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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 91/049

ICGEB ref. #: CRP/CHI-90-01

Project initiation: 1991

Project termination: 1995

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project

Studies of the stress response in biomining microorganisms. Possible implications in the improvement of the bioleaching process.

Keywords: bioleaching, stress response, heat shock, starvation, *Thiobacillus ferrooxidans*.

UNIDO contract # 91/049 **ICGEB ref. #:** CRP/CHI-90-01

Project initiation: 1991 **Project termination:** 1995

Principal investigator's name: Carlos A. Jerez

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Abstract:

The biomining or bioleaching of minerals is a process by which metals such as copper, gold and uranium are solubilized from the ores containing them by the action of acidophilic microorganisms. During this process, bacteria are subjected to different kinds of environmental stresses such as temperature or pH changes and lack of essential nutrients that may affect the activity of the bacteria, therefore, limiting the efficiency and rate of metal extraction. In response to these stressing conditions, bacteria are known to change the genetic expression of several cellular polypeptides, inducing stress proteins and others as a defense or remediating mechanism to overcome the adverse condition.

The main objective of this project was to know the stress responses of extreme acidophiles such as *Thiobacillus ferrooxidans* at the molecular level. This is not only interesting from the fundamental point of view, but will help also to monitor the in situ state of these microorganisms, making possible to improve their activity in industrial processes.

Experimentally, we studied the global gene expression changes by using two-dimensional polyacrylamide gel electrophoresis of the proteins synthesized in vivo and identified some of the polypeptides induced by microsequencing of their N-terminal ends. We analyzed in detail the proteins synthesized by *T. ferrooxidans* when subjected to heat shock (from 30° to 40°C), cold shock (from 30°to 10°C), pH stress (from 1.5 to 3.5 and from 3.5 to 1.5) and the lack of nutrients such as phosphate or changes in nutrients, such as from ferrous iron to elementary sulfur, which usually take place in bioleaching operations. Some proteins were identified as stress proteins or molecular chaperones, and others as part of the different response systems. Based on the results obtained, we begun to develop immunological methods that will allow to monitor the planktonic and ore-attached bioleaching microorganisms and their in situ physiological state in industrial operations.

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

OBJECTIVES

1. To know the components specifically induced in response to pH, temperature and nutrient changes.
2. To prepare either antibodies or nucleic acid probes de detect the components and the levels in which they are induced.
3. To assess the relative physiological condition of the bacteria in a given bioleaching operation (for example, if it is preferentially oxidizing sulfur or if starved for a given nutrient) by using the probes prepared. Decisions could then be made, if possible, to change the conditions to improve the local bacterial activity (e.g., add phosphate, etc).

Methodology:

Detection of proteins synthesized under stress conditions. To study the global regulatory responses of *T. ferrooxidans*, we employed analysis of the proteins synthesized under different stressing conditions by means of two-dimensional polyacrylamide gel electrophoresis of both non-labeled and radio-labeled bacteria. The chemolithotrophic microorganisms were labeled by growing them in the presence of either $Na_2{}^{14}CO_3$ or [^{35}S]methionine.

Analysis of some specific stress-induced proteins. The localization of some of the proteins that changed their levels under different conditions was done by preparing outer membrane proteins or by ^{125}I-labeling in the presence of Iodogen. Identification of some of the proteins was done by employing immunological reactions. Some of the proteins of interest, were identified by microsequencing of their amino-terminal ends. The polypeptides were extracted from Commassie Blue stained 2-D gels (one or more spots of the same protein to have around 100 pmoles of each species). After rehydration, they were concentrated by running them in an SDS-PAGE. After this, the protein was transfered to a membrane of polyvinylidene difluoride (PVDF) for direct automatic Edman microsequencing.

Preparation of polyclonal antibodies. Polyclonal antibodies against whole bacterial cells of *T. ferrooxidans* were employed to monitor the microorganisms under different conditions. Polyclonal antibodies were also prepared against surface proteins induced by the lack of phosphate.

