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USE OF PHOSPHOLIPIDS-LIPOSOMES FOR BIOREMEDIATION OF CONTAMINATED SOIL

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Executive Summary

Liposomes composed of soy bean lecithins induced repeatedly a high level of proliferation of microorganisms present in the soil contaminated or not with oil on a laboratory scale This effect can be translated in a potential bioremediation method of oil contaminated soil The object of this proposal are to analyze in depth the principle of liposome induced bioremediation optimize the liposomal composition and its mode of application The latter will be related to the precise nature of soils and degrading microorganisms The most efficient method to extract oil from contaminated soil is based on freon extraction Because freon is not environmentally friendly, we have developed an alternative method using hexane as extracting agent The use of liposomes as enhancers of bioremediation were combined with the use of oil degrading microorganisms commercially available and with microorganisms isolated in Kazakhstan Oil degrading bacteria isolated in Kazakhstan were characterized in Israel Growth curves of these bacteria on oil minimal medium were established Presence of genes specific to oil degradation were looked for by investigating the presence of plasmids linked to oil degradation in these strains Enhancement of bioremediation of oil contaminated soil by adding these bacteria were studied and did not show a great advantage on liposomal treatment alone The Kazakh bacteria were found to have an emulsifier activity rather a digestion activity on oil The bacterial population which increased during liposomal bioremediation of oil contaminated soil were studied and characterized by biochemical and Gas Chromatography techniques Specific bacteria were found to be enhanced by the presence of liposomes Most of the bacteria enriched during treatment of contaminated soil belonged to genera known to be able to use oil as a source of energy Being of natural origin the liposomes are non toxic and fully biodegradable We anticipate that this technology will also improve the availability of the oil to the microorganisms thereby permitting bioremediation even in emergencies as well as bioreclamation of contaminated soil The ultimate goals will be to upscale the liposome induced bioremediation conduct field trial tests and eventually use liposomes for bioreclamation of contaminated sites

B) Methods and Results

Research Objectives

In this proposal the overall aims were

Bioreclamation of sites contaminated with oil wastes using liposomes composed of natural phospholipids

The specific objectives were

-Optimization of the composition of the phospholipids to obtain maximal oil degradation

-Interaction between microorganisms and various liposomal composition and characterization of bacterial populations enhanced by liposomes

-Characterization of oil degrading bacteria isolated in Kazakhstan

In Israel at the Hebrew University Medical School the following tasks were realized

determine the amount of oil in contaminated soils

a) replacement of the freon-based extraction method by an hexane-based extraction method to

b) comparison of the efficacy of various liposome formulations in biodegradation of soil

c) evaluation of the bioremediation activity of the strains isolated in Kazakhstan

d) characterization of bacterial populations enhanced by liposomes

In Kazakhstan, at the Kazakh State University the following tasks were realized

a) isolation of bacteria growing in oil contaminated soil

b) characterization of bacteria growing on crude oil

c) Acquisition of PCR methodology for future characterization of oil grading bacteria.

B1 Source of soil, treatment and oil extraction

Source of soil

The soil both contaminated by crude oil and uncontaminated was given to as by Dr Yosefa Ben-asher of the Israeli Oil Office The source of soil was a field near the city of Rannana (Israel) that was contaminated by accident in 1992 The information regarding the amount of contamination was also given to us by Yosefa Ben-asher of the Israeli Oil Office In the experiments described in this paper we used soil contaminated by 16 636 ppm which in the text is referred as ~17,000 ppm oil contamination

Preparation of soil for bioremediation

Aliquots of 60 g or 150 g soil were used, all samples were watered so that 30% of the weight of the soil was liquid Samples with no liposomes were watered with sterile tap water and 0 4 mg $(NH_4)_2HPO_4$ per g soil was added Samples with 0 6% liposomes where watered with 10% SUV liposomes (S-100) diluted in sterile tap water and 600 μl of ($(NH_4)_2HPO_4$ (100 mg /ml) solution was added Samples with 0 1% liposomes where watered with 2 1 ml SUV liposomes (S-100) 57 9 ml sterile tap water and 0 4 mg $(NH_4)_2HPO_4$ per g soil was added $(NH_4)_2HPO_4$ was added as a source of Nitrogen

Incubation conditions

All soil samples where incubated at 30°C in a room with a permanent temperature control Incubation was done under aerobic condition in jars with a screw cap All samples were ventilated by mixing the soil once in two days All samples where watered after 10 days with 6% sterile tap water

Drying the soil in preparation for oil extraction

The samples we wanted to check for residual oil after incubation were too wet for oil extraction The problem was solved by pre drying the soil before oil extraction 40 g of soil were put in a chemical luminary hood for two days, then the soil was passed to a air vacuum at 40°C up to a point where the soil weight remained stable

