The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: October 14, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**Madhumita Ray**
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Location of experimental work to be carried out :

Building :	**TEB**	Room(s):	**313**
Building :	**Thompson Engineering Building**	Room(s):	**313A**
Building :		Room(s):	

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES: **NSERC Discovery/NSERC Engage**
GRANT TITLE(S): **Micropollutant and pathogen reduction in shudge due to advanced oxidation**
UNDERGRADUATE COURSE NAME(IF APPLICABLE):

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Sura Ali	**sali327@uwo.ca**	**June 1, 2011**

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

Biological agents will be used in two different types of bio-assays, the yeast estrogenicity assays (YES-assay) and a mutagenicity assays (Ames test).

The YES assay utilizes a recombinant yeast strain. Frozen glycerol stock culture will be stored in a --80 degree C freezer. Cultivation will occur in closed scintillation vials over night (5 mL) followed by 3-day incubation in sealed micro-titer plates. After assay completion all samples will be sterilized via autoclaving for 20 minutes at 120 degreeC. An autoclave is available in TEB 313. The sterility of samples is verified through weekly autoclave tests with Bacillus stearothermophilus spores (log available in TEB 313).

The Ames test ultilizes 5 strains of S. typhimurium. All strains are non-virulent histidine-deficiency mutants. Frozen stocks will be stored at -80 C. 1mL overnight cultures will be grown in closed scintillation vials followed by 5-day incubation in sealed micro-titer plates. All samples will be sterilized via autoclaving after completing the assays.

The wastewater plant effluent to be utilized at later stages of the work also has to be considered a potential source of biological material. However, the water that will be used is the clean effluent of a treatment plant. Water samples will immediately be sterilized via autoclaving, once received at Western and subsequently stored at 4 oC. Sterility of the water samples is required for the bio-assays to work accurately. The reason for using plant effluent is to evaluate the efficiency of the bioassays with a more complex water metrics, containing particles and organic carbon. However, at no point of time raw wastewater is used.

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

With the increasing world-wide demand for and pressure on natural resources, new cost effective technologies are required to provide sustainable development. Increasing presence of significantly large number of pharmaceutical drugs, hormones, personal care products, and a broad array of synthetic chemicals in wastewater treatment effluents in all over the world requires advance treatment of secondary effluent prior to reuse applications such as groundwater recharge of potable aquifers and augmentation of surface water sources [1]. Advanced oxidation processes (AOP) such as UV, UV/H2O2 and UV/O3, which produce hydroxyl radical in-situ are identified as viable technologies for removal of recalcitrant organics from various waste streams.The performance of the technologies will be assessed based on toxicological effects of micropollutants and their degradation products at various stages of advanced oxidative treatment using bioassays tests like yeast estrogen screen (YES) and Ames mutagenicity.

Companies such as Environmental Bio-detection Products, Inc. (EBPI) are specialized on developing rapid and regulatory conforming assays for the detection of biologically active compounds in water. EBPI is also specializing in the manufacture and custom application of simple, rapid, and cost effective methods for detection and monitoring of toxic, mutagenic and genotoxic materials. EBPI's assays are scaled-down to utilize a minimum sample volume, thereby also minimizing the costs for chemicals and required equipment. Their assay kits can be used to evaluate various toxicity parameters of wastewater or drinking water (acute or chronic toxicity, mutagenicity (Ames test), genotoxicity (ISO 13829), coliform concentration, etc.). However, the extremely low concentration of micropollutants renders these kits less accurate for their evaluation in water. The objective of the proposed work is to adopt the assay systems currently produced by EBPI towards the use on micropollutants and their metabolites by improving the analytical protocols used currently.

Commercially available EBPI assay kits will be evaluated for their use with micropollutnats such as bisphenol A. Initially pure water will be spiked with model micropollutants and YES and AMES test will be employed to determine parent compound toxicity. Thereafter, oxidation by-products of the micropollutants will be tested following the same bioassays. Tests will be conducted in different types of water such as influent and effluent from drinking water, and treated effluent from waste water treatment plants to charcaterize the effects of background water.