Reverse genetics and expression of some proteins induced under different growth conditions. To identify some of the genes of interest, whose expression varies during the different growth conditions, we started to employ reverse genetics. For this, we used the information generated from the N-terminal end sequences of the different proteins identified in 2-D gels. From these sequences, and the information of codon usage by *T. ferrooxidans* we synthesized the appropriate degenerate oligonucleotides. With this oligonucletides we generated the corresponding probes to identify and isolate the DNA fragments containing the genes in the genomic DNA from *T. ferrooxidans*.

RESULTS
(compare against the set objectives)

Heat shock response. We have previously studied the heat shock response of mesophilic bioleaching microorganisms such as *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and also of the thermophilic archaeon *Sulfolobus acidocaldarius*. We found that all these microorganisms apparently induced heat shock proteins. In *T. ferrooxidans* we found that the main proteins induced upon heat shock were the equivalents to *E. coli* DnaK and GroEL proteins (5, 13). This was established by immunological analysis (western blott) and by microsequencing of the N-terminal end of the corresponding proteins (5). These polypeptides showed a great identity with the *E. coli* chaperones (70% for GroEL and 80% with DnaK) and also had an oligomeric structure. GroEL proteins from both bacteria formed a 14-mer, whereas *E. coli* DnaK protein existed partially as a dimer and the *T. ferrooxidans* DnaK-equivalent showed only a monomeric nature under our experimental conditions (5). We also found that the levels of phosphorylation of DnaK and GroEL and other proteins greatly varied when *T. ferrooxidans* were subjected to heat shock and phosphate starvation. These results support the possible role of phosphorylation of chaperones in the sensing and regulation of the stress response in bacteria (Seeger, M., Osoric, G. and Jerez, C. A., submitted).

pH stress response. We found that a pH shift (3.5 to 1.5) also elicited a heat shock-like response in *T. ferrooxidans*. When *T. ferrooxidans* cells grown at pH 1.5 were shifted to pH 3.5, there were several changes in the general protein synthesis pattern, including a large stimulation of the synthesis of a 36,000 molecular weight protein (p36). The apparent low isoelectric point of p36, its location in the membrane fraction, and its cross-reaction with anti-OmpC from *Salmonella typhi* suggested that it may be a porin whose expression is regulated by extracellular pH (1).

Phosphate-starvation response. To cope with phosphate limitation, bacteria such as *E. coli* have evolved complex regulatory systems to assimilate phosphorous very efficiently. Phosphate regulation is complex, perhaps because phosphate is an essential nutrient. In the case of *T. ferrooxidans*, nothing was known about the proteins that may be involved in the phosphate scavenging and uptake mechanisms. We analyzed the molecular response of *T. ferrooxidans* to phosphate starvation. Cultivation of the bacteria in the absence of added phosphate not only reduced their growth rate and capacity to oxidize ferrous iron but induced a remarkable filamentation of the cells (7). Two-dimensional gel electrophoresis revealed at least 25 proteins whose levels of synthesis were increased upon phosphate limitation, as well as some polypeptides that were exclusively synthesized under the starving condition (6-8). One of the proteins induced by the lack of phosphate was an acid phosphatase with a pH optimum of about 3.8, and a M.W. of 26 kDa, which was apparently located in the periplasm. The N-terminal end sequence of the protein showed some homology with the known sequence of *Lysobacter enzymogenes* alkaline phosphatase and the *Escherichia coli* alkaline and acid (pH 2.5) phosphatases (7). The proteins induced by *T. ferrooxidans* upon phosphate starvation are probably part of a phosphate scavenging system utilized by the microorganisms in their natural environment, in which soluble forms of phosphorous could be limiting.

We found that phosphate starvation of *T. ferrooxidans* increased its capacity to attach to a sulfide mineral and to elemental sulfur (9). To establish if this effect was due to changes in the bacterial surface components, we analyzed the outer membrane proteins and the lipopolysaccharide (LPS) by employing SDS-PAGE and Western blotting with antisera against either whole cells or LPS from *T. ferrooxidans*. The amount of LPS in the cells starved for phosphate was about 25% higher when compared with control cells. However, only minor differences in the average size of the repeating units of the polymer were seen (9). These results, and the fact that several outer membrane proteins were induced in the phosphate-starved cells (4) suggest that not only LPS, but surface proteins and possibly hydrophobicity may play an important role in *T. ferrooxidans* attachment to minerals (9, 12).