Oil extraction

Oil extraction was performed on samples of 10 g soil The aliquots were mixed with 2 5 g anhydrous magnesium sulfate (to adsorb residual water in the soil) 120 ml of Freon (1 1 2

trichloro 1 2 2 tri floro ethylene boiling point 47°C) where added and mixed on a magnetic stirrer for 30 min After the stirring we waited 15 min for the solids to sink to the bottom of the Erlenmeyer 75 ml of the upper fluid where transferred to a clean flask where 5 g of silicic acid were added (to absorb polar lipids) The liquid was filtered through filtering paper (Whattman no 3) into a weighed colb evaporated and than weighed again The amount of residual oil was calculated after the evaporation To ensure that the extraction method was specific to oil and did not extract the phospholipids from the liposomes we compared levels of phospholipids in the extract. The method was specific for oil In parallel hexane was used as a substitute of freon and efficiency of extraction of the two methods was compared The results obtained in our laboratory showed that with proper homogenization of the contaminated soils similar extraction efficacy were obtained using hexane All freon-based procedure were replaced by the hexane-based procedure

B2 Liposome preparation

A 10% liposome solution from phospholipids extracted form soybeans was used The commercial name of the product used is S-100 (the number refers to the percent of phosphatidyl choline in the product) manufactured by Lipoid GmbH Ludwigshafen Germany DDW was added to 100 g of phospholipids to a total volume of 1 liter The mixture was homogenized by a High shearing homogenizer (Kinetica GmbH Littan-Luzern Switzerland) In this procedure multilamellar vesicles (MLV) are formed The MLV solution was transferred to a Rannie mini Lab 8 30H homogenizer at a pressure of 10 000 PSI This procedure makes liposomes that are small unilamellar vesicles (SUV liposomes) The SUV liposome solution was filtered for sterilization through a 0 2 μm polycarbonate filter (Haran et al 1993) Size evaluation was performed with a Coulter particle size analyzer (Coulter Electronics Higlean Florida USA) The liposome vesicle size was 30-60 nm (Barenholz & Amsalem 1993) Evaluation of phospholipid concentration in the liposome solution was performed by evaluating the amount of phosphate by a modification of the Bartllet Method (Barenholz & Amsalem 1993)

B3 Bacterial Growth and Characterization

Bacterial growth

Growth on liquid media was used for selective growth of oil degrading bacteria A minimal medium (MM) containing crude oil was used 1 litter of MM contained 5g NaCl, 1g NH_4NO_3 1g KH_2PO_4 0 2g $MgSO_4$, 0 02g CaCl 500 μl trace elements and 10 ml of crude oil Before preparing the medium all the salts where burnt at 180°C for 30 min , to eliminate carbon sources that the salts contained The final pH of the medium was pH7 (Stanier et al , 1986) Bacterial growth in MM was done at 30°C in aerobic conditions, all samples where shaken at a speed of 15 RPM for a period of 14 days Growth on solid medium for bacterial counts and for bacterial isolation was performed on petri dishes containing tryptic soy agar (TSA), composed of 15 g Bacto agar (Difco) and 30 g tryptic soy broth (Difco) in 1 Liter of DDW The petri dishes were incubated after applying the samples at 30°C for 48hr in aerobic conditions For

bacterial counts samples of 1 g soil was taken emulsified in saline diluted in logarithm dilution and seeded on petri dishes

Identification of bacteria

For characterization and isolation of the bacterial colonies 1 ml of the liquid medium after the incubation period was diluted and seeded on the petri dishes Isolated colonies were picked, re isolated and then characterized A gram staining was performed to control that the bacterial colony was a pure isolate In order to determine which method of characterization would be used primary characterization was obtained by performing a few basic tests Growth in aerobic and anaerobic conditions gram staining, oxidase and catalase activity Characterizing all types of bacteria was done by the method of Midi Microbial Identification System (MIS) A method in which the fatty acid profile of the bacteria is analyzed by gas chromatography (GC) The result is compared to a computerized reference library to determine the bacteria species (Wallace et al , 1988 Wallace et al , 1989) Identification of gram negative aerobic bacteria was performed in addition by a commercial kit (API 20 NE) The kit tests 8 biochemical tests and 12 carbon assimilation tests Determining the bacteria species was done by comparing the test results to a computerized reference library using the program Analytical Profile Index) Preparation of the bacterial stains for characterization was performed following the protocol of bioMérieux Vitek Inc Marcy-l'Etoile company (Overman et al , 1985)

B4 Purification and identification of the oil degrading strains brought from Kazakhstan

The two Kazakh students brought 45 strains with them to Israel Isolates of oil degrading bacteria were recultured and repurified on minimal medium containing oil as sole source of carbon and energy Stock bacteria were saved at -70°C From the stock collection the following tests were done on each strain Gram stain Motility Aerobic / Anaerobic Fermentative metabolism on Hugh-Leifson medium Growth on McConkey agar Oxidase test API non enteric NE API for Staphylococcus The results showed that from the 45 strains which were brought to Israel 22 were Yeast and only 23 were bacterial strains Preliminary identification revealed that 7 strains were Gram negative motile rods oxidase + and catalase variable with no pigment (pseudomonas like) Fifteen strains were Gram positive bacteria comprising 3 strains of Gram positive cocci (strain 27) and 12 strains of Gram positive irregular rods oxidase negative and catalase variable pigmented (yellow orange) and non motile To further identified strains 27 and 31 amino acid analysis of the bacterial cell wall was performed by TLC The technique for amino acid analysis of cell wall for strain 27 and 31 by TLC after alkali digestion of cell wall was done as follow Bacteria were grown on LB broth, harvested washed in DDW and the pellet was resuspended on 1 5 ml of 5% KOH (strain 27) and 1 ml for strain 31 The reaction mixture was incubated at 100°c for 35 minutes and then cooled An equal volume of DDW was added and the preparation was centrifuged 30 minutes at 10 000 g The cell walls were suspended in 250 ml of 6M HCl and incubated at 100°C for 18 h The samples were dried under vacuum and diluted in 50 ml of isopropanol filtered (0 2 mm) Four

