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The Effects of Oil and Dispersants on Polulations of Fungi

J.P. Sherry

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The Effects of Oil and Dispersants on Polulations of Fungi

J.P. Sherry

Final Report on Contract #OSS79-0001

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OBJECTIVES OF CONTRACT

1. To monitor the short and long term effects of oil and oil dispersant applications on the geo-aquatic and water mould components of oligotrophic freshwater ponds.

2. To monitor the long term effects of oil and oil dispersant applications on pond fungi capable of growth on two oil supplemented media: one containing microbially degraded, and the other non-degraded, Norman Wells crude oil as the sole carbon source.

3. To examine the ability of selected pond fungi to utilize Normal Wells crude oil as a sole carbon source.

4. To investigate the possibility of using induced mutations in fungi to assess the mutagenicity of water samples.

5. To initiate the development of a suitable fungal test system.

6. To analyze a set of water samples from the Love Canal region of the Niagara River for mutagenic activity.

SUMMARY

Methods are described for determining the effects of oil and oil dispersant applications on fungi in freshwater ponds. Detailed experimental data are presented describing the effects of oil and oil dispersant applications on the following mycological parameters: geo-aquatic fungi, water moulds, fungi capable of utilizing crude oil as a sole carbon source, and fungi capable of utilizing biodegraded oil as a sole carbon source. Thirty-nine fungal isolates were tested in pure culture for the ability to utilize Norman Wells crude oil as a carbon source: 12 were capable of so doing.

The scientific literature was reviewed with the purpose of selecting a fungal system for use in the analysis of water samples for mutagenic activity; several such systems are described. A single system, Saccharomyces cerevisiae D7, was selected for further development: test procedures, work schedules, and safety procedures are described for the D7 system.

The D7 test system was used to analyze a set of water samples from the Niagara River for mutagenic activity: no activity was detected.

CONCLUSIONS

1. Artificial pond systems are suitable for use in determining the effects of additives on aquatic fungal populations.

2. Fungi present in oligotrophic water posses the ability to degrade crude oil.

3. There are several fungal systems available that are apparently suitable for use in the analysis of water for mutagenic activity.

4. The *Saccharomyces cerevisiae* D7 system appears to be suitable for inclusion in a battery of short term microbial mutagenic tests.

5. Futher development work is warranted on the use of fungi to detect mutagenic activity in water samples.

1 INTRODUCTION

In this report I shall present a summary of the progress made and the problems encountered during my years' work on contract #OSS79-00011. The contract encompassed two main work areas: a study of the effects of oil and dispersants, with time, on the distribution and population densities of fungi in the research ponds at Bruce Point, and an evaluation of the feasibility of using induced mutations in fungi as indicators of the mutagenicity of water samples. It was also proposed that I participate in the preparation of an overview report on the oil degradation project. I shall deal with each of these topics in turn; firstly, however, I shall discuss the contract from an overall point of view.

1.1 Overall Comments

The bulk of the experimental work took place in the Microbiology Laboratories at NWRI under auspices of Mr. B.J. Dutka, the scientific authority for the contract. Excellent research facilities were made available in the Microbiology Laboratories and despite budget restrictions I was able to adapt existing equipment to cover most of my requirements, thus eliminating unnecessary capital expenditures: this was particularly true in the case of the yeast test.

I obtained all chemicals and growth media from either the Microbiology Laboratories chemical stores or from commercial sources; Dr. B.F. Scott, NWRI, provided the crude oil and oil dispersant. I encountered difficulties obtaining some mutagenic chemicals necessary for the development of the yeast test: the commercial synthesis of some carcinogenic chemicals such as Dimethylnitrosamine has been discontinued because of U.S. safety regulations.

Technical assistance was also provided by the Microbiology Section. The technician concerned, S. Kuchma, had worked with me on pregious projects (e.g., "Occurrence of Pathogenic Fungi in Bathing Beaches") and was familiar with basic mycological techniques; however, he had limited training in the advanced chemical, biochemical, and genetic techniques required for the yeast test. Although the extensive 'on the job' training required caused considerable delays, the situation may have been of mutual benefit to both technician and project: Scott has received a grounding in advanced mycological techniques, while the mutagenicity test procedures have been made very tight and, consequently largely operator-proof; this will be of benefit to other laboratories using our procedures to assess the mutagenicity of water samples.

Because the library at NWRI does not stock any of the standard genetics or mutagenesis journals, I did most of the literature survey work at the MacMaster University library.

I remained within the financial limits of the contract, and submitted brief progress reports for each of the months worked.

2 EFFECT OF OIL AND DISPERSANT ON FUNGI IN FRESH WATER PONDS

2.1 Introduction

Over the past forty years, the industrial societies have become increasingly dependent on oil and its products for their prosperity. Since the world's oil reserves appear to be confined to distinct geographic locations, this dependence has necessitated the transport of oil, usually in its crude form, from areas of production to areas of utilization. Inevitably, large quantities of oil must be shipped across water; consequently, the risks, and indeed instances,

of aquatic oil spills have increased with the development of the industrial nations.

Because a large sector of North America's industrial complex is concentrated in the Great Lakes basin, regular shipments of oil and its products occur therein throughout the shipping season. Yet, the possible effects of a major oil spill on either a simulated or natural Great Lakes ecosystem have yet to be determined.

In the event of such an oil spill the first line of defence must be the containment and subsequent mechanical cleanup of the spilt oil. However, weather conditions and the oil spill's location may preclude, or limit, the effectiveness of mechanical measures. In these instances other steps have to be taken to lessen the effects of the spill: the addition of chemical dispersants to the contaminated water may prove effective. A precondition for the use of chemical dispersants is that their effect on the ecosystem should be less deleterious than allowing the original oil to remain.

The present study was undertaken as part of a larger project designed to examine the distribution and effects of an oil dispersant application on freshwater ponds and to compare this with a pond treated with oil only. Outdoor, artificial ponds were selected for the experiment since they experience natural weather conditions and encourage a biotic succession, important factors in long term assessment. The following biological parameters were monitored: zooplankton, phytoplankton, bacteria, and fungi; only the fungal data are reported here.

The fungi are a large group of heterotrophic microorganisms, the majority of which are saprophytes and may be found wherever organic matter occurs. Both moulds and yeasts have been reported from

diverse aquatic ecosystems, where they may be present in both the water column and sediment. Those fungi found in natural waters may be divided into various groups, each showing a different degree of adaptation to life in the aquatic environment. The aquatic phycomycetes (water moulds) are probably the most highly adapted group: they occur in virtually all types of aquatic systems, and are characterized by the production of motile asexual reproductive spores: a distinct advantage to an organism that must live and reproduce in water. A variety of yeasts and moulds more commonly associated with the soil microflora have been consistently recovered from polluted and non-polluted waters (Cooke, 1957; Park, 1972a). Storm water runoff, land drainage, sewage treatment plant discharges, and air deposition may each contribute to the "geo-aquatic fungal" content of natural aquatic systems. Although the exact functional role of "geo-aquatic fungi" within the aquatic environment has yet to be elucidated, it is thought that they participate in the degradation and recycling of organic matter. Fungi from both terrestial (Perry and Cerniglia, 1973; Bemmann and Troeger, 1975; Shmotina, 1975) and aquatic (Ahearn and Meyers, 1976) environments can degrade oil.

In this project we concentrated our attention on the long term effects of oil and oil dispersant applications on the geo-aquatic and water mould components of the mycofloras of the experimental ponds. We also monitored the ponds for fungi capable of growth on two oil supplemented media: one containing microbially degraded, and the other non-degraded, Norman Wells crude oil as the sole carbon source.

2.2 Literature

A survey of the scientific literature was made under the following general headings:

a) The effect of oil on fungi in soil.

b) The effect of oil on fungi in aquatic environments.

c) The ability of fungi to degrade oil.

d) The effect of oil on bacteria in soil.

e) The effect of oil on bacteria in aquatic environments.

f) The ability of bacteria to degrade oil.

g) Mechanisms of microbial degradation of oil.

A computer search, biological abstracts, current contents, and review articles were used to obtain relevant references; copies of many of the articles were procured. A summary of the information contained in these references will be included in the publications arising from this study.

2.3 Materials and Methods

2.3.1 Ponds

The pond site was at the University of Toronto's Institute for Environmental Studies Field Station a Baie du Dore, near the

Douglas Point Nuclear Power Plant on Lake Huron. The five ponds measured 22.5 m x 9 m x 2 m deep and were lined with four layers of 6-mL black polyethylene sheeting. Water from nearby Lake Huron was pumped into the five ponds to a depth of 1.5 m; sufficient clean gravel, containing sand and silt, was then added to the ponds to provide a 5-cm deep sediment layer.

Initially, the ponds were allowed to stabilize for four months, after which the biota and water in each pond were monitored at least once a month until the project's termination. In late June, after examining the bacterial, fungal, phytoplankton, and zooplankton data, it was decided to use only four of the ponds in the experiment: ponds 1 and 3 were used for the oil and dispersant spills; pond 4 was used for the oil only spill; pond 2 was used as a control.

The oil spill was made by pouring six litres of Normal Wells crude oil onto the water surface of pond 4 from a height of one metre. The oil and dispersant spills were made as follows: one litre of Corexit 9527 dispersant was added to six litres of oil, hand stirred into thirteen litres of pond water, and poured onto the pond's water surface. Since each pond contained approximately 41 x 10^3 litres of water, the above treatments would produce concentrations of about 100 ppm oil and 20 ppm dispersant in the water phase - assuming that the dispersion process was completely successful.

Samples for mycological analysis were taken from near-surface, mid-depth, and near-bottom levels, and were immediately refrigerated and transported to the laboratory; all samples were processed within 30 hours of collection.

2.3.2 Geo-aquatic fungi

The geo-aquatic fungal content of the water samples was determined using a membrane filtration procedure described in Dutka (1976). Following triplicate filtration of appropriate sample portions, duplicate membrane filters (0.45 µm) were implanted onto mARGPA agar (Table 1), and then incubated at 15°C for five days. The third membrane filter was plated onto mSTMEA agar (Table 2), and was also incubated at 15°C for five days: mSTMEA proved useful in the selective enumeration of pink and cream coloured yeast colonies.

2.3.3 Oil utilizing fungi

Fungi capable of utilizing either crude or biodegraded oil as a sole carbon source were also enumerated by means of membrane filtration. Appropriate volumes of pond water were filtered through 0.45 µm membrane filters which were then plated onto freshly prepared basal agar medium (medium OBA, Table 3) containing either crude or biodegraded oil (2 mL/22 mL medium); control plates contained no oil. The inoculated oil media plates were incubated at 20°C for 21 days; fungal colonies were then enumerated in two categories: as total colonies and as well developed (strong growing) colonies. This subjective enumeration procedure was used to bias the data in favour of fungi that utilized the media incorporated oil as a carbon source, as opposed to fungi growing as background contaminants on impurities in the agar media.

Microbiologically degraded oil was prepared by addition of Normal Wells crude oil to a carbon-free chemically defined solution (10% V/V) (Liu and Dutka, 1972) in a 1-L erlenmeyer flask; a teflon membrane filter, through which 100 mL of pond water had been filtered,

was added as the microbial inoculum; no inoculum was added to the control flask. The flasks were loosely covered with tin foil to permit evaporation of the lighter volatiles, and were incubated for four months on a reciprocal shaker at 20°C in the dark. After incubation the degraded and non-degraded oil mixtures were sterilized in a microwave oven: these solutions were used as the sole carbon sources in medium OBA.

2.3.4 Enumeration of fungal colonies

Fungal colonies were enumerated using a steromicroscope (X10); all data are arithmetic mean values of triplicate or duplicate determinations and are expressed as colony forming units (CFU) per 100 mL.

2.3.5 Water moulds

The water mould content of the ponds was monitored using two methods: one quantitative and the other qualitative. The number of viable water mould propagules in the water samples were determined using a spread plate technique, and a hemp seed (*Cannabis sativa*) baiting technique was used to qualitatively recover water moulds.

a) Spread plate technique

One mL portions of each sample were spread onto the surface of predried plates of PSP agar; the inoculated plates were then incubated at 15°C for 48 hours. The number of typical phycomycete colonies growing on each plate was then enumerated; all potentially positive

colonies were transferred into pure culture on PSP agar (Table 4) and stored at 5°C for subsequent identification.

b) Baiting technique

Water moulds were qualitatively recovered from water samples as follows: 50 mL of water sample was placed into a previously sterilized battery jar; 100 mL solutions of NaCl (1% W/V), streptomycin (350 ppm) and sterile distilled water were added to the battery jar; 10 sterile, split hemp seeds were then added to each jar; the baited samples were incubated at 20°C for three days; 650 mL of sterile distilled water was then added to each jar and incubation was continued for a further four days; the number of colonized single seeds and clumps of colonized seed were then enumerated as units.

Individual colonized seeds and colonized seed clumps were transferred into pure culture on PSP agar and stored at 5°C for subsequent identification.

c) Identification of water mould isolates

All potentially positive water mould isolates from the PSP spread plates and the hemp seed baits were identified to generic level before tabulation as confirmed isolates.

The potential water mould isolates were grown on PSP agar at 15°C for 24 hours; six sterile, split hemp seeds were then placed on the agar surface, concentric with the colony margin; incubation was then continued

at 15°C for two more days, by which time the hemp seeds were usually well colonized. The colonized hemp seeds were then transferred into glass petri dishes: three seeds into each of two dishes containing distilled water and dilute salts solution (Table 5), respectively. After 24 and 48 hours the developing fungal colonies around each seed were examined microscopically for characteristic asexual reproductive structures; in order to induce asexual sporulation, it was sometimes necessary to repeat the above procedure or to incubate the cultures overnight at 5°C and then stand them at room temperature for four hours. The isolates were identified to generic level using standard mycological keys and taxonomic references.