Some of these polypeptides could be traced to the surface of the bacteria (4). One of these proteins was the *T. ferrooxidans* outer membrane protein (Omp40), whose level of expression was apparently regulated by the external pH and the concentration of phosphorus. The amino terminal sequence of Omp40 showed little identity with the *Escherichia coli* OmpC, OmpF or PhoE porins, but it was 38.5% identical to the outer membrane channel-forming protein NosA from *Pseudomonas stutzeri*, whose expression is also regulated environmentally (4).

We have prepared antibodies against whole cells of *T. ferrooxidans*, *L. ferrooxidans* and *T. thiooxidans* to monitor these cells under different conditions (2, 3, 11). We also prepared polyclonal antibodies against the outer membrane proteins induced under phosphate starvation that will be used for immunofluorescence to monitor the in situ state of bacterial cells in simulated industrial conditions (García, A., Varela, P. and Jerez, C. A. "Evolution of the planktonic and ore-attached populations of bioleaching microorganisms and determination of their in situ physiological state". Abstract book, 7th International Symposium on Microbial Ecology, Aug. 27-Sept 1st, Santos, Brazil, 1995)

Work plan and time schedule (originally envisaged)

RESULTS (continued...)

Changes in global expression of *T. ferrooxidans* when grown in sulfur. There is great interest in studying the structure, expression and regulation of the genes involved in the oxidation of ferrous iron and sulfur, since they make *T. ferrooxidans* and other bioleaching bacteria industrially important. Several proteins involved in ferrous ion oxidation by *T. ferrooxidans* are known, including rusticyanin, cytochromes a and c and other proteins. On the other hand, the proteins involved in sulfur oxidation have not been studied in detail and their cellular localization and exact roles are still unknown.

To identify some of the gene products involved in these oxidations, we have initiated the study of the changes in global gene expression in *T. ferrooxidans*, when the bacteria is grown on sulfur or iron. A few polypeptides were exclusively synthesized when the cells were grown in ferrous iron, and others were apparently synthesized only when *T. ferrooxidans* was grown in sulfur (10, 13).

By microsequencing of their N-terminal ends, and by specific Heme group staining, we have identified some of these proteins as cytochromes. In addition, we are preparing antibodies against some of the bacterial surface proteins that change their expression according to the environmental conditions. These antibodies will also allow us to monitor the in situ physiological state of the bioleaching microorganisms. This is important to establish, since due to the chemical and biological leaching of sulfides, elemental sulfur is generated which tends to coat mineral particles, interfering with the process.

Work plan and time schedule (originally envisaged)

1. Studies on the synthesis of different stress proteins. During the first and second years the plan contemplated the study of the response of *T. ferrooxidans* to stressing conditions of temperature, abrupt pH changes (in their growth range, between 1.5 and 3.5) and lack of the essential nutrient phosphate.

2. Characterization of some stress proteins. The plan contemplated during the three years the characterization of outer membrane proteins, periplasmic phosphatases and the molecular chaperones DnaK and GroEL.

3. Studies simulating industrial conditions. Studies with ores were planned during the three years to monitor the microorganisms in conditions similar to the actual bioleaching operations.

4. Isolation of some genes of interest. These studies were planned for the second and third years, once information on the proteins and their identification was obtained.

5. Preparation of antibodies and DNA probes. Antibodies against different components from *T. ferrooxidans* were planned for the second and third years.