microliters were run on TLC silica gel plates using as solvent a chloroform methanol ammonia water 8 8 3 1 mixture for 2 5 hours References Lysine ornithine alanine serine, glycine glutamic acid glucosamine were run in parallel The amino acid profiles of strains 31 and 27 were similar Lysine was found to be the major diaminoacid Strain 31 is coryneform with coccus cycle and contained lysine This analysis confirmed that strain 27 is a true Micrococcus and that strain 31 is a Corynebacterium-Coryneform

B5 Screening of bacterial strains for rapid growth on minimal medium with oil as a sole source of hydrocarbon and energy

Oil degrading bacteria isolated in Kazakhstan and characterized in Israel (see above) were inoculated on minimal medium containing 3% of Tengiz oil as sole source of carbon and energy Minimal medium composition NaCl 28 4 g, K_2HPO_4 4 74 g KH_2PO_4 0 56 g $MgSO_4$ 0 5 g $CaCl_2$ 0 1 g NH_4NO_3 2 5 g Trace elements 1 ml, Final pH adjusted to pH 7 1

This medium was sterilized by autoclave before adding as the sole source of carbon and energy 3 ml of Tengit crude oil The inoculated medium was incubated at 25°C for 15 days under gentle shaking (200 rpm) The following 6 stains (20 27, 30, 31, 33, 25) were selected from the vials in which the oil layer could be seen as broken the most rapidly (5-10 days)

B6 Identification of plasmids encoding genes responsible for oil degradation

The presence of plasmids linked to oil degradation was investigated in the strains which broke the oil layer the most rapidly Using the regular plasmid isolation technique of Birnboim, small plasmids were detected in strains 3 8 9, 10 20 25, 30 33 38

Methods for large-plasmid isolation were set up using as a control strain *Pseudomonas putida* which has a large plasmid (TOL plasmid) of 117 Kb size We were able to detect the TOL plasmid from *Pseudomonas putida* but no large plasmid was found in the rapid growing strains from Kazakhstan even when using in addition to gel electrophoresis Ultracentrifugation However hybridization of the DNAs of strains 20 and 33 with the labeled TOL plasmid in a Southern blot resulted in one positive band in each strain To confirm and identify the positive band we used PCR amplification based on a conserved gene present on the TOL plasmid We used primers derived from the *Xyl E* gene encoding a catechol 2 3-dioxygenase (Marques S and Ramos J L 1993 Mol Microbiol 9 923-929)

The following primers deduced from this gene were synthesized (NBI Annapolis USA)

F 5'-CACGTTGGCGGAAACAAACCTGAC-3'

B 5'-AGGTCAGCACGGTCATGAATCG-3'

These primers should amplify a fragment of 900 bp in size

PCR conditions used to amplify the Xyl gene were as follow

Primer 10 pmol + 100 mg/ml BSA + 2 5 mM dNTP

- Polymerase 0 3 U/ per reaction in manufacturer buffer

Cycles	94°C x 40 seconds followed by
26 cycles	60°C x 1 30 minute
	75°C x 2 30 minutes
Final	75°C x 10 minutes
	4°C x 10 minutes

After establishing with the TOL plasmid that the correct band was amplified (900 bp) we found that strains 20 and 33 produced by PCR a fragment of 1200 bp and not 900 bp as expected but which hybridized with the labeled *Xyl E* gene Therefore were able by PCR amplification to identify two strains with homologous genes to the *Xyl E* gene These strains are now being further characterized

B7 16S RNA PCR

Finally the first step for the detection of bacteria by PCR was performed Primers deduced from 16S RNA were used to amplify a specific DNA fragment from lysates of both Gram positive and Gram negative bacteria Six strains were checked by 16S RNA universal primers to evaluate the method The following universal primers (Bruce et al Appl Environm Microbiol 1992 58 3413-3416) which should amplify a 1500 bp fragment from the 16S RNA were synthesized at the Hebrew University

F 5'- AGAGTTTGATCCTGGCTCAG-3'

B 5'-AAGGAGGTGATCCAGCCGCA-3'

The amplification (PCR) conditions were as follow

Primers 20 picomole
BSA 100 mg/ml
Vent Polymerase 0 5U

Hot start	94°C x 3' followed by
26 cycles	94°C x 40 seconds
	60°C x 30 seconds
	75°C x 1 40 minute
Final	75oC x 5'
	4°C x 10'

This protocol was applied to 6 strains and we obtained the proper 1500 bp fragment for each strain

B8 Evaluation of the bioremediation activity of the strains isolated in Kazakhstan