2.3.6 Determination of the ability of selected pond fungi to utilize Norman Wells crude oil as a sole carbon source

a) Test fungi

The fungi listed in Table 6 were tested for their ability to utilize Norman Wells crude oil as a sole carbon source. These fungi were isolated from the indicated ponds (Table 6) by picking typical strong growing colonies from the surface of membrane filters, through which pond water samples had been passed and which had then been incubated on OBA agar containing crude oil, for one month at 20°C. The selected fungi were subcultured until pure on potato dextrose agar (PDA, Oxoid), and were then described (Table 6); moulds were stored on PDA slants at 5°C; yeasts were stored on yeast extract - malt extract agar (YM, Table 7) slants at 5°C.

b) Spore suspendions

Sporulating PDA cultures of the test moulds and YM agar cultures of the test yeasts were flooded with a sterile 0.1% (V/V) solution of Tween 80; after adequate agitation the resultant spore suspensions were filtered through several layers of sterile gauze to remove mycelium and debris. The spore suspensions were stored over night at 5°C before being used to inoculate the OUA agar slants (OUA, Table 8).

c) Oil preparation

Norman Wells crude oil was topped to 31% weight loss by exposure to a fresh air flow at room temperature: this measure ensured the removal of the potentially toxic volatile oil fractions.

d) Test procedure

Slants of oil utilization agar (OUA) were inoculated with spore suspensions of the test fungi: care was taken to spread the suspension evenly over the entire slant surface. The inoculated slants were incubated for 24 hours at 20°C to facilitate spore germination and adherence of the young mycelium to the agar surface. Crude oil was then pipetted into the tubes to a level half way up the agar slant, and the cultures were reincubated at 20°C for 30 days; then the oil was carefully removed using a pasteur pipette and growth was estimated by visual comparison with oil-free inoculated controls and an uninoculated slant that had contained crude oil.

2.3.7 Determination of the ability of selected pond fungi to utilize Norman Wells crude oil as a sole carbon source in the presence of Corexit 9527 oil dispersant

Test procedure

The procedure used was as previously described (Section 2.4(d)) with the exception that a 1:5 (by weight) mixture of Corexit 9527 dispersant in topped Norman Wells crude oil was added to the analytical and control tubes.

2.3.8 Determination of the ability of selected pond fungi to utilize Corexit 9527 as a sole carbon source

Test procedure

The procedure used was as previously described (Section 2.4(d)) with the exception that Corexit 9527 dispersant, either undiluted or diluted 1:5 (by weight) with sterile distilled water was added to the analytical and control tubes.

2.3.9 Determination of the toxicity of Corexit 9527 oil dispersant towards selected oil utilizing fungi

The toxicity of Corexit 9527 oil dispersant towards the following oil utilizing fungi was determined: OP1a&b, OP3a&b, OP5b, OP8b, OP9b, OP10, OP14a&b, OP15, OP16, OP17, OP23, OP25.

Four mm inoculum disks, cut from the margins of colonies of the test fungi growing on Czapek Dox agar (Table 9) were used to inoculate plates of test agar (X5): the test agar consisted of Czapek Dox agar containing the following Corexit concentrations (ppm): 0.19, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 800,

1600. The inoculated plates were incubated at 20°C; when the fungal colonies had nearly reached the edge of the control plates (no added dispersant), the diameters of the control and analytical colonies were measured; toxicity was calculated as the percentage growth inhibition when compared with the control colony.

2.4 Results

The results are presented in subsections: each consists of a title and data tabulation; the results will be discussed in detail in the publications arising from this study.

2.4.1 Effect of oil and oil dispersant mixtures on the geo-aquatic fungi of fresh water ponds

The raw and processed data obtained are presented in Tables 10 through 15.

2.4.2 Effect of oil and dispersant mixtures on pond fungi capable of utilizing crude oil as a sole carbon source

The raw and processed data obtained are presented in Tables 16 through 21.

2.4.3 Effect of oil and dispersant mixtures on fungi capable of utilizing biodegraded oil as a sole carbon source

The raw and processed data obtained are presented in Tables 22 through 27.

2.4.4 Effect of oil and oil dispersant mixtures on the phycomycete populations of fresh water ponds

The raw and processed data obtained are presented in Tables 28 through 31.

2.4.5 The ability of pond water fungi, to utilize Normal Wells crude oil as a sole carbon source

The raw and processed data obtained are presented in Tables 32 through 35.

2.5 Mycological Input into the Oil Study Report

The Materials and Methods section of this report forms the basis of the mycological section of the oil study Report #2; the Results section forms the basis of the oil study Report #3.

Portions of the data presented have already appeared in two publications (Scott et al., 1979; Dutka et al., 1980), and have been presented at two conferences (Dutka et al., 1980b and c).

3 USE OF FUNGI TO DETECT MUTAGENS IN WATER

3.1 Introduction

Over 80% of all human cancer is environmentally induced; implicated agents include UV light, viruses, and various forms of radiation and carcinogenic substances; carcinogenic chemicals cause a large proportion of human cancer and are believed to be responsible

for the current elevated cancer incidences. Carcinogenic chemicals are thought to act subcellularly by inducing aberrations in the genetic material, at either the molecular or chromosomal level: when such mutations lead to a loss of regulatory function, the result may be tumor growth and cancer.

When genetically active chemicals cause mutations in germ line or fetal cells, the offspring (if it does not abort) may be genetically and physiologically defective. Scientists in some of the advanced industrial nations are now seriously concerned about the impact of accumulated recessive mutations on mankind: the possble emergence - as a result of widespread contact with environmental mutagens - of high proportions of defective humans is no longer the concern of science fiction writers alone.

Thus, environmental mutagens may have a dual effect on man by increasing the instances of both cancer and birth defects.

Because of expense and time factors it is not feasible to use conventional mamalian tests to assess the potency of each of the 50,000 synthetic chemicals that are currently available - not to mention the 500 to 1,000 new chemicals that are put on the market each year. For this reason, several short term tests have been developed for the purpose of screening suspect chemicals for mutagenic activity: theoretically, if a chemical is carcinogenic it should also be capable of inducing mutations in test cells; when properly selected such mutations are easier to detect than cancer, which can take up to 20 years to develop. The most popular of the short term tests is the Ames' test which makes use of reverse mutations in the bacterium Salmonella typhimurium to detect mutagenicity in test chemicals: 90% of the carcinogens tested have given positive results in the Ames' test - an impressive correlation by any standards.

With so many mutagenic chemicals in every day use it is highly probably that many find their way into the aquatic environment through industrial and sewage treatment plant effluents, or through urban and rural land drainage: in fact, a number of carcinogenic chemicals have been identified in natural fresh water systems (Rokosh, 1980). Monitoring effluents and receiving waters for the presence of individual carcinogenic chemicals would be both excessively expensive and time consuming. It should be possible, however, to adapt short term mutagenicity screening tests for the detection of mutagenic activity in effluent, water, and sediment samples; positive samples could be further examined using analytical chemical techniques and, where necessary, mamalian carcinogenicity tests. The Ames' test has already been used for this purpose in the Microbiology Laboratories at NWRI.

There are, however, a number of weaknesses associated with the Ames' test: because bacteria are prokaryotic organisms they may respond differentyly than eukaryotic cells to mutagenic chemicals; the Ames' test does not, in its present form, permit the quantitative analysis of test samples; the Salmonella strains used are unstable: their spontaneous reversion rates are notoriously variable; a reverse mutation system is used to detect mutagenic activity: however, reverse mutations show high mutagen specificity, which limits their usefullness in broad spectrum screening techniques.

Because of the limitations of the Ames' test a decision was made to establish a battery of short term tests for the screening of water and effluent samples for mutagenic activity. The objectives of the present study were twofold:

1) to investigate the possibility of using induced mutations in fungi to assess the mutagenicity of water samples; and

2) to intiate the development of a suitable fungal test system.

3.2 Literature

A survey of the scientific literature was made under the following general headings:

a) Use of fungi/yeasts to detect mutagenicity in chemicals.

b) Use of fungi/moulds to detect mutagenicity in chemicals.

c) Use of Salmonella to detect mutagenicity in chemicals.

d) Use of Escherichia coli to detect mutagenicity in chemicals.

e) Use of microorganisms (moulds, yeasts, bacteria) to detect mutagenicity in water.

f) Use of microorganisms (moulds, yeasts, bacteria) to detect mutagenicity in effluents.

A computer search, biological astracts, current contents, and review articles were used to obtain relevant references; copies of many of the articles were procured; the failure of the NWRI library to purchase a subscription to the journal Mutation Research had a negative effect on this literature review, and necessitated several trips to the MacMaster University library. A summary of the information obtained will be included in the publications arising from this study.

3.3 Fungal Systems Used to Detect Mutagenic Activity in Chemicals

It was apparent from the literature review that several fungal systems have been used to detect the mutagenic activity of both chemicals and radiation emissions. Three organisms have been much used in such work: *Neurospora crassa*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*.

3.3.1 *Neurospora crassa* system

The haploid fungus *Neurospora* can form heterokaryons: colonies that contain more than one haploid nucleus of different genetic composition in their cytoplasm in each cell. Heterokaryons can be used to study many of the same genetic effects that occur in diploid organisms. When two different haploid strains with different biochemical requirements are combined to form a heterokaryon three types of conidia are produced: two different homokaryotic types that will grow only on supplemented media, and a heterokaryotic type that can grow on minimal medium; for example:

		Conidia
AB +	AB+	AB+ AB+ has AB requirement
plus	++C	++C ++C has C requirement
++C		AB+ ++C no requirement (unless a mutant)

The heterokaryotic conidia contain *at least* one haploid set of chromosomes of each genotype together in the same cytoplasm - not in the

same nucleus as in a diploid cell. Heterokaryons can be used to measure recessive lethal damage to cells. Recessive lethals in both heterokaryons and the diploid cells of higher organisms are caused by the same mechanisms: point mutations and and chromosome deletions.

An ingenious system, based on N. crassa heterokaryons, has been developed that not only provides a measurement of mutation induction resulting from gene inactivation at two specific loci, but can also provide an estimation of recessive lethal induction over the entire genome (de Serres and Malling, 1971). As far as is known, the technique is neither mutation nor gene specific: it detects all types of mutagenic activity resulting in the structural alteration of DNA; and, in addition, genetic damage to any gene in the genome is detectable.

The commonly used strain of N. crassa consists of two haploid components:

I) A hist-2 ad-3A ad-3B nic-2 + ad-2 + inos +.

II) A + + + + al-2 + cot + pan-2.

Only the heterokaryotic conidia of this strain will grow on minimal medium. Mutants at the ad-3A and ad-3B loci block sequential steps in the synthesis of purine causing the accumulation of purple pigment in the mycelium and an adenine requirement. The ad-2 block which precedes the ad-3 blocks was included in component I to prevent that component contributing to pigment accumulation: only inactivation of the ad-3A and ad-3B loci will cause purple colony formation. Therefore, pigment production is used as the criterion for a mutational event. The ad-3 system is ideally suited for inclusion in a screening program

designed to detect mutagenic activity in chemicals because it detects all types of mutagenic activity that cause structural alteration to DNA.

The estimate of recessive lethal damage over the entire genome is obtained by determining the percentage of heterokaryotic colonies with recessive lethal damage in one of the two components: component II is this case. Conidia from post treatment single colony isolates of heterokaryotic colonies are plated onto minimal medium supplemented with pantothenate. Colonies homokaryotic for component II should be small and compact, because of the cot/cot homozygous markers: any reduction in the number of such colonies inidcates recessive lethal damage to component II in the heterokaryotic culture.

The above system has been used to assess the mutagenicity of numerous chemicals: actinomycin D (Fisher, Malling, de Serres and Snyder, 1975), metronidazole and niridazone (Ong and Slade, 19078), aflatoxins (Matzinger and Ong, 1976), and nitrofurans (Ong, 1977).

3.3.2 Saccharomyces cerevisiae systems

Yeasts are genetically well understood organisms: much work has been done on their genetic composition and the damaging effects of mutagenic chemicals and radiation on it. Because yeasts are eukaryotic organisms, they resemble higher organisms in the manner in which their cytoplasm, nucleus, and genetic material are organized. Because of the extensive biochemical and physiological information available on the behaviour of yeasts in axenic culture it is possible to accurately control and manipulate them in the laboratory; yeasts can also be studied in haploid, diploid, or polyploid form: an advantage when designing different mutational systems. In short, yeasts combine the

practicality of bacteria with the genetic complexity and versatility of higher organisms: considerations which make them ideally suited for use in the screening of chemicals for genotoxic potential.

Several yeast test systems have been devised for the purpose of detecting mutagenic activity in chemicals: most of them using the brewers yeast S. cerevisiae. The most commonly used tests are summarized in Table 36; each of the tests utilizes one or more of the following genetic phenomena: reverse mutation, forward mutation, mitotic crossing over, and gene conversion.

a) Reverse mutation:

In reverse mutation systems a mutagen is used to revert a mutant locus to its wild type state: usually resulting in the loss of a nutritional dependency. In reversion tests the types of mutagen detectable are limited to those capable of acting on a specific locus in the test cells, often by means of a single selective mutation: such as a specific base pair substitution or frame shift mutation. Many mutagens, however, are restricted in their mode and locus of action; thus, some mutagens give false negative results in gene reversion tests. The use of a battery of reverse mutation systems would reduce this weakness, while adding considerably to the workload. Offsetting the above weaknesses are several advantages: the mutations monitored are caused by known mechanisms, and the results give a direct quantification of mutation induction - not merely an indirect estimation.

b) Forward mutation:

Forward mutation systems are not mutagen specific. Theoretically, any chemical capable of causing a one locus mutation from wild type should be detectable using a forward mutation test, irrespective of its mode of action. In practice, however, there are many technical weaknesses associated with currently used forward mutation systems: extensive replica plating is often required, and many of the marker genes are inadequately understood (Zimmermann, 1971).

c) Mitotic crossing over:

Mitotic crossing over is usually detected by the appearance in a heterozygous diploid of a recessive marker. It is necessary to demontrate the generation of the two reciptorcal products of the cross over in order to exclude the interference of other genetic phenomena such as point mutations, gene conversion, aneuploidy, etc. Many carcinogenic and mutagenic chemicals induce mitotic crossing over.

d) Gene conversion:

Mitotic gene conversion allows the expression of recessive genes; it involves the non-reciprocal exchange of genetic material between two homologous chromosomes. Mitotic gene covnersion frequencies in *S. cerevisiae* can be quantified by monitoring the restoration of a wild type gene and the loss of a nutritional requirement in a strain that contains two different, functionally defective, and non-complementing alleles of that locus.

Although the precise mechanism of gene conversion is not yet fully understood, it is known to involve localized chromosome pairing and the unilaterial transfer of pieces of chromatid of up to 1,000 nucleotides in length between homologous regions of chromatids. When a nutritional deficiency is eliminated the convertant will grow on a medium lacking the originally required growth factor.

Despite extensive investigations, mutagen specificity has yet to be associated with gene conversion systems. Because of this lack of mutagen specificity, it has been suggested (Murthy, 1979) that all chemicals shown to be non-mutagenic in the Salmonella reversion systems should be further tested for the induction of gene conversion in a suitable diploid yeast before their non-mutagenicity is confirmed.

