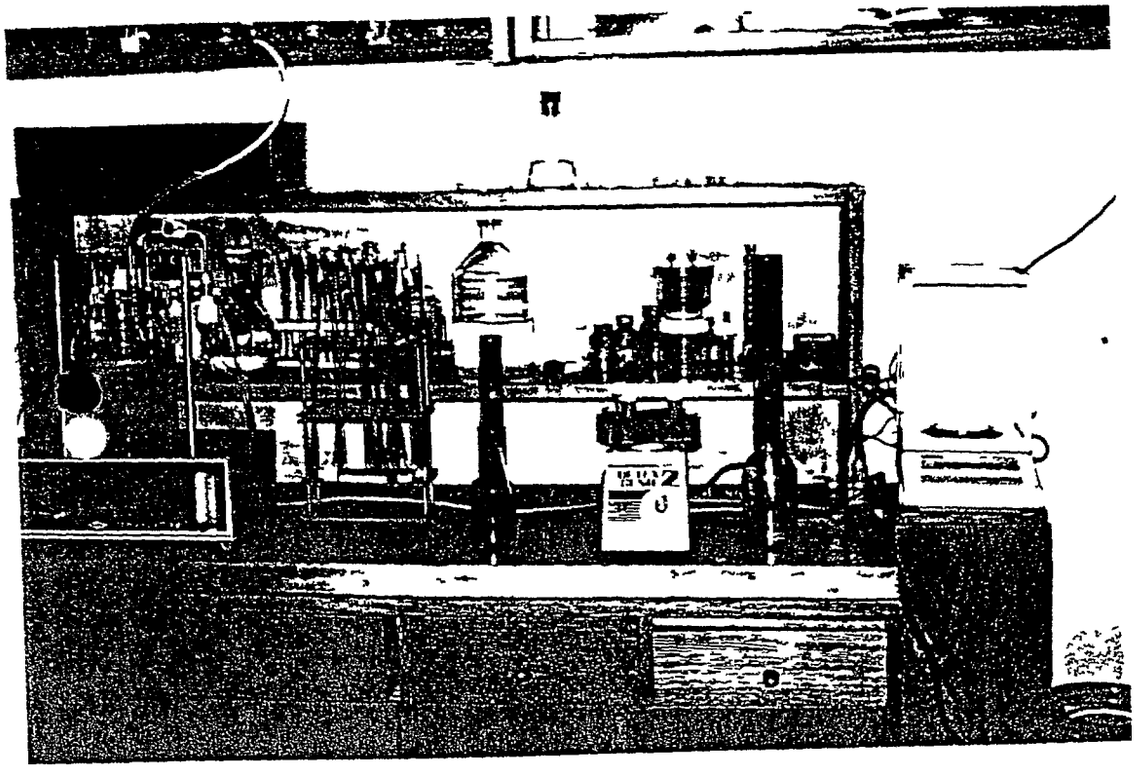

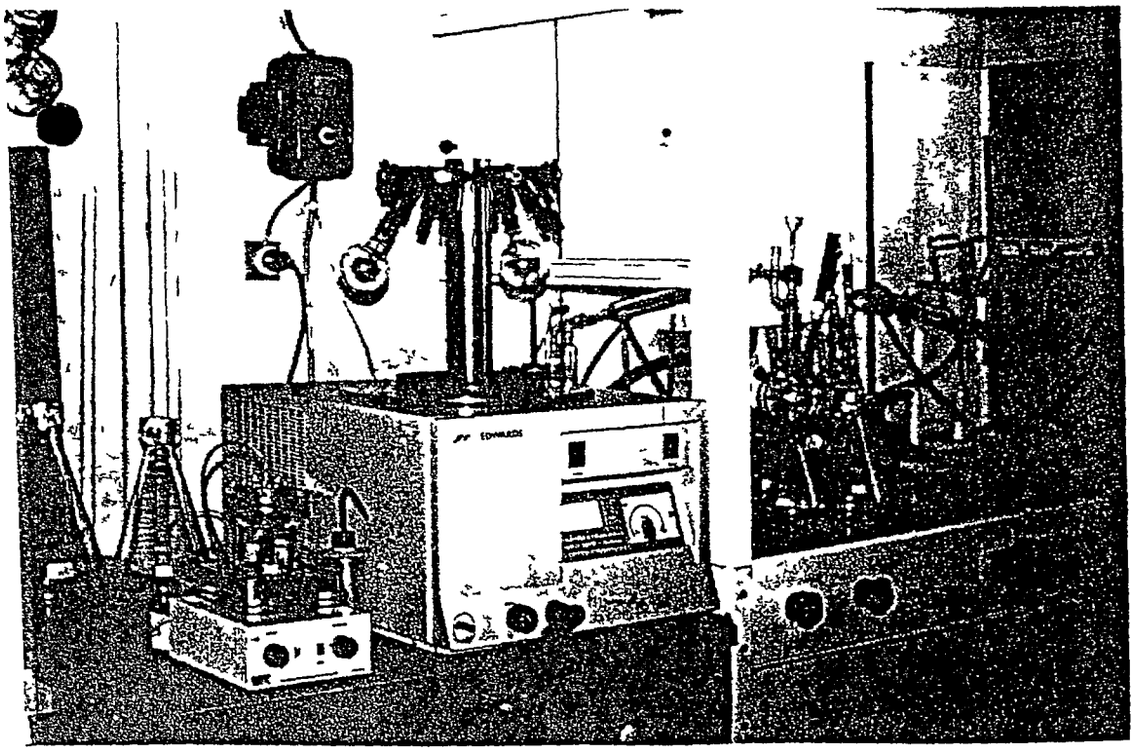
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