6. Use of antibodies and probes to monitor bioleaching operations. These studies were planned at the end of the proyect.

Work plan and time schedule (actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

	First year	Second year	Third year
Dates of payments reception	July 91	Feb 93	Jul 94

Activities:

1.	Studies on the synthesis of different stress proteins.	xxxxxxxx	xxxxxxxx	
2.	Characterization of some stress proteins.	xxxxxxxx	xxxxxxxx	xxxxxxxxx
3.	Preparation of antibodies against total bacteria and surface components.	xxxxxxxx		xxxxxxxxx
4.	Microsequencing of N-terminal ends of different polypeptides	xxxxxxxx	xxxxxxxxx	xxxxxxxxx
5.	Generation of DNA probes for reverse genetics			xxxxxxxxx
6.	Studies of monitoring bacteria grown in ores.			xxxxxxxxx
7.	Publications (numbers as in the list enclosed).	1-5	6-9	10-15

In general, most of the initial objectives planned were developed as expected. The characterization of some of the genes coding for proteins induced by the lack of phosphate in *T. ferrooxidans*, such as PhoB and PhoR and those apparently involved in preferential oxidation of sulfur was initiated at the end of the project and it will be further developed in future research projects.

We initiated collaboration with the group of Dr. Héctor Torres from Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI), Buenos Aires, Argentina.
We have had collaborative interaction with Dr. Tomás Vargas, from Facultad de Ciencias Físicas y Matemáticas, Universidad de Chile, specially for the studies involving ores and monitoring of microorganisms.
We collaborated with Dr. Ricardo Amils, from Universidad Autónoma de Madrid, since they visited our laboratory to learn the 2-D PAGE procedures we employ for the bioleaching microorganisms, and we learned pulse field gel electrophoresis technique in Spain.
We also had exchange with Dr. Börje Lindström, from the University of Umeã in Sweden where we performed the first microsequencing of the proteins studied.

PUBLICATIONS

1. **Amaro, A.M., Chamorro, D., Seeger, M., Arredondo, R., Peirano, I. and Jerez, C.A.** (1991) Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacterium *Thiobacillus ferrooxidans*. J. Bacteriol. **173**: 910-915.

2. **Jerez, C.A. and Arredondo, R.** (1991) A sensitive immunological method to enumerate *Leptospirillum ferrooxidans* in the presence of *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. **78**: 99-102.

3. **Jerez, C.A. and Arredondo, R.** (1991) Dot-immunobinding assay for specific and fast detection and enumeration of different bioleaching microorganisms. In Bioleaching: from molecular biology to industrial applications (Badilla, R. Vargas, T. and Herrera, L., eds.) Ed. Universitaria, pp. 89-94.

4. **Jerez, C.A., Seeger, M. and Amaro, A.M.** (1992) Phosphate starvation affects the synthesis of outer membrane proteins in *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. 98: 29-34.

5. **Varela, P. and Jerez, C.A.** (1992) Identification and characterization of GroEL and DnaK homologues in *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. 98: 149-154.

6. **Seeger, M. and Jerez, C.A.** (1992) The lack of phosphate affects global gene expression in *Thiobacillus ferrooxidans*. Geomicrobiol. J. 10: 227-237.

7. **Seeger, M. and Jerez, C.A.** (1993) Phosphate-starvation induced changes in *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. 108: 35-41.

8. **Seeger, M. and Jerez, C.A.** (1993) Response of *Thiobacillus ferrooxidans* to phosphate limitation. FEMS Microbiol. Revs. 11: 37-42.

9. **Amaro, A.M., Seeger, M., Arredondo, R., Moreno, M. and Jerez, C.A.** (1993). The growth conditions affect *Thiobacillus ferrooxidans* attachment to solids. In Biohydrometallurgical Technologies, Vol II. (A.E. Torma, M.L. Apel and C.L. Brierley, eds.). The Minerals, Metals & Materials Society pp. 577-585.

10. **Osorio, G., Varela, P., Arredondo, R., Seeger, M. Amaro, A.M. and Jerez, C.A.** (1993) Changes in global gene expression of *Thiobacillus ferrooxidans* when grown in elemental sulfu.. In Biohydrometallurgical Technologies, Vol II. (A.E. Torma, M.L. Apel and C.L. Brierley, eds.). The Minerals, Metals & Materials Society pp. 565-575.