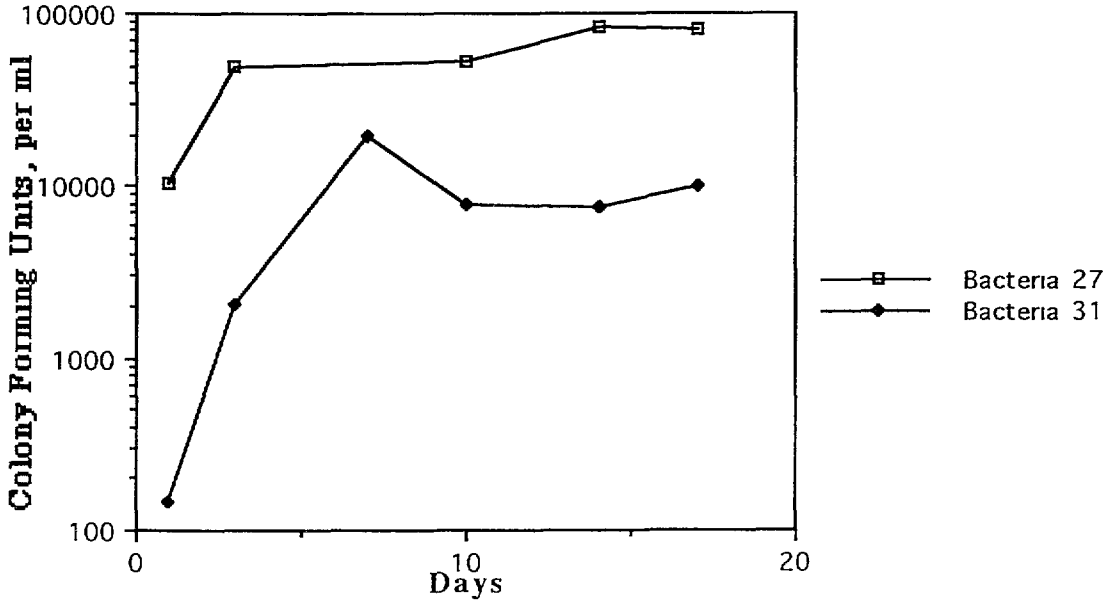
Among the 45 strains brought to Israel from Kazakhstan 25 strains have been examined and three have been definitively identified as *Micrococcus rubeum Corynebacterium spp* and *Acinetobacter spp* The three characterized strains isolated in Kazakhstan were added to the above mentioned systems Their contribution to bioremediation of oil contaminated soils was evaluated Using the bacteria from Kazachastan seems to improve the degradation a little but the results were not very conclusive The bacterial count in these experiment did not allow to distinguish between the indigenous bacteria and the exogenous bacteria so we could not check if the bacteria added succeeded to grow in our samples, or to compete with the indigenous bacteria

To evaluate the growth of the added exogenous bacteria in the soil soils were sterilized and then were artificially contaminated In all these experiments there was no positive correlation between the bacterial count and the oil degradation This may be due to the fact that

the bacteria have an ability to live in a medium containing oil (in this case soil) but they use poorly the oil as a source of carbon and energy

Although strains 10 27 and 31 did not improve the oil degradation they were found in liquid medium good oil emulsifier Therefore the effect of added bacteria (Micrococus spp strain 27 and Arthrobacter strain 31) on oil bioremediation in liquid medium (10 ml of minimal medium and 100 µl of oil) was evaluated Bacterial isolate 27 showed a moderate growth in the first two weeks The growth was very moderate in the period of the experiment Bacteria number 31 grew significantly between days one and three and continued growing until day 7 and then after a small decrease remained stable

Bacterial growth



Oil degradation was also evaluated in these experiments performed in liquid medium The results showed that there was no significant oil degradation the difference between the samples with bacteria and the control was hardly noticed (see table below) This may by related to the poor bacterial replication Even though there was no significant oil degradation there was a significant visual change in the oil In the control samples the oil above the medium was in one shiny layer and the medium below stayed clear In the samples with the bacteria the oil seemed to be emulsified in the medium and unshiny, the medium became turbid This suggests that the bacteria produced emulsifiers which changed the physical state of the oil Some of the emulsifier may contribute to the oil weight inducing an artifact to the determination of the oil weight

Residual oil

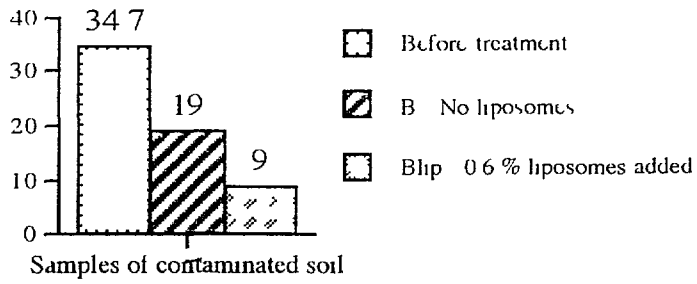
sample/week	0 (mg)	1 (mg)	2 (mg)	3 (mg)
control	45 2	45 2	42	42
2(bac 27)	44 3	35 06	39 16	39 2
3(bac 31)	36 1	45 9	40 06	35 96