Test strains acquired:

Before selecting a strain of S. cerevisiae for use in the analysis of water for mutagenic activity, I acquired cultures of the following strains: D3, D4, D5, D7, S211a, S238 and S288a. I was unable to obtain a culture of JD1, despite repeated requests to its developer.

System selected:

Strain D7 was selected for use in the initial feasibility studies because it combines gene conversion, mitotic crossing over, and gene reversion systems in the one strain. The genotype and phenotype of D7 are described in Table 36.

4 SYSTEM DEVELOPMENT

4.1 Long Term Storage of Test Strains

Permanent stock cultures of the acquired strains were prepared as follows: tubes containing 5 mL of YEPG broth (Table 37) were inoculated with the test strain and were incubated at 28°C for 24 hours; 0.8 mL of broth culture and 0.07 mL of dimethyl sulfoxide were then placed into 2-mL sterile tubes which were then sealed; the tubes were then stored in liquid nitrogen. Cultures were revived by removing the tube from the liquid nitrogen and scraping some of the frozen culture in YEPG broth; the inoculated broth was incubated at 28°C.

4.2 Routine Storage of Test Strains

Two methods were used for the routine maintenance of stock cultures: agar slant cultures and low density broth cutures. Agar slant stock cultures were prepared by streaking the surface of appropriately supplemented YEPG agar slants (Table 37) with fresh broth grown cell suspensions; the inoculated slants were incubated for 24 hours at 28°C and were then transferred to 5°C. Broth stock cultures were prepared by inoculating tubes of YEPG broth - 5 mL/tube - with a single loop of a 24-hour YEPG broth grown cell suspension; the inoculated broth cultures were stored at 5°C. Both the above procedures were repeated at least once every three months.

4.3 Checking Test Strains

The test strains were tested periodically for the retention of those genetic markers essential to the various yeast tests.

4.3.1 Haploid strains

Haploid strains were tested on a routine basis for the presence of diploid cells which can result from mating type locus mutations and subsequent conjugation. Inoculum from late log cultures of the haploid strains, in YEPG broth, were streaked onto the surface of sporulation agar (Table 38); the inoculated plates were incubated at 25°C and were examined after 24 and 48 hours for the presence of asci: when present, asci inidcate that diploidization has occurred and that the strain has, therefore, deteriorated.

4.3.2 Diploid strains

Diploid strains were tested on a routine basis for accumulations of haploid cells resulting from meiosis. Inoculum from late log cultures of the haploid strains in YEPG broth were streaked onto the surface of sporulation agar; the inoculated plates were incubated at 25°C, and were examined after 24 and 48 hours for the presence of asci: the complete failure or inability of large numbers of cells to produce asci indicates that meiosis has occurred in the stock culture, and that the strain has, therefore, deteriorated.

4.3.3 Strain D7

Approximately 200 cells of strain D7 were inoculated into each of 10 test tubes containing supplemented YEPG broth. The inoculated tubes were incubated for two days at 28°C on a reciprocal shaker; 0.1 mL aliquots were then removed and plated onto plates of T7 agar (SCM [Table 39]minus tryptophan) and 7b agar (SCM minus isoleucine) - 10 plates were spread in each case. The inoculated plates

were incuabted at 28°C, and the broth cultures were stored at 5°C. After four days the convertant colonies on the T7 agar plates, and the revertant colonies on the 7b plates were enumerated. Stock cultures that gave high spontaneous convertant or revertant frequencies were discarded; broth cultures that gave low frequencies were used to prepare fresh stock cultures.

4.4 Media Related Problems

In the initial stages of this project considerable difficulty was encountered growing strain D7 on both SCM and T7 agars. The problems were eventually traced down to the accidental elimination of one or more essential amino acids from the media; the problem did not reoccur after the media preparation procedures were redesigned. The theory that the other amino acids in the media would compensate for a missing one is erroneous, needless to say.

4.5 Preparation of Growth Curve for Strain D7

Two-day old colonies of D7, grown on SCM at 28°C were used to prepare a 2,000-cell/mL suspension of the yeast in sterile distilled water. Triplicate tubes, each containing 5 mL of supplemented SCM broth, were inoculated with 0.1 mL portions of the cell suspension; the inoculated tubes were incubated at 28°C on a reciprocal shaker (Eberbach). At the time intervals indicated in Figure 1, the number of viable cells in each tube was determined using a spread plate technique on SCM agar; these data were used to prepare a growth curve (Figure 1). The data presented in Figure 1 show that, under the specified growth conditions, the cultures are in the early stationary phase after 40 hours.

4.6 Safety Procedures

Because of the potential health hazards involved in working with mutagenic chemicals and potentially mutagenic water samples, strict safety procedures were adhered to at all times in the laboratory. Of particular concern was the question of how to deal with liquid mutagenic wastes; on instruction from the scientific authority for the project, I devised safety procedures for dealing with liquid mutagenic wastes in the Microbiology laboratories (Appendix #1). After a comprehensive discussion the proposed safety procedures were accepted at a section meeting.

4.7 Determination of Spontaneous Convertant Frequencies for Strain D7

Ten tubes of SCM broth (5 mL/tube) were each inoculated with 200 cells of strain D7, and were then incubated at 28°C on a reciprocal shaker. After 40 hours incubation 0.1 mL aliquots of each culture were spread onto plates of T7 agar - five plates per culture; the inoculated plates were incubted for three days at 28°C, and then the number of spontaneous convertant colonies on each plate was enumerated. The results of a typical determination are listed in Table 40; according to this determination, cultures #3, 6, 7, 8, 9, and 10 would be most suitable for use in a mutagenicity assay. The above procedure was used to select suitable D7 cultures for all mutagenicity tests.

4.8 Effect of Spread Plate Incubation Time on the Determination of Spontaneous Convertant Frequencies

The effect of spread plate incubation time on the detrmination of spontaneous convertant frequencies was determined using the

above procedure, with the exception that the spread plates were examined for convertant colonies after three and six days incubation at 28°C; the numbers of viable cells plated were determined using a spread plate technique on SCM agar. The results in Table 41 show that although increased incubation time does increase the number of spontaneous convertants enumerated, it does not affect the overall selection of suitable cultures: culture #3 in this case, would be rejected.

The mean number of convertants per 10^7 cells plated was 99.4: this compares favourably with the value of 105 reported by Zimmermann (1975).

4.9 Determination of the Number of Spontaneous Mutants at the ade2 and ilv1 Loci of Strain D7

The number of spontaneous mutants at the ade2 and ilv1 loci were occassionally determined. SCM7 broth cultures of D7 - inoculated with 200 cells per tube - were incubated with shaking at 28°C for 40 hours; 0.1 mL aliquots were then plated onto SCM7 agar (X10 plates per tube), and 1 mL aliquots were plated onto 7b agar; both sets of plates were then incubated at 28°C for seven days, when the number of revertant colonies on the 7b agar plates and the number of aberrant colonies on the SCM7 agar plates were determined. The number of spontaneous mutants at both loci were always less than four per 10^7 cells.

Test procudure development:

A preliminary test procedure was devised; it was used to test the response of strain D7 to the known mutagen ethyl methane sulphonate. Cells of D7 were exposed to the indicated concentrations of EMS (Table 42) in phosphate buffer pH 7, for four hours at 28°C. Two conclusions were drawn fromt the results presented in Table 42: the

numbers of cells recovered from the test were too low; and excessively large percentage of the aberrant colonies were of a hairline nature (20 to 30%). These observations suggested the following modifications to the test procedure: the centrifugation speed during the cell washing procedure was increased from 5,000 rpm for 10 min to 10,000 rpm for 20 min; a cluster removal step was introduced to the test procedure in an effort to reduce the number of hairline colonies.

Test of modifications #1 and 2:

A fresh D7 cell suspension of 3×10^7 cells per mL was prepared and put through the cell washing and cluster removal procedures. Microscopic examination of the resultant cell suspension showed a complete absence of cell clusters, which are the cause of hairline colonies. The final cell yield, while an improvement over the previous result, was still too low: 42% of the theoretical maximum yield. For this reason two further modifications were introduced to the procedure: the centrifugation conditions were changed to 15,000 rpm for 30 min; and a method was devised for draining the washing buffer from the centrifuge tubes, without disturbing the sedimented cells, by means of a vertical suction apparatus.

Test of modifications #3 and 4:

The foregoing modifications were tested using the proposed procedure. Four separate trial runs gave yields of 84.3, 77.4, 74.5 and 81.6% of the theoretical suspension yield; this improvement in cell yields was considered adequate for the time being.

4.10 Mutation Induction in S. cerevisiae D7: Test Procedure

The above modifications and the preliminary test procedure were combined to give the test procedure outlined in Appendix 2. The

laboratory work schedule required to maintain this test procedure on a week-to-week basis is described in Table 43.

With the D7 test procedure, it is imperative that the backup culture maintenance steps be strictly adhered to, so as to avoid unnecessary time delays: failure to inoculate a few broth tubes on a Thursday can result in the loss of a week's production.

4.11 Test of Revised D7 Yeast Test Procedure

The response of strain D7 to EMS was determined using the revised test procedure. Cells of D7 were exposed to the indicated EMS concentrations (Table 44) in phosphate buffer at pH 7 for four hours at 28°C. Several observations were made on the results presented in Table 44:

a) The survival rates observed are erratic; 2% EMS should cause an approximately 40% killing rate in D7 under the described test conditions.

b) The numbers of cross over and total aberrant colonies enumerated were too low for the described test conditions.

c) The numbers of convertant colonies enumerated were consistent with the described test conditions.

d) The numbers of revertant colonies enumerated on the 2% treatment plates were consistent with the described test conditions; however, the numbers enumerated following treatment with the lower EMS concentrations were too high: an indication that suppressor mutants were erroneously enumerated as revertants on these plates.

The above observations suggested that the EMS solutions may not have been prepared correctly; close examination of the techniques used in the preparation of the mutagen solutions supported this conclusion. Consequently, the test solution preparation procedures were revised and the above test was repeated.

4.12 Repeat Test of Revised Yeast Test Procedure

The results presented in Table 45 show that improvements were obtained in the mitotic crossing over and the gene reversion results: both of which were now more consistent with the described test conditions. The survival rates, however, were still erratic; we did not come to grips with the cause of this problem until after the terms of the present contract had expired. However, I believe the problem has now been solved.

4.13 Preliminary Attempt to Incorporate a Metabolic Activation System into the D7 Test Procedure

Because many chemicals require metabolic activation before becoming active in microbial mutagenesis tests, a metabolic activation system was devised. The essence of the activation system is the addition of liver microsomes that have been isolated from Aroclor dosed rats to the test system. One mL portions of S-9 mix (Appendix 3) were added to the appropriate D7 test tubes in the place of the 1 mL portions of phosphate buffer. The metabolic activation system has been incorporated into the revised D7 test procedure.

2-Acetyl aminofluorene - the chemical used as a positive control for the metabolic activation system in the Ames' test - was

tested for its mutagenic activity towards D7 in the presence of the S-9 mix. The results presented in Table 46 indicate that 2-AAF is non-mutagenic towards D7: none of the D7 test systems showed a positive response. This test was repeated with greater replication (X2) and similar negative results were obtained.

A search of the literature then revealed that two chemicals would probably be more suitable as positive controls for the metabolic activation system: dimethyl nitrosamine and diethyl nitrosamine. An extensive search of the commercial chemical supply houses revealed that DMN is no longer available because of its potent carcinogenicity; a quantity of the less mutagenic DEN was purchased: because it was not delivered until after mid-March, it was not investigated under the terms of the present contract.

4.14 Mutagenic Analysis of Some Niagara River Water Samples Using the D7 Test Procedure

On November 3 and December 5, 1979, water samples were collected from the following locations in the Love canal region of Niagara River:

Sample #	Location
263	In Lake Ontario 1 km from mouth of river.
264	In Lake Ontario 10 km from mouth of river.
265	2 km below Love canal.
266	Devils Hole, New York.
267	Lewiston, New York.
268	Youngston, New York.

The samples were sored in the dark at 4°C, and were prepared for analysis as follows: samples were firstly sterilized by filtering through 0.2 μm membrane filters; portions of each sample were then

evaporated, under vacuum, at 55°C to one-fifth and one-tenth of their original volumes; the samples were refiltered through 0.2 µm membrane filters and were tested for sterility by spread plating onto nutrient agar plates which were then incubated at 20°C for five days.

The samples were then tested for mutagenic activity using the revised D7 test procedure; the samples were also analyzed uding the Ames' test. the results presented in Tables 47 to 49 show that the samples were not mutagenic towards D7 at the concentrations tested.

APPENDIX 1

LIQUID MUTAGENIC WASTES

If dealt with incorrectly, liquid mutagenic wastes (LMW) can present a serious health hazard to laboratory workers: skin contact, vapour or aerosol inhalation, or ingestion can result in the occurrence of diseases that arise from damage to genetic material, often only after prolonged incubation periods. It is imperative, therefore, that safety procedures governing the processing of LMW be carefully devised; once accepted by the relevant parties, such procedures should be strictly adhered to by all personnel working with liquid mutagens.

A central problem must be faced in devising a protocol for the safe processing and handling of LMW: the risk of spillage must be eliminated. All other aspects of the protocol derive from the solution to this problem. Not surprisingly, the problem can be solved by dealing directly with its cause: avoid the accumulation of mutagenic wastes in liquid form; this can be achieved by absorbing all LMW onto a solid carrier material immediately after their generation. Consequently, the risk of a LMW spill no longer exists. Should a spillage of the absorbed waste occur, it can be dealt with using standard laboratory procedures for the removal of solid chemical spills.

The following safety procedure is based upon a central concept: absorbed mutagenic wastes present fewer safety hazards than do LMW.

Suggested Procedure for Dealing with LMW

1. Place carrier substance into a glass beaker: carrier substance may be tissue paper or wood chips; sawdust or cat litter will not suffice because of dust generation and adherence to the beakers walls.

2. Place beaker and carrier in the fume hood where work will occur.

3. Put one plastic disposal sack inside another; then put both sacks in a container designed for storage of hazardous waste materials.

4 As soon as any LMW is generated, pour it into the glass beaker.

5. Transfer the contaminated carrier substance into the plastic sacks before it becomes saturated; close the waste container.

6. Place fresh carrier substance in the beaker.

7. Repeat steps 4 to 6 as often as is necessary.

8. At the end of the work period, seal the plastic sacks and transfer to waste pick-up point.

9. The beaker may be either rinsed into the disposal sacks or discarded.

10. Observe maximum safety procedures during each of the above steps.

At no stage should any liquid mutagenic wastes be present in a work area.