11. **Amaro, A. M., Hallberg, K. B., Lindström, E. B. and Jerez, C. A.** (1994) An immunological assay for the detection and enumeration of thermophilic biomining microorganisms. Appl. Environ. Microbiol. **60**: 3470-3473.

12. **Arredondo, R., García, A. and Jerez, C. A.** (1994) The partial removal of lipopolysaccharide from *Thiobacillus ferrooxidans* affects its attachment to solids. Appl. Environ. Microbiol. **60**: 2846-2851.

13. **Jerez, C. A., Varela, P., Osorio, G., Seeger, M., Amaro, A. M. and Toledo, H.** Differential gene expression of *Thiobacillus ferrooxidans* under different environmental conditions. Minerals Bioprocessing II. (R. W. Smith, D. Holmes, eds.). The Minerals, Metals & Materials Society. In press.

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ .....
2) consumables	US$	2) consumables	US$ 8,263.58
3) training	US$	3) training	US$ .....
4) literature	US$	4) literature	US$ 1,433.22
5) miscellaneous	US$	5) miscellaneous	US$ 303.20
TOTAL GRANT	**US$**.....	**TOTAL**	**US$** 10,000.00

Please itemize the following budget categories (if applicable)

Capital equipment

-Microcentrifuge US$ 3,727.43
-Olympus fluorescence microscope and photomicrography attachments US$ 16,635.42
-Power supply and electrophoresis chamber US$ 2,561.65
-Laser printer US$ 2,161.88
-Gel dryer, UV transilluminator, magnetic stirrer and photographic system US$ 9,504.05

Training (provide names, duration of training, host laboratory)

- a) Carlos A. Jerez attended as Organizer and Convener of the Coloquium "Molecular Biology and Biochemistry of Acidophilic Chemolithotrophs: Applications on Bacterial Leaching of Ores" and the additional presentation of a poster, American Society for Microbiology General Meeting, New Orleans, USA, 26-30 May, 1992.
b) Presentation of a poster by C. A. Jerez in the symposium "Advances in Modern Biotechnology, La Habana, Cuba, 8-12 June, 1992. Both events US$ 3,000

Presentation of part of the work done in the project by Carlos A. Jerez in:
International Biohydrometallurgy Symposium, Jackson Hole, Wyoming, U.S.A., August 22-25, 1993. US$ 2,646.60. 7th International Congress of Bacteriology and Applied Microbiology, Prague, Czech Republic, July 3-8, 1994. Partial financing of US$ 600

Literature

-Geomicrobiology Journal, Special issue: Geomicrobial Agents and Processes for Bioleaching Metal Sulfide Ores.
-Ausubel et al., Short Protocols in Molecular Biology, 1992, John Wiley & Sons, Inc.
-Charackliss and Marshall, Biofilms, 1990, John Wiley & Sons, Inc.
-Bergey's Manual of Determinative Bacteriology, 1994, Williams and Wilkins.
-Watson et al. Recombinant DNA, 1992. W. H. Freeman and Company and other specialized texts.
-Subscription to Molecular Microbiology, Applied and Environmental Microbiology and other specialized journals.

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.

CONICYT

UNIDO STATEMENT OF ACCOUNTS DATE : AUGUST 25 1995

CONTRACT : Nº 91 / 049

INSTITUTION : UNIVERSIDAD DE CHILE

PROYECT TITLE : STUDIES OF THE STRESS RESPONSE IN BIOMINING MICROORGANISMS. POSSIBLE IMPLICATIONS IN THE IMPROVEMENT OF THE BIOLEACHING PROCESS.

PROYECTS LEADER : DR. CARLOS A. JERez C.