B9 Comparison of the efficacy of various liposome formulations in biodegradation of soil contaminated with various amount of oil

The different experiments in bioremediation of soils contaminated by oil show that there are certain conditions that improve the bioremediation there seems to be an advantage in using SUV composed of S-100 soy phosphatides over SUV composed of S-40 soy phosphatides By comparing soil with similar contamination of oil (~15,000 ppm), Liposomes S-100 allowed as much as 75% degradation of the oil whereas using Liposomes S-40 there was only 57% decrease in oil contamination However bioremediation with added SUV composed of S-40 was better than the control soil without SUV The advantages of Liposomes S-100 was also clear in the bacterial count The bacteria grew continuously in the period of the experiment or there amount stayed stable whereas when Liposomes S-40 were used the bacteria grew rapidly during the first three days and then there was a moderate continuing decrease in their amount.

In the first experiment presented, soil was contaminated in the lab for controlled contamination The soil was contaminated to a level of 10,000 ppm (10 mg crude oil per 1 gr soil), the soil was then incubated for two weeks in room temperature, so that the oil would be well absorbed and the population of oil degrading microorganisms will be enriched Soil was then separated to two aliquots one that liposomes 0 6% were added and the other without liposomes Both samples contained 60 gr soil and were treated as described in materials and methods Oil extraction's was performed before starting the experiment and after 21 days of incubation Before treatment the oil extraction results were 34 7 mg/10 g soil After the 21 day incubation period soil treated with liposomes the oil amount was reduced by ~75% to 9 mg/10 g soil (s d ±0 73) in the soil with no liposomes the oil amount was reduced by ~40% to 19 9 mg/10 g soil (s d ±1 05) (fig 1)

Fig 1 Residual oil in soil contaminted in the laboratory by 10 000 ppm oil after 21 days incubation

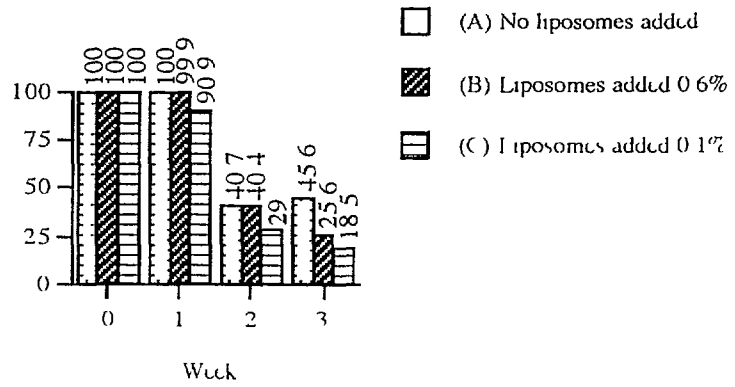


Legend figure 1 Before treatment the soil contained 34 7 mg/10g soil of crude oil In soil treated with no liposomes after 21 days incubation 19 mg/10g soil of crude oil resided in the soil (B) In the soil treated with the liposomes 9 mg/10g soil of crude oil resided in the soil (Blip) after the same period

The bioremediation experiment with soil contaminated in the field by ~17 000 ppm crude oil was improved when we added liposomes after an incubation period of three weeks (fig 2) In all

samples there seems to be no improvement after one week of incubation there is no reduction in the amount of oil in the soil (soil without liposomes 40 7 mg/10g soil of s d ±2 03 soil with 0 6% liposomes 32 3 mg/10g soil of s d ±1 29, and soil with 0 1% liposomes 29 46 mg/10g soil s d ±2 91) After two weeks there seems to be a significant progress in the bioremediation in all samples In the samples with no liposomes added there was ~60% reduction in the oil contamination (13 2 mg/10g soil of s d ±1 37) In the samples with the higher liposome concentration (0 6%) there was also a reduction of ~60% in the oil contamination (13 1 mg/10g soil of s d ±0 4), in the samples with the lower liposome concentration (0 1%) the oil contamination reduced by ~70% (9 4 mg/10g soil of s d ±1 62) After three weeks at the end of the incubation period the bioremediation in soil treated with liposomes (at two concentration) seems to improve though the bioremediation in soil treated with no liposomes didn't improve (samples with no liposomes 14 8 mg/10g soil of s d ±1 02) When using liposomes at the lower concentration (0 1%) there was ~80% reduction in the oil contamination (6 mg/10g soil of s d ±0 66), ~75% reduction in the oil contamination was achieved when using liposomes at the higher concentration (0 6%) (9 96 mg/10g soil of s d ±1 16)