The above suggestions are based on procedures originally developed by Litton Bionetics Inc: they should be used as a basis for discussion by the concerned parties: scientific staff, technical staff, management, safety committee, and media and washup room staff. Hopefully, such a discussion will result in an adequate and acceptable solution the LMW problem.

J.P. Sherry

February 28, 1980

APPENDIX 2

MUTATION INDUSTRION IN SACCHARO MYCES CEREVISIAE D7: TEST PROCEDURE

1. Inoculate tubes of D7 broth using stock cultures of *S. cerevisiae* D7; incubate inoculated tubes at 28°C with shaking.

2. After 24 hours, streak the broth culture onto plates of SCM7 and incubate them at 28°C for 48 hours.

3. Pick a typical small colony and prepare a cell suspension in 1.0 mL sterile distilled water. Adjust the cell titre to 2×10^3/mL.

4. Inoculate 10 tubes of D7 broth (5 mL per tube) with 0.1 mL aliquots of the D7 cell suspension. Incubate at 28°C with shaking.

5. When cultures reach early stationary phase, spread 0.1 mL aliquots onto plates of SCMT7 (x5). Incuate plates at 28°C for 48 hours. Store tube cultures at 4°C.

6. Count the number of convertant colonies growing on the SCMT7 agar plates; discard cultures that give an excessive number of convertants.

7. Discard the supernatant liquid from the selected cultures. Resuspend the yeast cells in sterile distilled water; centrifuge at 10,000 rpm for 20 minutes; discard the supernatant; repeat this wash procedure once. Resuspend the final cell pellet in 5 mL of sterile 0.1 M potassium phosphate buffer at pH 7.0.

Preparation of phosphate buffer:

KH_2PO_4, MW = 136.09; 0.2 M solution contains 27.22 g/L

K_2HPO_4, MW = 174.18; 0.2 M solution contains 34.84 g/L

	K_2HPO_4	KH_2PO_4	distilled H_2O
pH 7.0	61.0 mL	39.0 mL	100 mL
	0.122 M	0.078 M	OM = 0.1M

Adjust pH with KOH.

8. Dilute the yeast cell suspension 1:10 with phosphate buffer.

9. There should be less than 15% clusters in the cell suspension.

10. Clusters are removed by centrifuging slowly for five minutes at a speed at which it takes 20 minutes to sediment all the yeast cells.

11. Check the supernatant for cell clusters.

12. Repeat cluster removal step (No. 10), if necessary.

13. Prepare stock suspension of 3×10^7 cells/mL.

14. Store stock suspension at 4°C for a minimum period.

15. Pipette 3.5 mL portions of test solutions into test tubes (x2 per treatment). Place tubes in a shake water bath at 28°C and allow 15 minutes for temperature equilibrium. Use a buffer blank and a known mutagen (e.g., EMS) as control (Appendix 1).

16. Five minutes before test commencement, add 1 mL S-9 mix or 1 mL buffer to appropriate tubes.

17. Start treatment by pipetting 0.5 mL portions of cell stock suspension into the test tubes.

18. Incubate, with shaking, at 28°C.

19. Stop treatment by addition of 10 mL sterile, ice cold water; store this mixture in an ice bath for 30 minutes.

20. Spin down yeast cells in a refrigerated centrifuge (15,000 rpm for 30 minutes).

21. Resuspend pellet, in ice cold sterile distilled water, by thoroughly agitating at high speed; centrifuge, discard supernatant, repeat once.

22. Resuspend final cell pellets in 5 mL sterile distilled water (per original test tubes).

23. i) Plate 0.1 mL aliquots of final cell suspension onto SCMT7 (x5) - theoretical yield should be 2 x 10^5 cells per plate.

    ii) Plate 1 mL aliquots of final cell suspension onto SCM7b (x5) - theoretical yield should be 2 x 10^6 cells per plate.

    iii) Plate 0.1 mL aliquots of a 1 in 10 dilution of the final cell suspension onto SCMT7 (x5) - theoretical yield should be 2 x 10^4 cells per plate.

    iv) Plate 0.1 mL aliquots of: undiluted, -1, -1, and -3 dilutaions of the final cell suspension onto SCM7 (x40).

24. i) After three days' incubation at 28°C score survivors on SCM7.

ii) After three days' incubation at 28°C score convertants on SCMT7.

iii) After seven days' incubation at 28°C score revertants on SCMT7b.

iv) After seven days' incubation at 28°C, followed by two days at 4°C, score mitotic cross overs on SCM7.

APPENDIX 2

METABOLIC ACTIVATION PROCEDURE:

Each mL of S-9 mix has the following composition:

S-9 liver microsomal fraction (Litton Bionetics)	0.5 mL
$MgCl_2$ (0.4 M), prepared in stock solution	0.02 mL
KCl (1.65 M), prepared in stock solution	0.02 mL
Glucose-6-phosphate (1 M)	0.005 mL
NADP (0.1 M)	0.04 mL
Sodium phosphate buffer, pH 7.4 (0.25 M)	0.4 mL
Sterile distilled H_2O	0.015 mL

a) Thaw liver suspension and mutagenic chemicals.

b) Prepare G-6-P, NADP, and buffer solutions.

c) Mix above solutions MINUS S-9 FRACTION together; filter sterilize and add to the liver fraction.

d) Carry out test as described in the revised D7 test procedure.

PREPARATION OF EMS CONTROL SOLUTIONS

Prepare a 2.86% stock solution of EMS in 0.1 M phosphate buffer at pH 7.0 (28.6 mL EMS + 971.4 mL buffer). Dilute as follows:

Initial EMS Concentration (% v/v)	Reaction Concentration (% v/v)	Stock Volume (mL)	Buffer Volume (mL)	Stock #	Test Solution Volume (mL)	Buffer or S9 Volume (mL)	Cell Suspension Volume (mL)
2.86	2.0	3.5	0	1	3.5	1.0	0.5
	1.5	75	25	1	3.5	1.0	0.5
	1.0	50	50	1	3.5	1.0	0.5
	0.5	25	75	1	3.5	1.0	0.5

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TABLE 1. CONSTITUENTS AND PREPARATION OF MODIFIED AUREOMYCIN ROSE BENGAL GLUCOSE PEPTONE AGAR (MARGPA)

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Glucose	10.0
Peptone	5.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.5
Agar	20.0
Rose bengal	0.035
Aureomycin HCl	0.2

- Soak and dissolve all ingredients except Aureomycin in 800 mL distilled water for 15 min, then bring to the boil to dissolve. Sterilize by autoclaving at 121°C for 15 min, then cool to 42 to 46°C, pH 5.4 (approximately).
- Prepare antibiotic solution separately and add to the cooled medium.
- Dispense in 6 to 8 mL quantities into petri dishes (50 x 10 mm).

TABLE 2. CONSTITUENTS AND PREPARATION OF MODIFIED STREPTOMYCIN TERRAMYCIN MALT EXTRACT AGAR (MSTMEA)

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Malt extract	30.0
Peptone	5.0
Agar	15.0
Streptomycin	0.2
Terramycin HCl	0.2

- Soak and dissolve all ingredients except streptomycin and terramycin in 800 mL distilled water for 15 min, then bring to the boil to dissolve. Sterilize by autoclaving at 121°C for 15 min, then cool to 42 to 46°C, pH 5.4 (approximately).
- Prepare antibiotic solution separately and add to the cooled medium.
- Dispense in 6 to 8 mL quantities into petri dishes (50 x 10 mm).

TABLE 3. CONSTITUENTS AND PREPARATION OF OIL BASAL AGAR (OBA)

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
(i) BASAL MEDIUM	
NH_4NO_3	1.0
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.1
$CaCl_2$	0.1
$FeSO_4 \cdot 7H_2O$	0.01
KH_2PO_4	1.0
Trace metal solution	1 mL
Agar (Oxoid purified)	20.0
Rose bengal	0.035
(ii) TRACE METAL SOLUTION	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.1
$Na_2B_4O_7 \cdot 10H_2O$	0.09
$ZnSO_4 \cdot 7H_2O$	1.0
$MnCl \cdot 4H_2O$	0.06
$CuSO_4 \cdot 5H_2O$	0.35
2NHCl	2.5 mL

a) Filter sterilize (0.2 μm) trace metal solution.
b) Add constituents of basal medium (i) to IL distilled H_2O.
c) Heat to dissolve.
d) Adjust pH to 5.4 and dispense into boiling tubes (22 mL/tube).
e) Autoclave (10 psi/20 min).
f) Cool in water bath to 50 to 55°C.
g) Add 2 mL of test oil per tube.
h) Add filter sterilized aureomycin (200 μg/mL final concentration) to each tube.
i) Mix thoroughly and pour into plastic petri dishes.

TABLE 4. CONSTITUENTS AND PREPARATION OF PSP AGAR

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
$Na_2HPO_4 \cdot 12H_2O$	0.596
KH_2PO_4	2.04
Yeast extract (Difco)	2.0
Glucose	10.0
Agar	20.0

Adjust pH of the above medium to 6.2 and aseptically add 0.5 g/L sodium benzylpenicillin and 0.5 g/L streptomycin sulphate.

TABLE 5. DILUTE SALTS SOLUTION

STOCK SOLUTION AND VOLUME	COMPONENTS	WEIGHT (g)
#1 500 mL	$(NH_4)_2HPO_4$	66.04
	KH_2PO_4	68.05
	K_2HPO_4	87.09
#2 250 mL	$CaCl_2 \cdot 2H_2O$	18.38
	$MgCl_2 \cdot 6H_2O$	25.42

Add 0.5 mL of stock solution #1 and 0.1 mL of stock solution #5 per litre of distilled water. Autoclave at 121°C for 15 min.

TABLE 6. FUNGAL ISOLATES USED IN OIL UTLIZATION STUDIES

CULTURE #	SOURCE POND #	SUPERFICIAL DESCRIPTION*
OP1a	1 - 3	Mould, heavily sporulating, green reverse black with white margin.
OP1b	1 - 3	Mould, slightly floccose, yellow-brown powdery surface, reverse colourless.
OP2a	1 - 3	Yeast, slightly mucoid, shiny, coral pink.
OP2b	1 - 3	Mould, small colony (2 to 4 cm diam), mucoid spots on colony surface, dark green-black colour.
OP3a	1 - 3	Mould, small colony, no apparent sporulation, light green, reverse dark brown.
OP3b	1 - 3	Mould, rings of floccose mycelium, yellow with green sporulation areas, reverse yellow.
OP4a	1 - 3	Yeast, dark brown-black, very mucoid, common on oil containing media.
OP4(ii)	1 - 3	Mould, fine surface growth, moist mycelium, yellow, reverse yellow.
OP4b	1 - 3	Mould, large colony, floccose, green sporulation areas.
OP5a	1 - 3	Mould, floccose, grey-green, reverse grey-brown.
OP5b	1 - 3	Mould, heavy green sporulation on colony surface, reverse yellow.
OP6a	1 - 3	Yeast, mucoid, shiny, coral pink.
OP7a	1 - 3	Mould, grey-green floccose mycelium, many pycnidia, reverse grey-brown with white margin.
OP7b	1 - 3	Yeast, mucoid, shiny, black-brown.
OP8a	1 - 3	Yeast, mucoid, brown-black.
OP8b	1 - 3	Mould, full plate colony, heavy sporulation over entire surface, reverse light green.

* After 14 days incubation on PDA at 20°C.

Continued...../

TABLE 6 (CONT'D). FUNGAL ISOLATES USED IN OIL UTLIZATION STUDIES

CULTURE #	SOURCE POND #	SUPERFICIAL DESCRIPTION*
OP9b	1 - 3	Mould, full plate colony, heavy sporulating over entire surface, reverse light green.
OP10a	1 - 3	Mould, large colony floccose, green sporulation areas.
OP10b	1 - 3	Yeast,shiny, cream coloured.
OP11a	2	Yeast, slightly raised, cream.
OP12a	2	Yeast, mucoid, shiny, black-brown.
OP12b	2	Yeast, mucoid, shiny, black-brown.
OP13b	2	Yeast, cream.
OP14a	2	Mould, fluffy mycelium, grey-green, reverse grey.
OP14b	2	Mould, fluffy mycelium, white.
OP15a	2	Mould, large colony, floccose, green sporulation areas.
OP16	4	Mould, large colony, dark green sporulation areas, reverse black.
OP17	4	Mould, ¾ plate, heavy sporulation, light green, reverse brown, yellow diffusate in agar.
OP18	4	Mould, concentric regions of grey-green and colourless mycelium, mucoid sporulation areas near colony centre, reverse black with white margin.
OP19	4	Yeast, cream, matte, rough, contoured.
OP20	4	Mould ¾ plate, purpoe sporulation area, reverse green-black, margin white.
OP21	4	Mould, ¾ plate, depressed centre, black with white margin.
OP22	4	Yeast, mucoid, shiny, coral pink.
OP23	4	Mould, floccose, dark green with white margin, reverse black.

* After 14 days incubation on PDA at 20°C.

Continuted...../

TABLE 6 (CONT'D). FUNGAL ISOLATES USED IN OIL UTLIZATION STUDIES

CULTURE #	SOURCE POND #	SUPERFICIAL DESCRIPTION*
OP24	4	Yeast, mucoid, dark brown.
OP25	4	Mould, floccose, green with white margin, reverse black with white margin.
OP1c	1 - 3	Mould, floccose, pycnidia at colony centre, black, reverse black with white margin.
OP2c	1 - 3	Yeast, mucoid, shiny, coral pink.
OP3c	1 - 3	Yeast, mucoid, shiny, coral pink.

* After 14 days incubation on PDA at 20°C.

TABLE 7. CONSTITUENTS AND PREPARATION OF YEAST EXTRACT - MALT EXTRACT AGAR (YM)

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Yeast extract	3.0
Malt extract	3.0
Peptone	5.0
Glucose	10.0
Agar	25.0

Autoclave at 121°C for 15 min.

TABLE 8. CONSTITUENTS AND PREPARATION OF OIL UTILIZATION AGAR (OUA)

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Yeast nitrogen base (Difco)	6.7
Agar (Difco Noble)	20.0

Dissolve the agar in 900 mL distilled water; make up to 1 L; autoclave; cool to 45°C and add filter sterilized yeast nitrogen base; pour as slants in screw capped tubes (25 mm X 150 mm).

TABLE 9. CONSTITUENTS AND PREPARATION OF CZAPEK DOX AGAR

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Sodium nitrate	2.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.01
Sucrose	30.0
Agar	20.0

If glass distilled water is used, trace elements must be added: $ZnSO_4 \cdot 7H_2O$, 0.01 g; $CuSO_4 \cdot 5H_2O$, 0.005 g.