REPORT FOR THE PERIOD STARTING : FEBRUARY 25 1994 ENDING : FEBRUARY 25 1995
YEAR 1

I N C O M E : U.S.$. 10.000,00

UNIDO FUNDS RECEIVED (DATE) : JULY 28 1994
INTEREST EARNED : 0

TOTAL INCOME: U.S.$. 10.000,00

E X P E N D I T U R E

I T E M	ALLOCATED IN U.S.$.	RECEIVED IN U.S.$.	EXPENDITURE IN U.S.$.	ITEM BALANCE BROUGHT FWD.
SMALL EQUIPMENT	[illegible]	[illegible]	[illegible]	[illegible]
CONSUMABLES & CHEMICALS	7,000.00	7,000.00	8,263.53	-1,263.53
[illegible]			[illegible]	
[illegible]			[illegible]	
EDUCATION & TRAINING	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]				
[illegible]				
[illegible]				
LITERATURE	[illegible]	[illegible]	[illegible]	[illegible]
			[illegible]	
OTHER EXPENSES	[illegible]	[illegible]	[illegible]	[illegible]
T O T A L	10,000.00	10,000.00	9,999.52	0.48

POOR QUALITY

CONICYT

We certify that the above expenditures were employed to support the research planned in UNIDO Contract N° 91/049, in agreement with UNIDO guidelines and the allowed items

Dr. CARLOS JEREZ
Proyect leader.

Srta. ROXANY BARAHONA
Financial Officer

CONICYT

U N I D O — STATEMENT OF ACCOUNTS — DATE : JUNE 16 1994

CONTRACT : Nº 91 / 049

INSTITUTION : UNIVERSIDAD DE CHILE

PROYECT TITLE : STUDIES OF THE STRESS RESPONSE IN BIOMINING MICRCORGANISMS. POSSIBLE IMPLICATIONS IN THE IMPROVEMENT OF THE BIOLEACHING PROCESS.

PROYECTS LEADER : DR. CARLOS A. JEREZ

REPORT FOR THE PERIOD STARTING : FEBRUARY 25 1993 ENDING : FEBRUARY 25 1994.

I N C O M E : U.S.$. 35.000,00

UNIDO FUNDS RECEIVED (DATE) : FEBRUARY 16 1993
INTEREST EARNED : 0

TOTAL INCOME: U.S.$. 35,000,00

E X P E N D I T U R E

I T E M	ALLOCATED IN US.$.	RECEIVED IN US.$.	EXPENDITURE IN US.$.	ITEM BALANCE BROUGHT FWD.
SMALL EQUIPEMENT	14.000,00	14.000,00	16.635,42	(2.635,42)
Microscopio			12.512,00	
Gastos de Aduana			4.123,42	
CONSUMABLES & CHEMICALS	13.000,00	13.000,00	13.401,55	(401,55)
Chemicals			6.587,23	
Miscellaneous			6.814,32	
EDUCATION & TRAINING	4.000,00	4.000,00	3.246,60	753,40
(travel and per diem)				
Airplane Tickets			1.521,00	
Stay Expenses			1.725,60	
LITERATURE	1.000,00	1.000,00	669,65	330,35
Books			669,65	
OTHER EXPENSES	3.000,00	3.000,00	1.046,43	1.953,57
Inscrip. Symposium			1.046,43	
T O T A L	35.000,00	35.000,00	34.999,65	0,35

UNIVERSIDAD DE CHILE - FACULTAD DE MEDICINA - DIVISION DE CIENCIAS MEDICAS NORTE

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UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION

UNIDO CONTRACT Nº 91/049 REFERENCE Nº CRP/CHI90-01)

FINANCIAL STATEMENT

FOR THE PERIOD 07/91 TO 07/92

NAME OF PRINCIPAL INVESTIGATOR: CARLOS JEREZ GUEVARA.

INSTITUTION: UNIVERSIDAD DE CHILE

PROJECT TITLE: Studies of the stress response in biomining microorganisms. Possible implications in the improvement of the bioleaching process.

AMOUNT RECEIVED FOR PERIOD 07/91 TO 07/92 US$ 35.000.-

BUDGET CATEGORY	
SMALL EQUIPMENT	18.000.-
CONSUMABLES & CHEMICALS	10.000.-
EDUCATION & TRAINING	3.000.-
LITERATURE	1.000.-
OTHER EXPENSES	3.000.-
TOTAL EXPENDITURES	35.000.-

FINANCIAL OFFICER: FERNANDO NAVAS M. (Signature)

(print)

DATE: 10/05/92 PHONE: 7370081/5213