Fig 2 Kinetics of bioremediation in soil contaminated by ~17,000 ppm oil



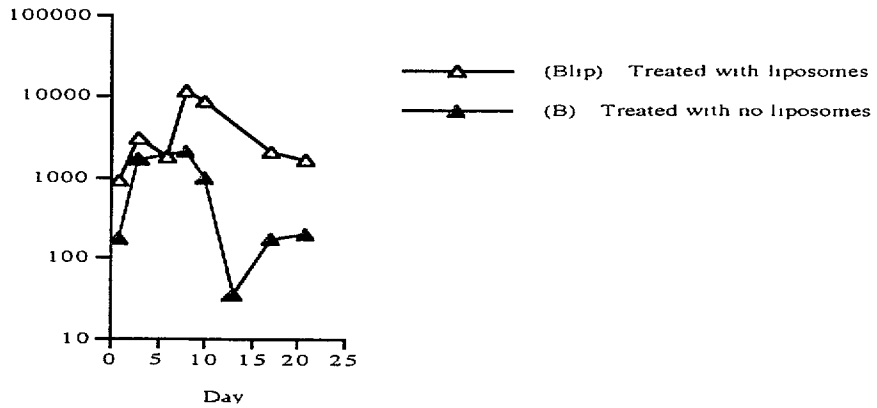
Legend figure 2 In all three soil samples A B and C after one week of treatment there seems to be no biodegradation After two weeks of bioremediation both in soil treated with no liposomes (A) and in the soil treated with 0 6% liposomes (B) there is 60% biodegradation the soil treated with 0 1% liposomes there is a 70% reduction in the amount of oil (C) After 3 weeks in sample (C) there is no progress in the boiremediation compared with the prior week in sample (B) the amount reduced is ~75% and in soil (C) the amount reduced is ~80%

B10 Comparison of the influence of various liposome formulations on bacterial growth

In the soil contaminated in the lab by 10 000 ppm crude oil the number of colony forming units (CFU) when treated with liposomes is higher during most of the experiment compared with the soil treated with no liposomes (figure 3) The CFU in the soil treated with liposomes at day one is ~1x108 compared with ~1x107 in the soil with no liposomes Up till day 7 the amount of CFU in both seems to be similar ~1 5x108 in the following days till the end of the experiment the number of CFU in the soil with liposomes is higher then in the soil with no liposomes The CFU in the soil with liposomes is in day 8 ~1x109 and reduces gradually to

~1x10⁸ till the end of the experiment day 21 In the soil with no liposomes the CFU in day 9 is ~1x10⁸ then reduces till day 12 to ~1 5x10⁷ then again the CFU reach till the end of the experiment ~1x10⁷ The standard deviation in all soil aliquots for the CFU was up to ±1x10⁵

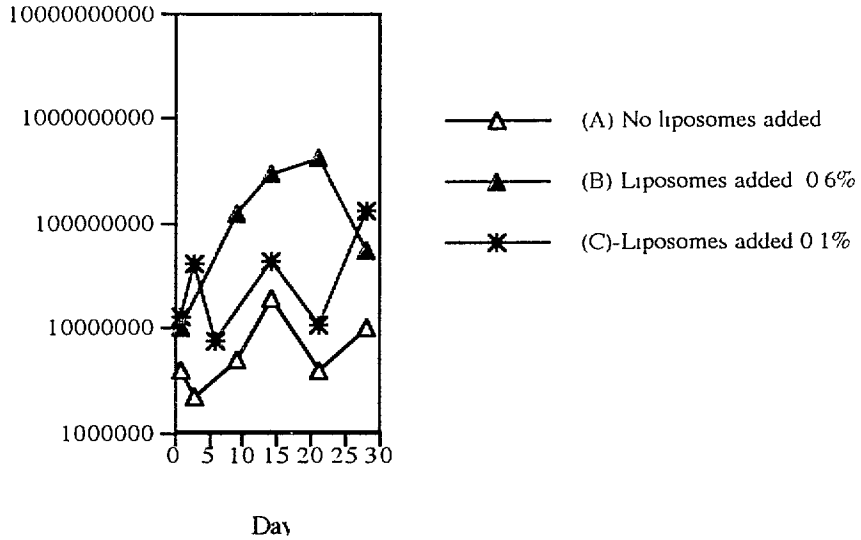
Fig 3 Bacterial growth in soil contaminated by in the laboratory by 10 000 ppm oil



Legend figure 3 In (Blip) soil treated with liposomes the number of CFU in day 1 was ~1x108 and it increases to ~1x109 till day it, then it is reduced gradually to ~1x108 till day 21 the end of the experiment In (B) soil treated with no liposomes at day 1 the number of CFU is ~1x107 then it reaches it's maximum ~1x108 at day 9, till day 12 it is reduced to ~1 5x106 then the CFU are increased up ~1x107 to at the end of the experiment day 21

In the soil contaminated accidentally in the field by 17 000 ppm crude oil the results are similar to those of the soil contaminted in the lab (described above) here too the number of CFU is generally higher in the samples treated with liposomes compared with the samples treated with no liposomes In Soil samples with 0 6% liposomes added the number of CFU in day 1 was ~1x10⁷ it increases gradually up to ~1x10⁹ day 21 then it decrease to ~1x10⁸(Fig 4) In the soil with 0 1% liposomes added the number of CFU in day one seems to be very high ~1x10⁷ then it increases till day 6 to ~1 5x10⁷ At day 14 the number of CFU is lower then ~1x10⁸ then it decreases to the same number as in day 6 and increases again till day 28 to above ~1x10⁸ CFU In the soil treated with no liposomes the number of CFU at day 1 was ~1 5x10⁶ the number of CFU increases to ~1 5x10⁷ at day 14 it then decreases to ~1 5x10⁶ and increases again to ~1x10⁷ at day 28 An increasing number of CFU in oil contaminated soil with added liposomes were shown in many experiments with soil contaminated with different amounts of oil, both in the soil contaminated "naturally" and in soil contaminated in situ (data not shown)