TABLE 10. NUMBER OF VIABLE FUNGI IN WATER SAMPLES (CFU/100 mL)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
		216	168	149	128	106	77	56	29	0	7	14	28	41	55	69	83	97	111	139	167	202	230	251	296	322	350	385
1	S	1360	900	103	50	337	1140	223	640	54	173	40	680	300	1020	1480	700	1340	400	6500	4800	620	-	400	183	70	380	2480
		780	800	74	67	273	1040	250	660	88	213	27	-	300	640	1180	400	1380	340	6100	5680	800	-	800	217	87	400	2360
						303									660				380	5800	4720	920	-	860	113	67	440	2480
1	M	103	170	42	25	93	460	137	207	43	220	40	420	90	1300	1280	500	1220	400	1320	1840	520	-	520	460	60	46	82
		157	170	53	29	97	820	153	230	47	187	33	380	53	1300	1420	480	1020	520	1600	1700	580	-	620	500	39	47	66
						109									460	960			420	1100		600	-	600	400	34	55	78
1	B	620	190	40	40	48	277	323	207	63	303	33	260	50	1020	1120	680	1060	320	1680	1260	207	-	300	260	40	24	53
		600	173	43	42	48	340	277	247	57	227	27	220	50	1220	1140	680	1020	460	1940	1400	267	-	320	200	52	35	47
						50									760	840			380	1380	1540	177	-	160	140	53	33	54
2	S	240	93	70	55	2060	2700	500	180	29	34	156	180	25	227	1800	127	860	340	760	1180	500	217	3320	120	30	52	59
		360	117	87	62	2160	3300	600	233	28	39	139	200	43	180	1920	140	1360	300	760	1120	400	270	3280	120	32	51	53
						2540													320	800	1020	600	287	3040	300	27	54	50
2	M	88	33	20	9	175	840	600	190	17	41	41	100	51	210	423	97	380	260	860	190	83	87	1060	147	44	55	20
		79	47	37	12	144	780	680	190	24	30	50	180	46	157	383	117	420	380	640	163	70	153	1520	80	25	66	24
						165									177				160	820	163	73	117	1020	113	27	63	24
2	B	143	1000	44	36	190	500	820	197	56	29	27	180	62	180	547	77	380	147	760	150	80	47	223	113	28	42	35
		177	200	56	39	237	580	680	230	67	40	39	140	68	237	420	82	460	133	1000	113	74	70	180	120	22	34	29
						193									110				147	980	147	71	52	200	157	28	21	27
3	S	360	96	48	170	1100	920	340	360	22	87	29	340	300	900	3340	1200	800	127	1300	460	101	31	340	300	93	51	1140
		380	108	66	200	1120	900	360	540	31	93	35	220	300		2300	1320	960	130	1100	520	113	38	460	180	83	61	980
						900													150	1480	680	124	31	480	240	33	49	1100
3	M	153	58	15	37	57	780	197	280	9	118	18	420	21	1000	3040	2340	720	70	1040	280	28	26	210	180	80	31	21
		113	52	16	43	53	940	183	277	16	89	28	320	28	780	3040	2240	600	50	1140	320	20	18	200	183	107	47	26
						53									760	3160			83	1340	300	23	18	180	140	73	47	21
3	B	65	55	27	107	71	440	110	213	38	108	30	440	200	1160	3740	1840	680	97	1140	140	49	14	180	380	103	57	17
		82	72	33	95	60	560	200	250	39	97	32	520	360	1020	4000	2440	860	100	1440	220	34	21	157	280	113	45	18
						70									820	4120			107	1380	300	36	22	143	300	93	42	16

Continued..../

TABLE 10 (Continued). NUMBER OF VIABLE FUNG I IN WATER SAMPLES (CFU/100 mL)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
4	S	150	170	34	33	9080	1260	1000	307	63	90	100	520	140	203	3180	280	760	520	800	480	520	400	7600	240	240	87	960
		187	173	37	41	8400	640	700	263	71	–	70	500	220	–	3540	160	780	500	760	760	520	400	7800	280	140	130	960
						10200													480	740	640	640	400	8360	320	240	83	900
4	M	51	70	36	17	360	2280	680	280	53	81	143	360	100	227	3980	123	340	400	680	480	37	43	3080	220	100	57	34
		69	110	39	20	163	2180	620	263	50	–	130	500	100	190	2760	–	280	360	680	540	32	57	2560	300	183	48	35
						190									200	2300			340	980	660	26	46	2200	260	157	50	38
4	B	110	80	48	41	63	2620	920	150	65	67	123	480	40	207	1900	360	420	520	1020	360	39	19	1140	1220	130	45	19
		100	153	39	49	61	1940	760	263	38	70	147	700	57	163	1540	240	520	460	860	440	31	29	1640	1140	143	42	24
																1700			600	840	260	31	42	1560	900	170	49	18
5	S	1840	107	32	113	1020	3200	680	203	42	90	160	440	67	–	–	177	–	–	–	–	–	–	–	–	–	–	–
		2380	117	41	96	1160	3900	600	187	51	37	160	420	63	–	–	100	–	–	–	–	–	–	–	–	–	–	–
						1220												–	–	–	–	–	–	–	–	–	–	–
5	M	520	21	8	35	170	360	580	150	29	83	69	380	207	–	–	153	–	–	–	–	–	–	–	–	–	–	–
		240	22	10	25	170	600	380	157	25	67	51	400	–	–	–	150	–	–	–	–	–	–	–	–	–	–	–
						170												–	–	–	–	–	–	–	–	–	–	–
5	B	–	27	7	13	64	580	580	177	22	40	42	420	540	–	–	153	–	–	–	–	–	–	–	–	–	–	–
		–	24	4	13	80	720	480	227	22	43	45	440	920	–	–	167	–	–	–	–	–	–	–	–	–	–	–

TABLE 11. NUMBER OF VIABLE FUNGI IN THE POND WATER SAMPLES (DATA ARE EXPRESSED AS ARITHMETIC MEAN VALUES OF REPLICATE SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	1070	850	89	59	304	1090	237	650	71	193	34	680	300	773	1330	550	1360	373	6133	5067	780	-	687	171	75	407	2440
1	M	130	170	48	27	100	640	145	219	45	204	37	400	72	1020	1220	490	1120	447	1340	1770	567	-	580	453	44	49	75
1	B	610	182	42	41	49	309	300	227	65	265	30	240	50	1000	1033	680	1040	387	1667	1400	217	-	260	200	48	31	51
2	S	300	105	79	59	2253	3000	550	207	29	37	148	190	34	204	1860	134	1110	320	773	1107	500	258	3213	180	30	52	54
2	M	84	40	29	11	161	810	640	190	21	36	46	140	49	181	403	107	400	267	773	172	75	119	1200	113	32	61	23
2	B	160	600	50	38	207	540	750	214	62	35	33	160	65	176	484	80	420	142	913	137	75	56	201	130	26	32	30
3	S	370	102	52	185	1040	910	350	450	27	90	32	280	300	900	2820	1260	880	136	1293	553	113	33	427	240	70	54	1073
3	M	133	55	16	40	54	860	190	279	13	104	23	370	25	847	3080	2290	660	68	1173	300	24	21	197	168	87	42	23
3	B	74	64	30	101	67	680	155	232	39	103	31	480	280	1000	3953	2140	770	101	1320	220	40	19	160	320	103	48	17
4	S	169	172	36	37	9227	950	850	285	67	90	85	510	180	203	3360	220	770	500	767	627	560	400	7920	280	207	100	940
4	M	60	90	38	19	238	2230	650	272	52	81	137	430	100	206	3013	123	310	367	780	560	32	49	2613	260	147	52	36
4	B	105	117	44	45	62	2280	840	207	52	69	135	590	49	185	1713	300	470	527	907	353	34	30	1447	1087	148	45	20

TABLE 12. NUMBER OF VIABLE FUNGI IN THE POND WATER SAMPLES (DATA ARE EXPRESSED AS GEOMETRIC MEAN VALUES OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	439	297	56	40	114	600	218	319	59	219	34	403	103	924	1188	568	1166	401	2393	2324	458	-	470	249	54	85	211
2	M	159	136	49	29	422	1095	642	203	34	36	61	162	48	187	713	105	571	230	817	289	141	120	919	138	29	47	33
3	B	154	71	29	91	156	810	218	308	24	99	28	368	128	914	3250	1835	765	98	1260	332	48	24	238	235	86	48	75
4	S	102	122	39	32	514	1690	774	252	57	80	116	506	96	198	2588	201	482	459	816	499	85	84	3105	429	165	62	88

TABLE 13. FREQUENCY OF OCCURRENCE OF MAXIMUM NUMBERS OF GEO-AQUATIC FUNGI IN SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES FROM EACH OF THE EXPERIMENTAL PONDS

POND No.	FREQUENCY OF OCCURRENCE OF MAXIMUM GEO-AQUATIC FUNGAL COUNTS (EXPRESSED AS A % OF THE TOTAL # OF SAMPLES FROM EACH POND)		
	SURFACE	MID-DEPTH	BOTTOM
1	73	15	12
2	70	7	22
3	67	7	26
4	63	7	30

TABLE 14. FREQUENCY OF OCCURRENCE OF MINIMUM NUMBERS OF GEO-AQUATIC FUNGI IN SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES FROM EACH OF THE EXPERIMENTAL PONDS

POND No.	FREQUENCY OF OCCURRENCE OF MINIMUM GEO-AQUATIC FUNGAL COUNTS (EXPRESSED AS A % OF THE TOTAL # OF SAMPLES FROM EACH POND)		
	SURFACE	MID-DEPTH	BOTTOM
1	15	35	50
2	11	52	44
3	19	52	30
4	15	44	44

TABLE 15. RELATIONSHIP BETWEEN FUNGAL LEVELS IN THE TREATED AND CONTROL PONDS

	PERCENTAGE OF SURVEYS IN WHICH MEAN* FUNGAL LEVELS EXCEEDED CONTROL POND LEVELS		
	POND No. 1	POND No. 3	POND No. 4
Pre Spill (-215 to 0 days)	66.7	44.7	66.6
Post Spill (0 to 165 days)	100	81.8	72.7
Post Spill (200 to 385 days)	71.4	42.9	71.4

* Geometric mean of fungal levels in surface, mid-depth, and bottom water samples.

TABLE 16. NUMBER OF VIABLE FUNGI IN WATER SAMPLES CAPABLE OF GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CABON SOURCE (DATA ARE EXPRESSED AS CFU/100 mL)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	0	135	5	12	-	160	-	-	33	2	0	0	11	10	640	110	600	150	600	0	0	-	390	97	20	180	1800
1	M	0	31	14	11	-	80	-	-	20	0	-	0	4	60	240	20	30	50	0	0	0	-	170	110	21	5	53
1	B	0	96	0	0	-	80	-	-	0	6	-	0	0	0	110	70	280	0	0	0	0	-	0	10	33	0	43
2	S	0	70	37	4	-	0	-	-	22	0	-	12	0	0	0	40	270	50	690	0	0	0	2300	0	0	30	0
2	M	0	0	0	0	-	0	-	-	0	0	0	13	10	0	0	50	40	0	220	0	10	73	700	0	0	0	3
2	B	0	228	-	0	-	0	-	-	0	0	0	12	0	3	0	77	40	0	700	0	20	10	0	20	0	0	0
3	S	0	62	26	0	-	0	-	-	0	3	0	50	7	170	470	30	480	30	650	0	0	0	610	50	49	5	423
3	M	0	24	4	9	-	120	-	-	0	0	0	0	0	0	470	150	0	10	600	0	1	15	290	20	54	0	0
3	B	0	17	21	54	-	177	-	-	3	3	4	0	0	130	360	0	0	40	150	20	0	0	0	0	0	0	0
4	S	0	72	-	15	-	0	-	-	-	3	0	80	0	0	140	0	240	0	200	530	80	100	2300	0	0	0	1157
4	M	0	22	13	11	-	0	-	-	-	0	80	120	2	0	260	0	0	0	570	0	0	-	2250	40	0	0	3
4	B	0	22	30	-	-	180	-	-	6	1	40	0	2	0	290	0	120	0	50	100	0	0	100	0	-	0	30

TABLE 17. NUMBER OF VIABLE FUNGI IN WATER SAMPLES CAPABLE OF GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CABON SOURCE (DATA ARE EXPRESSED AS A PERCENTAGE OF THE NUMBERS OF VIABLE FUNGI ENUMERATED ON mARGPA AGAR)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	15.9	6	20	-	15	-	-	46	1	0	0	4	1	48	20	44	40	10	0	0	-	57	57	27	44	74
1	M	-	18.2	29	41	-	13	-	-	44	0	-	0	6	6	20	4	3	11	0	0	0	-	29	24	48	10	71
1	B	-	53	0	0	-	26	-	-	0	2	-	0	0	0	11	10	27	0	0	0	0	-	0	5	69	0	84
2	S	-	67	47	7	-	0	-	-	76	0	-	6	0	0	0	30	24	16	89	0	0	0	72	0	0	58	0
2	M	-	0	0	0	-	0	-	-	0	0	0	9	20	0	0	47	10	0	28	0	13	61	58	0	0	0	13
2	B	-	38	-	0	-	0	-	-	0	0	0	8	0	2	0	96	10	0	77	0	27	18	0	15	0	0	0
3	S	-	61	50	0	-	0	-	-	0	3	0	18	2	19	17	2	55	22	50	0	0	0	14	21	70	9	39
3	M	-	44	25	23	-	14	-	-	0	0	0	0	0	0	15	7	0	15	51	0	4	71	15	12	62	0	0
3	B	-	27	70	53	-	26	-	-	8	3	13	0	0	13	9	0	0	40	11	9	0	0	0	0	0	0	0
4	S	-	42	-	41	-	0	-	-	-	3	0	16	0	0	4	0	31	0	26	85	14	25	29	0	0	0	123
4	M	-	24	34	58	-	0	-	-	-	0	58	28	2	0	9	0	0	0	73	0	0	-	86	15	0	0	8
4	B	-	19	68	-	-	8	-	-	12	1	30	0	4	0	17	0	26	0	6	28	0	0	7	0	0	0	0

TABLE 18. PERCENTAGE OF VIABLE FUNGI IN THE PONDS CAPABLE OF GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS GEOMETRIC MEAN VALUES OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	25	6	9	-	17	-	-	13	1	-	0	3	2	22	9	15	8	2	0	0	-	12	19	45	8	76
2	M	-	14	6.8	2	-	0	-	-	4	0	-	8	3	1	0	51	13	3	58	0	7	10	16	2	0	4	2
3	B	-	42	44	11	-	7	-	-	2	2	2	3	1	6	13	2	4	24	30	2	2	4	6	6	16	2	3
4	S	-	27	48	49	-	2	-	-	12	1	12	8	2	0	8	0	9	0	22	13	2	5	26	2	0	0	10

TABLE 19. NUMBER OF VIABLE FUNGI IN WATER SAMPLES CAPABLE OF STRONG GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CABON SOURCE (DATA ARE EXPRESSED AS CFU/100 mL)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	15	8	-	60	-	-	-	1	0	0	14	40	330	100	360	120	630	0	0	-	70	40	30	150	0
1	M	-	-	2	1	-	80	-	-	-	2	0	0	2	0	140	80	0	30	190	0	0	-	0	20	0	7	23
1	B	-	-	0	0	-	20	-	-	-	7	10	0	0	50	110	50	220	0	0	0	0	-	0	30	14	0	50
2	S	-	-	22	18	-	140	-	-	-	0	-	0	0	0	0	50	180	50	800	0	0	120	700	10	0	0	3
2	M	-	-	6	3	-	60	-	-	-	0	5	4	5	0	0	90	0	0	0	0	7	27	350	0	0	0	0
2	B	-	-	-	0	-	147	-	-	-	0	0	0	0	0	0	87	0	0	470	0	23	0	0	20	13	0	0
3	S	-	-	15	63	-	80	-	-	-	3	0	0	2	50	310	0	600	30	850	0	0	0	430	43	3	0	143
3	M	-	-	6	10	-	280	-	-	-	2	0	0	0	20	350	40	20	0	350	0	0	0	230	0	63	0	0
3	B	-	-	20	53	-	76	-	-	-	2	3	0	0	80	290	20	0	40	0	120	0	0	0	40	0	0	3
4	S	-	-	50	7	-	0	-	-	-	6	0	80	0	0	160	0	230	0	200	490	90	0	0	0	0	0	430
4	M	-	-	0	1	-	60	-	-	-	0	80	60	2	20	170	0	0	0	0	0	0	40	0	10	0	6	0
4	B	-	-	15	0	-	140	-	-	-	3	30	0	3	10	290	0	90	70	50	90	0	0	0	100	0	0	20

TABLE 20. NUMBER OF VIABLE FUNGI IN WATER SAMPLES CAPABLE OF STRONG GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CABON SOURCE (DATA ARE EXPRESSED AS A PERCENTAGE OF THE NUMBERS OF VIABLE FUNGI ENUMERATED ON mARGPA AGAR)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	17	14	-	6	-	-	-	1	0	0	5	5	25	18	26	32	10	0	0	-	10	23	40	37	0
1	M	-	-	4	4	-	13	-	-	-	1	0	0	3	0	11	16	0	7	14	0	0	-	0	4	0	14	31
1	B	-	-	0	0	-	6	-	-	-	3	33	0	0	5	11	7	21	0	0	0	0	-	0	15	29	0	98
2	S	-	-	28	31	-	5	-	-	-	0	-	0	0	0	0	37	16	16	103	0	0	47	22	6	0	0	6
2	M	-	-	21	27	-	7	-	-	-	0	11	3	10	0	0	84	0	0	0	0	9	23	29	0	0	0	0
2	B	-	-	-	0	-	27	-	-	-	0	0	0	0	0	0	108	0	0	51	0	31	0	0	15	50	0	0
3	S	-	-	29	34	-	9	-	-	-	3	0	0	1	6	11	0	68	22	66	0	0	0	101	18	4	0	13
3	M	-	-	38	25	-	33	-	-	-	2	0	0	0	2	11	2	3	0	30	0	0	0	117	0	72	0	0
3	B	-	-	67	52	-	11	-	-	-	2	10	0	0	8	7	9	0	40	0	55	0	0	0	13	0	0	18
4	S	-	-	139	19	-	0	-	-	-	7	-	16	0	0	5	0	30	0	26	78	16	0	0	0	0	0	46
4	M	-	-	0	5	-	3	-	-	-	0	58	14	2	10	6	0	0	0	0	0	0	82	0	4	0	12	0
4	B	-	-	34	0	-	6	-	-	-	4	22	0	6	5	17	0	19	13	6	25	0	0	0	9	0	0	100

TABLE 21. PERCENTAGE OF VIABLE FUNGI IN THE PONDS CAPABLE OF STRONG GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS GEOMETRIC MEAN VALUES OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	4	4	-	8	-	-	-	1	3	1	2	3	14	13	8	6	5	0	0	-	2	11	11	8	14
2	M	-	-	24	9	-	10	-	-	-	0	3	1	2	0	0	69	3	3	17	0	7	10	9	4	4	0	2
3	B	-	-	42	35	-	15	-	-	-	2	2	1	1	5	9	3	6	10	13	4	0	0	23	6	7	0	6
4	S	-	-	17	5	-	3	-	-	-	3	11	6	2	4	8	0	8	2	5	12	3	4	0	3	0	2	17

TABLE 22. NUMBER OF FUNGI IN WATER SAMPLES CAPABLE OF GROWTH ON A MEDIUM CONTAINING BIODEGRADED NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS CFU/100 mL)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	110	49	13	-	160	-	-	16	8	0	0	7	0	590	230	170	150	750	0	150	-	750	457	0	100	2900
1	M	-	93	14	21	-	80	-	-	0	0	-	0	5	0	520	100	0	110	660	0	0	-	170	260	3	4	6
1	B	-	101	0	0	-	80	-	-	0	0	0	0	0	0	0	60	160	0	0	100	0	-	-	50	12	0	18
2	S	-	34	18	3	-	0	-	-	0	-	136	7	0	0	0	120	300	0	440	0	110	0	700	30	40	10	117
2	M	-	13	0	0	-	0	-	-	0	3	0	26	3	0	0	0	0	80	790	0	0	93	150	0	0	0	10
2	B	-	266	-	0	-	510	-	-	0	0	2	5	0	10	100	77	0	0	80	0	0	0	0	100	0	0	6
3	S	-	64	16	0	-	0	-	-	0	0	0	60	2	110	320	180	60	0	700	0	0	0	810	170	0	9	573
3	M	-	15	6	18	-	120	-	-	0	0	0	70	4	10	220	120	0	0	250	50	0	15	210	30	0	5	1
3	B	-	10	8	57	-	177	-	-	0	2	9	0	1	80	320	130	10	0	350	20	0	0	0	20	0	0	0
4	S	-	137	69	19	-	0	-	-	0	0	0	0	4	0	360	0	140	0	500	1380	120	0	1800	0	0	0	907
4	M	-	15	0	27	-	0	-	-	0	7	20	0	5	0	90	0	0	0	0	60	0	17	2050	570	50	0	0
4	B	-	57	16	100	-	180	-	-	0	25	60	90	5	0	270	50	70	0	1050	70	0	0	50	0	-	0	22

TABLE 23. NUMBER OF VIABLE FUNGI IN WATER SAMPLES CAPABLE OF GROWTH ON A MEDIUM CONTAINING BIODEGRADED NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS A PERCENTAGE OF THE NUMBER OF VIABLE FUNGI ENUMERATED ON mARGPA AGAR)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	13	55	22	-	15	-	-	23	4	0	0	2	0	44	42	13	40	12	0	19	-	109	267	0	25	119
1	M	-	55	29	78	-	13	-	-	0	0	-	0	7	0	43	20	0	25	50	0	0	-	29	57	7	8	8
1	B	-	55	0	0	-	13	-	-	0	0	0	4	0	0	0	9	15	0	0	7	0	-	0	25	25	0	35
2	S	-	32	23	5	-	0	-	-	0	-	92	19	0	0	0	90	27	0	57	0	22	0	22	17	133	19	217
2	M	-	33	0	0	-	0	-	-	0	8	0	3	6	0	0	0	0	30	102	0	0	78	13	0	0	0	43
2	B	-	44	-	0	-	94	-	-	0	0	6	21	0	6	21	96	0	0	9	0	0	0	0	77	0	0	20
3	S	-	63	31	0	-	0	-	-	0	0	0	19	1	12	11	14	7	0	54	0	0	0	190	71	0	17	53
3	M	-	27	38	45	-	14	-	-	0	0	0	0	16	1	7	5	0	0	21	17	0	71	107	11	0	12	4
3	B	-	16	27	56	-	26	-	-	0	2	29	0	0	8	8	6	1	0	27	9	0	0	0	6	0	0	0
4	S	-	80	192	51	-	0	-	-	0	0	0	0	2	0	11	0	18	0	65	220	21	0	23	0	0	0	96
4	M	-	17	0	142	-	0	-	-	0	9	15	0	5	0	3	0	0	0	0	11	0	35	78	219	34	0	0
4	B	-	49	36	222	-	8	-	-	0	36	44	15	10	0	16	17	15	0	116	20	0	0	3	0	-	0	110

TABLE 24. PERCENTAGE OF VIABLE FUNGI IN THE PONDS CAPABLE OF GROWTH ON A MEDIUM CONTAINING CONTAINING BIODEGRADED NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS GEOMETRIC MEAN VALUES OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	34	12	12	-	14	-	-	3	2	0	0	2	0	12	20	6	10	8	2	3	-	15	72	6	6	32
2	M	-	36	4	2	-	5	-	-	0	3	8	6	2	2	3	21	3	3	37	0	3	4	7	24	5	3	57
3	B	-	30	32	14	-	7	-	-	0	1	3	7	3	5	9	7	2	0	31	5	0	4	27	17	0	6	6
4	S	-	41	19	19	-	2	-	-	0	7	9	2	5	0	8	3	6	0	20	36	3	3	18	8	6	0	22

TABLE 25. NUMBER OF VIABLE FUNGI IN WATER SAMPLES CAPABLE OF STRONG GROWTH ON A MEDIUM CONTAINING BIODEGRADED NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS CFU/100 mL)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	56	9	-	180	-	-	25	0	0	0	3	0	480	80	110	150	630	0	10	-	440	180	0	70	300
1	M	-	-	7	3	-	140	-	-	2	2	0	0	5	0	390	20	0	120	390	100	0	-	10	60	5	0	6
1	B	-	-	0	0	-	100	-	-	10	2	0	0	0	0	230	20	110	0	0	50	20	-	0	30	14	0	15
2	S	-	-	20	11	-	140	-	-	0	-	103	0	0	0	0	80	260	20	400	0	70	0	500	40	33	0	93
2	M	-	-	0	1	-	60	-	-	15	0	5	37	3	0	0	0	0	40	670	0	0	47	0	0	0	0	7
2	B	-	-	-	0	-	87	-	-	0	0	0	0	0	0	60	87	0	0	20	0	0	0	0	60	13	0	0
3	S	-	-	1	93	-	180	-	-	4	0	0	10	0	50	430	20	90	0	1100	0	0	0	530	53	0	0	233
3	M	-	-	9	9	-	300	-	-	0	0	0	0	0	10	290	60	20	0	250	60	0	2	100	0	0	6	0
3	B	-	-	9	43	-	336	-	-	17	2	0	0	0	10	350	70	40	0	100	100	2	0	0	30	0	0	0
4	S	-	-	26	14	-	20	-	-	0	1	5	0	0	0	200	0	160	0	400	1360	90	0	0	0	0	0	430
4	M	-	-	0	0	-	0	-	-	0	5	10	0	3	0	210	0	0	0	0	0	0	12	650	0	50	1	0
4	B	-	-	0	34	-	120	-	-	0	0	0	10	6	0	180	50	0	0	900	50	0	0	100	0	-	0	2

TABLE 26. NUMBER OF FUNGI IN WATER SAMPLES CAPABLE OF STRONG GROWTH ON A MEDIUM CONTAINING BIODEGRADED NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS A PERCENTAGE OF THE NUMBERS OF VIABLE FUNGI ENUMERATED ON mARGPA AGAR)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	63	15	-	17	-	-	35	0	0	0	1	0	36	15	8	40	10	0	1	-	64	105	0	17	12
1	M	-	-	15	11	-	22	-	-	4	1	0	0	7	0	32	4	0	27	29	6	0	-	2	13	11	0	8
1	B	-	-	0	0	-	32	-	-	15	1	0	0	0	0	22	3	11	0	0	3	9	-	0	15	29	0	29
2	S	-	-	25	19	-	5	-	-	0	-	70	0	0	0	0	60	23	6	52	0	14	0	16	22	110	0	172
2	M	-	-	0	9	-	7	-	-	71	0	11	26	6	0	0	0	0	15	87	0	0	39	0	0	0	0	30
2	B	-	-	-	0	-	16	-	-	0	0	0	0	0	0	12	109	0	0	2	0	0	0	0	46	50	0	0
3	S	-	-	2	50	-	20	-	-	15	0	0	4	0	6	15	2	10	0	85	0	0	0	124	22	0	0	22
3	M	-	-	56	23	-	35	-	-	0	0	0	0	0	1	9	3	3	0	21	20	0	10	51	0	0	14	0
3	B	-	-	30	43	-	49	-	-	44	2	0	0	0	1	9	3	5	0	8	45	5	0	0	9	0	0	0
4	S	-	-	25	38	-	2	-	-	0	1	6	0	0	0	6	0	21	0	52	217	16	0	0	0	0	0	46
4	M	-	-	0	0	-	0	-	-	0	6	7	0	3	0	7	0	0	0	0	0	0	24	25	0	34	2	0
4	B	-	-	0	76	-	5	-	-	0	0	0	2	12	0	11	17	0	0	99	14	0	0	7	0	-	0	10

TABLE 27. PERCENTAGE OF VIABLE FUNGI IN THE PONDS CAPABLE OF STRONG GROWTH ON A MEDIUM CONTAINING NORMAL WELLS CRUDE OIL AS THE SOLE CARBON SOURCE (DATA ARE EXPRESSED AS GEOMETRIC MEAN VALUES OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	10	5	-	23	-	-	13	1	0	0	2	0	29	6	4	10	7	3	2	-	5	27	7	3	14
2	M	-	-	5	6	-	8	-	-	4	0	9	3	2	0	2	19	3	4	21	0	2	3	3	10	18	0	17
3	B	-	-	15	37	-	32	-	-	9	1	0	2	0	2	11	3	5	0	24	10	2	2	18	6	0	2	3
4	S	-	-	3	14	-	2	-	-	0	2	3	1	3	0	8	3	3	0	17	14	3	3	6	0	6	1	8