Fig 4 Bacterial growth in soil contaminated by ~17 000 ppm oil



Legend figure 4 (A) in soil with no liposomes added the number of CFU starts with ~1 5x106 then it decreases by 0 25 log at day 3 it increases to ~1 5x107 till day 14 then it decreases to ~1 5x106 and increases again to ~1x107 (B) number of CFU in soil treated with 0 6% liposomes in day 1 is ~1x107 then it increases till day 21 to ~1 5x108 and decreases to ~1x108 at the end of the experiment (C) number of CFU in the soil with 0 1% liposomes added is in day 1 as in (B) then it increases by 0 5 log at day 3 and decreases again to the same CFU as in day 1 at day 7 The number of CFU increases again to ~1 5x107 at day 14 then it is decreased to ~1x107 at day 21 and increases again to the number of ~1x108 CFU

B10 Characterization of bacterial populations enhanced by liposomes

Soil contaminated with oil were incubated with and without liposomes and stirred manually every second days for 30 days Samples were streaked on plates for bacterial growth and treated by hexane to determine the amount of oil degraded Bacteria were identified by API 20 NE and by GC analysis of fatty acids with a HP GC 5890 apparatus Some bacteria were present in all the soil samples with or without liposomes treatment as shown in the table below

Bacteria that appeared in all soil samples during all the experiment

Bacteria	Method of identification
Micrococcus luteus	GC
Orchrobactrum anthropi	GC & API 20E
Pseudomonas cepacia	API 20E
Bacillus megaterium	GC
Pseudomonas stutzeri	GC
Alcaligenes xylosoxydans	GC & API 20E
Nocardia asteroides	GC

Other bacteria appeared in the soil when the liposomes were added during the whole period of the experiment as shown in the following table

Bacteria that appeared in the liposomes treated soil

Bacteria	Method of identification
Staphylococcus hominis	GC
Micrococcus kristinae	GC
Micrococcus varians	GC
Alcaligenes piechaudi	GC
Bacillus lateroporns	GC

Finally, we characterized the bacteria that grew during four weeks of bioremediation in soil contaminated accidentally by ~17 000 ppm crude oil both in aliquots treated with 0 1% liposomes and in aliquots treated without liposomes The bacteria colonies that where picked for characterization where the prevalent bacterial colonies that had a minimum of ten colony on the petri dish In the first three weeks of incubation all bacteria which grew in the soil without liposomes grew also in the soil with liposomes (9 species) In the soil treated with liposomes 5 additional bacteria species grew, after four weeks of incubation in the soil treated with liposomes another seven bacteria species grew in addition to the other fourteen that appeared in the weeks before (total of 21 bacteria species) This phenomenon did not occur in the soil that wasn't treated with liposomes no additional species where located other than the ones found during the first three weeks (table 1)

Table 1 Bacteria species characterized during 4 weeks of bioremediation in soil contaminated accidentally by 17,000 ppm crude oil, treated with (0 1%) and without liposomes

No	Bacteria	Method of characterizing	Samples with no liposomes during 4 weeks of experiment	Samples with liposomes during 3 weeks of experiment	Samples with liposomes on the 4th week of experiment
1	*Micrococcus luteus**	GC	+	+	+
2	*Ortobactrum anthropi*	GC & API20NL	+	+	+
3	*Pseudomonas cepacia**	API20NE	+	+	+
4	*Bacillus megaterum**	GC	+	+	+
5	*Psedomonas stutzeri**	GC	+	+	+
6	*Alcaligenes xylosoxydans**	GC & API20NE	+	+	+
7	*Xantomonas maltophilia**	API20NE	+	+	+
8	*Sphman pancimobilis*	API20NE	+	+	+
9	*Nocardia asteroides**	GC	+	+	+
10	*Staphylococcus hominis*	GC	-	+	+
11	*Micrococcus kristinae**	GC	-	+	+
12	*Micrococcus varians**	GC	-	+	+
13	*Alcaligenes piechaudi**	GC	-	+	+
14	*Bacillus laterosporus*	GC	-	+	+

15	*Staphylococcus epidermidis*	GC	-	-	+
16	*Staphylococcus cohini*	GC	-	-	+
17	*Staphylococcus warneri*	GC	-	-	+
18	*Bacillus circulans**	GC	-	-	+
19	*Corynebacterium aquaticum**	GC	-	-	+
20	*Listera seeligeri*	GC	-	-	+
21	*Micrococcus lylae**	GC	-	-	+

* Bacteria that are mentioned in the literature as capable of degrading oil components

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The Department of Microbiology and the Industry in general in Kazakhstan has benefited from up to date methodology in bacteriology and from advanced equipment acquired through this grant In addition PCR technology was introduced through training of a two Kazakh post-graduate student in Israel and by equipment acquisition