TABLE 28. NUMBER OF HEMP SEEDS COLONIZED PER 50 mL POND WATER SAMPLE (DATA ARE GEOMETRIC MEANS OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	SAMPLE NUMBER AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	1.3	0	0	0	0	1	1	0	7.2	0	1.3	3.2	3.3	5.3	1.6	1.4	2.6	2.2	1	1.6	0	0	0	2.6	2.6	2.0	1.4
2	M	0.7	0	0	1.3	0	0	1.6	3.8	2.2	1.3	1.4	1.4	1	1.3	0	0	1.6	2.1	0	0	0	0	0	1.6	1	1	0
3	B	0	0	0	0	0	0	3.6	2.4	3.1	0	0	2.3	2.3	3.4	2.1	1	1.8	1.6	0	0	0	0	0	2.5	1.3	1.6	1
4	S	1.9	0	0	0	1.4	2.8	2.9	1.8	7.7	1	1.3	0	2.5	1.8	1	1	2.0	2.0	1	0	0	0	0	3.1	2.5	1	0

TABLE 29. SPREAD PLATE ESTIMATE OF THE NUMBERS OF PHYCOMYCETES IN POND WATER SAMPLES (DATA ARE GEOMETRIC MEANS OF SURFACE, MID-DEPTH, AND BOTTOM WATER SAMPLES)

POND No.	DEPTH	NUMBER OF PHYCOMYCETES (CFU/3 mL)																										
		SAMPLE NUMBERS AND COLLECTION DAY																										
		1 216	2 168	3 149	4 128	5 106	6 77	7 56	8 29	9 0	10 7	11 14	12 28	13 41	14 55	15 69	16 83	17 97	18 111	19 139	20 167	21 202	22 230	23 251	24 296	25 322	26 350	27 385
1	S	-	-	0	0	0	1	0	0	1.3	0	1.3	3.3	1.9	0	1.3	1	0	1	1	0	0	0	0	1.3	1.3	1.4	1.6
2	M	-	-	0	0	0	0	0	0	0	1.3	1	1	0	0	0	0	2.4	0	0	0	0	0	0	1.3	0	1	0
3	B	-	-	0	0	0	1.6	2.3	1.4	0	0	1	6.7	1.4	1.4	1	1.9	0	2.5	0	0	0	0	0	1.8	1	1.3	1.3
4	S	-	-	0	0	0	1.3	0	1.4	0	1.4	0	1	0	1.8	1	2.3	0	1	1.3	0	0	0	0	1.4	1	1	1.3

TABLE 30. ESTIMATE OF PHYCOMYCETE CONTENT OF POND WATER SAMPLES USING (i) HEMP SEED BAITING AND (ii) A SPREAD PLATE TECHNIQUE

POND No.	SURVEY GROUP			
	1 - 9	10 - 11	12 - 18	24 - 27
	(i) Baiting Estimate (Number of Seeds Colonized per 50 mL Sample			
1	1.2	0.65	2.8	2.2
2	0.5	1.35	1.1	0.9
3	0.5	0	2.1	1.6
4	1.3	1.15	1.5	1.7
	(ii) Spread Plate Estimate (CFU/50 mL)			
1	5.5	6.5	20	23
2	0	11.5	0.72	10
3	13	5	32	23
4	7	7	17	20

TABLE 31. GENERIC IDENTITY OF PHYCOMYCETES ISOLATED FROM POND WATER SAMPLES

SURVEY #	POND NUMBER AND GENERA ISOLATED			
	1	2	3	4
1	Sap[1](1)[3], Pythium(3), Aphan[2](1)	Sap(2)		Sap(8)
2				
3				
4		Sap(2)		
5				Sap(3)
6	Sap(1)		Sap(3), Achlya(1)	Sap(11)
7	Sap(1)	Sap(3) Pythium(1)	Sap(23)	Sap(10) Achlya(1)
8		Sap(10)	Sap(12)	Sap(7)
9	Sap(23)	Sap(10)	Sap(9)	Sap(23)
10		Sap(11)		Sap(8)
11	Sap(5)	Sap(5)	Sap(1)	Sap(2)
12	Sap(16)	Sap(5)	Sap(21)	Sap(1)
13	Sap(16)	Sap(1)	Sap(13)	Sap(8)
14	Sap(5)	Sap(2)	Sap(17)	Sap(7)
15	Sap(5)		Sap(1)	Sap(2)
16	Sap(3)		Sap(8)	Sap(9)
17	Sap(8)	Sap(17)	Sap(5)	Sap(5) Pythium(1)
18	Sap(8)	Sap(6)	Sap(14)	Sap(9)
19	Sap(2)			Sap(3) Pythium(2)
20	Sap(4)			Sap(6)
21				
22				
23				
24	Sap(11) Pythium(1)	Sap(5)	Sap(14) Pythium(1)	Sap(15)
25	Sap(9) Achlya(3)	Sap(1)	Sap(2) Pythium(1)	Sap(9)
26	Sap(12)	Sap(3)	Sap(7)	Sap(2)
27	Sap(6) Pythium(1)		Sap(3)	Sap(2)

[1] Saprolegnia.
[2] Aphanomyces.
[3] Total number of isolates from hemp seed baits and spread plates.

TABLE 32. ABILITY OF FUNGAL ISOLATES TO UTILIZE NORMAL WELLS CRUDE OIL

ISOLATE NO.	GROWTH ON AGAR SURFACE		
	AIR	INTERFACE	SUBMERGED
OP1a	0	1	1
OP1b	0	3	2
OP1c	0	0	0
OP2a	0	0	0
OP2b	0	0	0
OP2c	0	0	0
OP3a	0	0	1
OP3b	1	4	2
OP3c	0	0	0
OP4a(i)	0	0	0
OP4a(ii)	0	0	0
OP4b	0	0	0
OP5a	0	0	0
OP5b	2	4	2
OP6a	0	0	0
OP7a	0	0	0
OP7b	0	0	0
OP8a	0	4	0
OP8b	0	4	2
OP9b	0	4	2
OP10a	0	0	2
OP10b	0	0	0
OP11a	0	0	0
OP12a	0	0	0
OP12b	0	0	0
OP13b	0	0	0
OP14a	0	1	1
OP14b	2	4	2
OP15a	0	2	0
OP16	0	0	1
OP17	0	0	2
OP18	0	0	0
OP19	0	0	0
OP20	0	0	0
OP21	0	0	0
OP22	0	0	0
OP23	0	3	2
OP24	0	0	0
OP25	0	3	3

Legend: 0, no growth; 1, growth greater than control; 2, growth greater than 1 but not continuous over agar surface; 3, growth covers agar surface; 4, agar covered by a thick mat of mycelium.

TABLE 33. ABILITY OF FUNGAL ISOLATES TO UTILIZE COREXIT OIL DISPERSANT

ISOLATE NO.	GROWTH ON AGAR SURFACE		
	AIR	INTERFACE	SUBMERGED
OP1a	C	C	C
OP1b	O	C	C
OP1c	O	C	C
OP2a	C	C	C
OP2b	C	C	C
OP2c	C	C	C
OP3a	C	C	C
OP3b	C	C	C
OP3c	C	C	C
OP4a(i)	C	C	C
OP4a(ii)	C	C	C
OP4b	C	C	C
OP5a	O	C	C
OP5b	C	C	C
OP6a	C	C	C
OP7a	C	C	C
OP7b	C	C	C
OP8a	C	C	C
OP8b	C	C	C
OP9b	C	C	C
OP10a	C	C	C
OP10b	C	C	C
OP11a	C	C	C
OP12a	C	C	C
OP12b	C	C	C
OP13b	C	C	C
OP14a	C	C	C
OP14b	C	C	C
OP15a	O	C	C
OP16	C	C	C
OP17	C	C	C
OP18	C	C	C
OP19	C	C	C
OP20	C	C	C
OP21	O	O	O
OP22	C	C	C
OP23	C	C	C
OP24	C	C	C
OP25	C	C	C

Legend: C, growth less than control; O, no growth.

TABLE 34. ABILITY OF FUNGAL ISOLATES TO UTILIZE COREXIT OIL DISPERSANT DILUTED WITH FIVE PARTS OF WATER (BY WEIGHT)

ISOLATE NO.	GROWTH ON AGAR SURFACE		
	AIR	INTERFACE	SUBMERGED
OP1a	=C	0	0
OP1b	C	0	0
OP1c	=C	0	0
OP2a	=C	0	C
OP2b	=C	0	0
OP2c	0	0	0
OP3a	C	0	0
OP3b	C	0	0
OP3c	=C	0	0
OP4a(i)	=C	0	0
OP4a(ii)	=C	0	0
OP4b	=C	0	0
OP5a	=C	0	0
OP5b	=C	0	0
OP6a	=C	0	0
OP7a	=C	0	0
OP7b	=C	0	0
OP8a	=C	0	0
OP8b	=C	0	0
OP9b	=C	0	0
OP10a	=C	0	0
OP10b	=C	0	0
OP11a	=C	0	0
OP12a	=C	0	0
OP12b	=C	0	0
OP13b	=C	0	0
OP14a	=C	0	0
OP14b	=C	0	0
OP15a	=C	0	0
OP16	=C	0	0
OP17	=C	0	0
OP18	=C	0	0
OP19	C	0	0
OP20	=C	0	0
OP21	=C	0	0
OP22	=C	0	0
OP23	=C	=C	=C
OP24	0	0	0
OP25	=C	0	0

Legend: C, growth less than control; =C, growth equal to control; 0, no growth.

TABLE 35. ABILITY OF FUNGAL ISOLATES TO UTILIZE NORMAL WELLS CRUDE OIL IN THE PRESENCE OF COREXIT OIL DISPERSANT

ISOLATE NO.	GROWTH ON AGAR SURFACE		
	AIR	INTERFACE	SUBMERGED
OP1a	=C	0	0
OP1b	=C	0	0
OP1c	=C	0	0
OP2a	=C	0	0
OP2b	=C	0	0
OP2c	=C	0	0
OP3a	C	0	0
OP3b	C	0	0
OP3c	=C	0	0
OP4a(i)	=C	0	0
OP4a(ii)	C	0	0
OP4b	C	0	0
OP5a	=C	0	0
OP5b	=C	0	0
OP6a	=C	0	0
OP7a	C	0	0
OP7b	=C	0	0
OP8a	=C	0	0
OP8b	=C	0	0
OP9b	=C	0	0
OP10a	=C	0	0
OP10b	=C	0	0
OP11a	=C	0	0
OP12a	=C	0	0
OP12b	C	0	0
OP13b	C	0	0
OP14a	=C	0	0
OP14b	=C	0	0
OP15a	=C	0	0
OP16	=C	0	0
OP17	=C	0	0
OP18	=C	0	0
OP19	=C	0	0
OP20	=C	0	0
OP21	=C	0	0
OP22	=C	0	0
OP23	=C	0	0
OP24	=C	0	0
OP25	C	0	0

Legend: C, growth less than control; =C, growth equal to control; 0, no growth.

TABLE 36. SACCHAROMYCES CEREVISIAE STRAINS USED IN THE DETECTION OF MUTAGENS

STRAIN	GENOTYPE	PHENOTYPE	MUTATION	REFERENCE
D3	his8 ade2 cyh4 + + +	Red sector in a white colony.	Mitotic cross over.	Brusick & Mayer, 1973. Mayer, Hybner & Brusick, 1976
D4	ade2-1 trp5-12 ade2-2 trp5-27	Adenine dependence to adenine independence. Tryptophan dependence to tryptophan independence.	Gene conversion.	Zimmermann, 1971.
D5	ade2-40 ade2-119	ade2-40 - absolute adenine requirement, red colony. ade2-119 - leaky adenine requirement, pink; complement in diploid - white.	Mitotic cross over.	Zimmermann, 1973.
D7	ade2-40 trp5-12 ilv1-92 ade2-119 trp5-27 ilv1-92	As D5. As D4. Isoleucine dependence to isoleucine independence.	Mitotic cross over. Gene conversion. Reverse mutation.	Zimmermann, Kern & Rosenberger, 1975.
D6	his4 0a + 0α ade-3 + 0 leu1 trp5 cyh2 met13 + ade-6 0 + + + + ade2-40 ade2-40	Diploid colonies are red cycloheximide sensitive. Monosomic colonies are white and cycloheximide resistant.	Aneuploidy.	Parry & Zimmermann, 1976.
S138	met 138	Methionine dependence to methionine independence.	Reverse mutation by frameshift mechanism.	Brusick & Mayer, 1973.
S211α	met 211	Methionine dependence to methionine independence.	Reverse mutation by base pair substitution.	Brusick & Mayer, 1973.
S288α	α ρ^+ can^S	Canavanine sensitive to canavanine resistant.	Forward mutation by frameshift and base pair substitution.	Brusick & Mayer, 1973.
JD1	A ade2-1 ser1 his8 his4C trp5-u9 α + + + his 4ABC trp5-u6	Histidine dependence to histidine independence. Tryptophan dependence to tryptophan independence.	Gene conversion.	Parry, 1977.
XV185-14C	a ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10 A + + + + + +	Lysine dependence to lysine independence. Threonine dependence to threonine independence. Histidine dependence to histidine independence.	Reverse mutation.	Shahin & von Borstel, 1976.

TABLE 37. CONSTITUENTS OF YEPG AGAR AND SUPPLEMENTED YEPG AGAR

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Yeast extract	10.0
Peptone	20.0
Glucose	20.0
Agar	15.0

YEPG agar should be enriched with appropriate growth factors for the maintenance of auxotrophic strains of S. cerevisiae: this precaution reduces the accumulation of prototrophic mutants which have a selective advantage in old cultures.

STRAIN	SUPPLEMENT	CONCENTRATION (mg/L YEPG)
D5	Adenine sulfate	5
D4	L-Tryptophan	10
	Adenine sulfate	10
D3	Adenine sulfate	10
S288a	-	-
S211a	L-Methionine	10
S138	L-Methionine	10
D7	Adenine sulfate	4
	Tryptophan	10
	Isoleucine	30

TABLE 38. CONSTITUENTS OF SPORULATION AGAR

CONSTITUENT	CONCENTRATION (g/L distilled H_2O)
Potassium acetate	10.0
Bacto yeast extract	1.0
Glucose	0.5
Agar	20.0

TABLE 39. CONSTITUENTS AND PREPARATION OF SYNTHETIC COMPLETE MEDIUM (SCM)

CONSTITUENT	CONCENTRATION (g/L DISTILLED H_2O)
Yeast nitrogen base w/o amino acids (Difco)	6.7
Adenine sulfate	0.005
L-Arginine-HCl	0.010
L-Histidine-HCl	0.010
L-Isoleucine	0.060
L-Leucine	0.060
L-Lysine-HCl	0.010
L-Methionine	0.010
L-Tryptophan	0.010
L-Valine	0.030
Uracil	0.010
Glucose	20.0
Agar	15.0

Convertants and revertants are detected on SCM lacking one or more of the standard ingredients.