The collaboration between the two laboratories has enable to accomplish the proposed studies according to the original plan The Kazakh investigators have isolated oil degrading bacteria in Kazakhstan These bacteria were characterized by classical and advanced methodologies which were transferred to the Kazakh scientists Our methods in bacterial identification of bacteria were transferred to Kazakhstan and this will allow to continue the identification of the relevant bacteria involved in bioremediation The PCR methodology acquired during this grant is now available in Kazakhstan

This grant allowed the establishment of E-Mail which has changed dramatically the possibilities of the Kazakh Scientis to communicate with the world A PCR machine (MJ research) micropipettes (Gilson) with tips were purchased by the Kazakh scientists and will allow the use of PCR for population study of bacteria.

Specific bacteria were found to be enhanced by the presence of liposomes Most of the bacteria enriched during treatment of contaminated soil belonged to genera known to be able to use oil as a source of energy These bacteria with the liposomes studied during this grant could be a new basis for efficient bioremediation of soil contaminated by oil in Kazakhstan and elsewhere in the world

In conclusion, the technology and the research generated by this grant will enable further development in the study of bioremediation in Kazakhstan

PROJECT PRODUCTIVITY

The collaboration between the two laboratories has enable to accomplish the proposed studies according to the original plan The Kazakh investigators brought their expertise in isolating numerous new oil degrading microorganisms which were characterized with the expertise of the Israeli investigators The bacteria isolated in Kazakhstan are being tested in a new system for their increased ability to degrade oil in the presence and in the absence of liposomes This may result in the description and use of new oil degrading bacteria which might contribute to the improvement of bioremediation of contaminated soils with oil

FUTURE WORK

To improve the bioremediation in future experiments, different combinations and amounts of bacteria should be tested In addition, we found that the minimal medium used by us and defined by our colleagues in Kazakhstan contains a high amount of salt, especially NaCl (20g/l) New tests on growth and bioremediation were done using a minimal medium with only 5g/l of NaCl The data showed that in fact most of the strains were not able to degrade oil but were good emulsifiers Indeed in Kazhakhstan our colleagues confirmed our finding It is clear now for them that the strains # 27, 31 10 are only emulsifiers and do not show a significant petroleum degradation activity They suppose, that during the growth on the petroleum and using a petroleum hydrocarbons as a source of C and energy these strains can produce a hydrocarbons of different chemical structure and/or molecular weights This can be a cause why, we can not see a significant oil' fraction removal from the soil samples in the experiments As for the growth of the strains 27 31 on the petroleum in liquid culture, They think, that these bacteria do attach tightly to the oil drops during the growth and are not easily countable by common methods Considering that first, these strains are emulsifiers and second, that the petroleum emulsification facilitate other bacteria to degrade petroleum mixture, they suggest, continuing using the bacteria 27, 31 10 in the bioremediation experiments and try a combination with other strains even with *Pseudomonas putida* and see how they can facilitate soil bioremediation In Kazakhstan, our colleagues are now looking for additional strains which will be selected using a modified minimal medium as described above For continuing of the work with the mentioned strains it will be necessary to perform a gas-chromatography analysis of the petroleum after their growth to identify which oil fraction they are using and which emulsifiers they do produce in order to identify their emulsification activity Preparation for field trial in Kazhastan which could not be achieved in the time allocated to this project should be considered as a future project and the potential sources of financing such a project should be looked at

COLLABORATION, TRAVEL, TRAINING AND PUBLICATIONS

As planned our collaborators in Kazakhstan have isolated oil degrading bacteria and have brought them in Israel We managed to obtain visas for two post doctoral students from the Kazakh State University They arrived in April 1994 in Israel and worked for a full year at the Hebrew University (March 1995) They left with equipment (PCR) and materials (primers enzymes media various reagents) which allow them to continue the work in Kazakhstan The Kazakh principal investigators Professors Bersimbaev and Shigaeva visited Israel to discuss the progress achieved during the first year and to assess the training of their students

Two post doctoral Kazakh students were trained in our laboratory for a full year on scientific literature research (use of computerized library, Medline etc), on bacteriology (methods of identification) and on molecular biology for the PCR studies

Professor M Shigaeva and Professor R I Bersimbev from the Kazakh State University visited our laboratory in Israel by the end of August 1994 They were impressed by our facilities (library Xerox Scientific equipment etc) During their stay we managed to provide them

the scientific papers they needed They were able to discuss and see the progress of their two students They requested to extend the training period of their students in our laboratory for an additional six months to allow them to get more training They even asked about the possibility for their students to do their Ph D at the Hebrew University We agreed to extend the stay of the students until March 1995, but we could not, for financial and technical reasons (Language Ph D requirement at Hebrew University) commit ourselves to tutor these students for a Ph D at the Hebrew University, however, we agreed to be involved in their future research in Kazakhstan

Strengthening of developing country institutions This has been achieved in two levels

A) Training of two Ph D students from Kazakh State University in up to date bacteriology and molecular biology

B) Introduction of PCR technology done by using instruments bought through this grant and which were taken to Kazakh State University at the end of the training period

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