TABLE 40. DETERMINATION OF SPONTANEOUS CONVERTANT FREQUENCIES OF *S. CEREVISIAE* D7 CULTURES

TUBE #	# OF CONVERTANT COLONIES PER PLATE					
	REPLICATE 1	REPLICATE 2	REPLICATE 3	REPLICATE 4	REPLICATE 5	Mean
1	*	*	*	*	*	*
2	111	98	106	100	110	105
3	195	95	102	80	101	94.6
4	102	107	85	112	118	105
5	383	384	433	417	516	427
6	83	96	97	100	103	96
7	93	67	81	78	65	77
8	91	87	84	87	92	88
9	82	100	102	89	-	93
10	68	80	67	81	101	79

* Fungal contamination.

TABLE 41. EFFECT OF SPREAD PLATE INCUBATION TIME ON THE DETERMINATION OF SPONTANEOUS CONVERTANT FREQUENCIES

CULTURE #	# OF CONVERTANT		VIABLE CELLS PLATED (10^7 X CFU)
	INCUBATION TIME (days) 3	INCUBATION TIME (days) 6	
1	107	118	1.2
2	95	115	1.2
3	200	200	1.1
4	101	120	1.4
5	92	109	0.8
6	99	117	1.3
7	81	108	0.75
8	87	103	0.94

TABLE 42. FIRST TRIAL OF D7 TEST PROCEDURE: RESPONSE OF D7 TO EMS

CONCENTRATION (% V/V)	SURVIVORS			CONVERTANTS (CFU/PLATE)	REVERTANTS (CFU/PLATE)
	(CFU/PLATE)*	CROSSOVERS	TOTAL ABERRANT		
Control	63.5	0	2(1270)**	0.2	0
0.5	118.7	7	16(1662)	0.2	0
1.0	107.7	15	67(2154)	0.2	2
1.5	82.7	14	80(1488)	0	1
2.0	1.5	0	0(29)	0	0

* Undiluted.
** Colonies scored.

TABLE 43. D7 YEAST TEST - WORK SCHEDULE

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
Week #2 Pick two typical colonies from SCM7 plates. Prepare dilution series. Perform cell counts (X10). Prepare stock suspension of 2×10^3 cells per mL. Recount. Store stock at 5°C. Store D7 stock slants at 5°C.	**Each Week** Prepare media orders.	**Week #2** Inoculate 10 tubes of D7 broth with 0.1 mL of stock cell suspension at 4 pm. Incubate for 40 hours at 28°C on shaker. Restore stock suspension at 5°C.	**Week #1** Inoculate three tubes of D7 broth from stock slants. Incubate for 24 hours at 28°C with shaking.	**Week #1** Streak SCM7 plates (X3) from D7 broth tubes. Incubate over weekend at 28°C. Streak three fresh slants of D7 agar. Store broth tubes at 5°C.
Week #3 Count the T7 plates from Week #2. Select cultures suitble for use in test. Wash cells. Prepare serial dilutions. Perform cell counts (X10). Prepare 3.0×10^7 cells/mL stock suspension. Confirm cell count. Dry required number of T7, SCM7, and 7b plates. Label all plates. Store stock suspension at 5°C.	**Week #3** Prepare EMS solution. Prepare S9 liver microsomal mix if necessary. Set up test solutions. Inoculate tubes with stock suspension. During test exposure, prepare buffer dilution tubes, ice slurry, centrifuge tubes etc. Stop test. Wash Test cells. Suspend final pellets and store at 5°C.	**Week #3** Spread plate all test samples onto T7 (X10), SCM7 (X40). Incubate upright at 28°C. Clean lab.	**Week #2** Dry T7 plates for Friday. Maintain stocks of phosphate buffer (pH 7.0). Check on media stocks for next test.	**Week #2** At 8 am spread 0.1 mL samples from each tube (X10) onto SCM7 agar (X5). Incubate over weekend at 28°C. Store the 10 broth cultures at 5°C. Prepare lab for test on Week #3. Prepare any solutions required for Week #3. Sterilize pipet tips, etc.
Week #4 Count all T7 plates from Week #3. Count SCM7 plates from Week #3.		**Week #4** Transfer SCM7 plates from Week #3 to 5°C. Recount T7 plates. Recount SCM7 plates where required.	**Week #3** Invert all spread plates from Wednesday. Dispose of mutagenic wastes.	**Week #4** Count mitotic crossovers on SCM7 plates from Week #3.

TABLE 44. TRIAL OF REVISED TEST PROCEDURE: RESPONSE OF D7 TO EMS

CONCENTRATION (% V/V)	SURVIVORS				CONVERTANTS PER 10^5	REVERTANTS PER 10^6
	(X 10^3)	(%)	CROSSOVER (%)	TOTAL ABBERANT (%)		
Control	68(2736)	100	0	0	0	0(0)
0.5	98(3900)	144	0.21(8)	1.38(54)	8.2(80)	81.6(400)
1.0	78(3112)	115	0.71(22)	3.5(108)	42.3(330)	87.1(340)
1.5	106(4240)	156	1.01(43)	5.4(230)	97.2(1030)	139.6(740)
2.0	79(3081)	116	1.04(32)	6.39(197)	178.5(1410)	207.5(820)

Numbers in parenthesis: actual colony counts.

TABLE 45. REPEAT TRIAL OF REVISED TEST PROCEDURE: RESPONSE OF D7 TO EMS

CONCENTRATION (% V/V)	SURVIVORS				CONVERTANTS PER 10^5	REVERTANTS PER 10^6
	(X 10^3)	(%)	CROSSOVER (%)	TOTAL ABBERANT (%)		
Control	80.3(3212)	100	0	0.06(2)	0.89(7.1)	0.5(2.0)
0.5	76.5(3060)	95.3	0.26(8)	2.03(62)	18.8(144)	9(34)
1.0	70.5(2820)	87.8	0.53(15)	3.9(110)	50.2(354)	32(111)
1.5	91.6(3662)	114.1	1.47(54)	6.2(227)	73.47(673)	155(7110)
2.0	110.9(4436)	138	1.21(54)	7.25(332)	135.3(1500)	204(1140)

Numbers in parenthesis: actual colony counts.

TABLE 46. RESPONSE OF D7 TO METABOLICALLY ACTIVATED 2-ACETYLAMINO FLUORENE

CONCENTRATION (μg/mL)	SURVIVORS (X 10^3)	SURVIVORS (%)	SURVIVORS CROSSOVER (%)	SURVIVORS TOTAL ABBERANT (%)	CONVERTANTS PER 10^5	REVERTANTS PER 10^6
Control	123.4(1851)	100	0.05(1)	0.2(3)	0.8(5)	7.8(48)
0.1	136.4(2047)	110.5	0	0.1(2)	0.7(5)	5.4(37)
1.0	108.2(1623)	87.6	0.06(1)	0.4(6)	1.6(9)	4.2(23)
10.0	121.8(1828)	98.7	0.05(1)	0.2(3)	1.1(7)	6.1(37)
200.0	123.5(1853)	100	0.1(2)	0.1(2)	0.3(2)	3.7(23)

Numbers in parenthesis: actual colony counts.

TABLE 47. RESPONSE OF D7 TO NIAGARA RIVER WATER SAMPLES #263 AND #264

SAMPLE	CONCENTRATION (X times)	SURVIVORS (X 10^3)	SURVIVORS (%)	SURVIVORS CROSSOVER (%)	SURVIVORS TOTAL ABBERANT (%)	CONVERTANTS PER 10^5	REVERTANTS PER 10^6
Blank	-	193(7720)	100	0(0)	0.01(1)	0.83(16)	0.4(4)
Control	2.0% EMS	93(3720)	48	1.6(58)	7.8(291)	191(1780)	240(1115)
263	1	194(7760)	101	0.01(1)	0.03(2)	0.6(12)	0(0)
263	5	181(7240)	94	0(0)	0.03(2)	0.3(5)	0.2(2)
263	10	199(7960)	103	0.01(1)	0.01(1)	0.5(9)	0.2(2)
264	1	194(7760)	101	0(0)	0.06(5)	0.4(8)	0.2(2)
264	5	184(7360)	95	0(0)	0.05(4)	0.5(10)	0.1(1)
264	10	224(8960)	116	0(0)	0.03(3)	0.03(7)	0.2(2)

Numbers in parenthesis: actual colony counts.

TABLE 48. RESPONSE OF D7 TO NIAGARA RIVER WATER SAMPLES #265 AND #266

SAMPLE	CONCENTRATION (X times)	SURVIVORS (X 10^3)	SURVIVORS (%)	CROSSOVER (%)	TOTAL ABBERANT (%)	CONVERTANTS PER 10^5	REVERTANT PER 10^6
Blank	-	159(6360)	100	0(0)	0.06(4)	0.1(2)	0.3(2)
Control	2.0% EMS	59.2(2368)	37	1.1(25)	7.5(178)	194(1151)	115(340)
265	1	113.2(4528)	71	0(0)	0.02(1)	0.8(9)	1.1(6)
265	5	93.8(3752)	59	0(0)	0(0)	0.6(6)	0(0)
265	10	105(4200)	66	0(0)	0(0)	0.5(5)	0(0)
266	1	155(6200)	97.5	0(0)	0.03(2)	0.5(7)	0.3(1)
266	5	52(2080)	33	0.05(1)	0.10(2)	0.6(3)	0.4(1)
266	10	126(5040)	79	0(0)	0.02(1)	0.5(6)	0.2(2)

Numbers in parenthesis: actual colony counts.

TABLE 49. RESPONSE OF D7 TO NIAGARA RIVER WATER SAMPLES #267 AND #268

SAMPLE	CONCENTRATION (X times)	SURVIVORS (X 10^3)	SURVIVORS (%)	CROSSOVER (%)	TOTAL ABBERANT (%)	CONVERTANTS PER 10^5	REVERTANTS PER 10^6
Blank	-	150.2(6008)	100	0(0)	0.03(2)	0.2(3)	0.01(3)
Control	2.0% EMS	64.9(2596)	43.2	1.3(33)	8.6(222)	163(1059)	113(369)
267	1	168.3(6732)	112	0(0)	0.03(2)	0.8(13)	0(0)
267	5	85(3400)	56.5	0(0)	0.06(2)	1.1(9)	0.7(3)
267	10	30(1200)	20.0	0.8(1)	0.25(3)	0	1.3(2)
268	1	122(4880)	81.2	0(0)	0.04(2)	0.6(7)	0.3(2)
268	5	65.5(2620)	43.6	0(0)	0(0)	0.6(4)	0.3(1)
268	10	96.4(3856)	64.2	0(0)	0.03(1)	1.0(10)	0.2(1)

Numbers in parenthesis: actual colony counts.

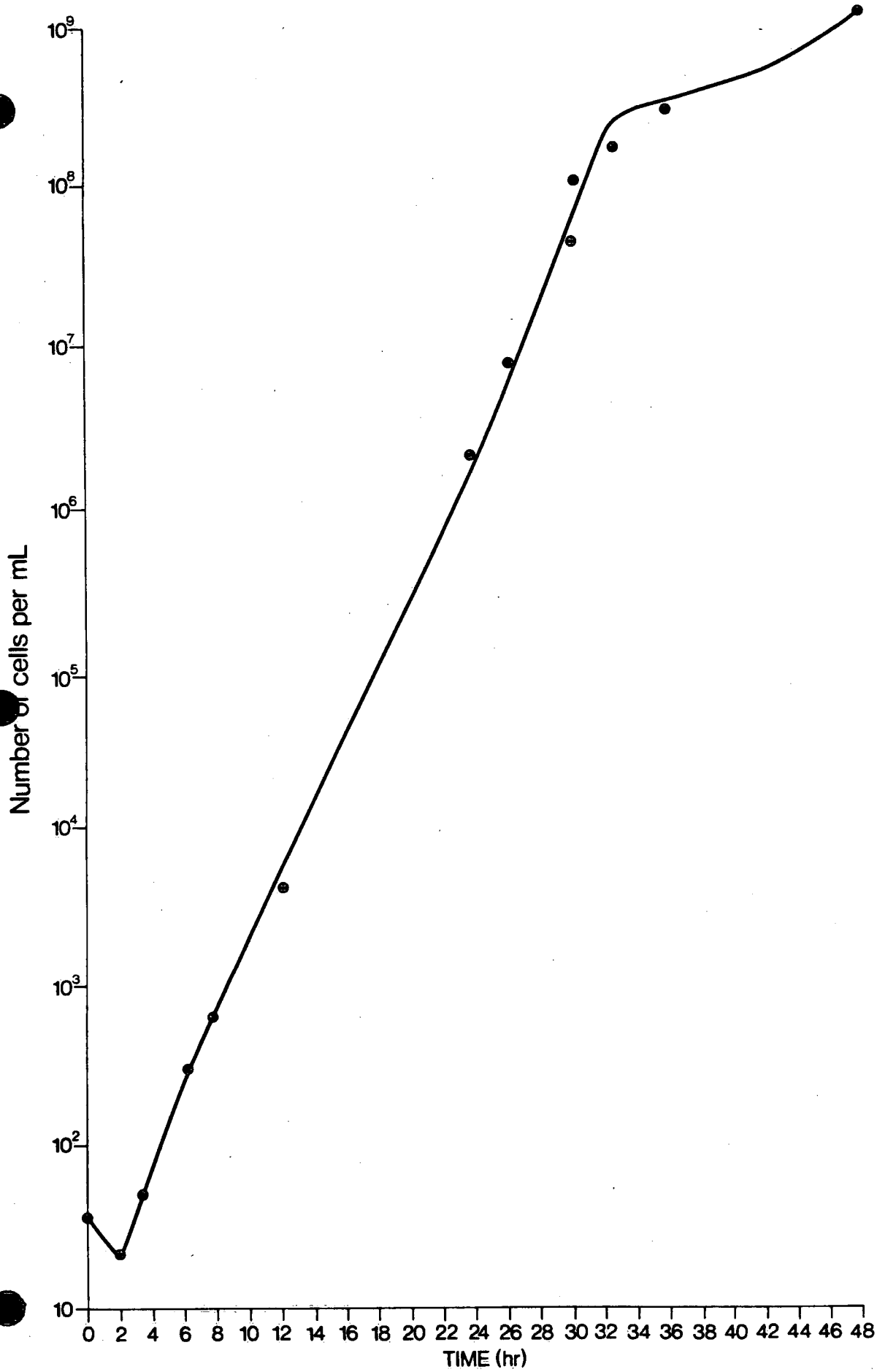

Figure 1 Growth curve for strain D7

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