The Ames assay is based on histidine deficient mutants of Salmonella Typhimurium strains. The specific strains are not regulated in Canada and can be purchased freely from assay kit manufacterers such as EBPI. Virulent Salmonella typhimurium are classified as containment level 2, the so-called Ames strains are not virulent and are specifically excepted from level 2 containment by the Swiss authorities (see atched documentation). The maximum cultivation volume of the strains is 1mL and will be conducted in level 2 approved facilities (TEB313a), to ensure extra caution.

The YES assay uses a yeast strain carrying a human estogen receptor. A colorimentic response can be detected if incubated in the presence of an indicator and estrogenic compounds. The YES test requires the cultivation of no more than 5mL yeast at a time. All bioassays will be conducted in micro-titer plates.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☒ NO
If YES, please give the name of the species _____
What is the origin of the microorganism(s)? _____

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
Salmonella Typhimurium TA100	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.001**	**EBPI**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
S.Typhimurium TA98	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.001**	**EBPI**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
S.Typhimurium TA 97a	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.001**	**EBPI**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
S.Typhimuirum TA 97b	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.001**	**EBPI**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
S.Typhimurium TA102, TA 1535	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.001**	**EBPI**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
Saccharomyces cerevisiae BJ 1991	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.005**	**Trojan UV**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
Wastewater treatment plant effluent	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**1**	**City of London**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: **The composition of the waste water treatment plant is unknown, however, it is treated water, assumed safe by the city of London. It is further sterilized upon receiving it.**

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☐ YES ☒ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☐ Yes ☐ No		Not applicable
Rodent	☐ Yes ☐ No		
Non-human primate	☐ Yes ☐ No		
Other (specify)	☐ Yes ☐ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☐ Yes ☐ No			
Rodent	☐ Yes ☐ No			
Non-human primate	☐ Yes ☐ No			
Other (specify)	☐ Yes ☐ No			

****Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)***

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☐ 1 ☐ 2 ☐ 2+ ☐ 3

Additional Comments: _____

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☐ YES ☒ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/UNKNOWN**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

Additional Comments: _____

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☐ YES ☒ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☐ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?

**** Please attach a Material Safety Data Sheet or equivalent if available.***
***** Please attach a plasmid map.***
******No Material Safety Data Sheet is required for the following strains of E. coli:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☐ YES, complete table below ☐ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction

**** Please attach a Material Safety Data Sheet or equivalent.***

4.3.1 Will virus be replication defective? ☐ YES ☐ NO

4.3.2 Will virus be infectious to humans or animals? ☐ YES ☐ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☐ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☒ NO ☐ YES, specify
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☒ NO ☐ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: _____

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 7.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☐ YES ☒ NO If **NO**, please proceed to section 8.0

7.2 Name of animal species to be used

7.3 AUS protocol #

7.4 List the location(s) for the animal experimentation and housing.

7.5 Will any of the agents listed in section 4.0 be used in live animals
☐ NO ☐ YES, specify:

7.6 Will the agent(s) be shed by the animal:
☐ YES ☐ NO, please justify:

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 9.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☐ YES ☒ NO If **NO**, please proceed to Section 10.0

9.2 If YES, please name the toxin(s) or hormones(s)
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone

9.4 How much of the toxin or hormone is handled at one time*?

9.5 How much of the toxin or hormone is stored*?

9.6 Will any biological toxins or hormones be used in live animals? ☐ YES ☐ NO
If **YES**, Please provide details:

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: _____

10.0 Insects

10.1 Do you use insects? ☐ YES ☒ NO - If **NO**, please proceed to Section 11.0

10.2 If YES, please give the name of the species.

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention? ☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

10.6 Please describe the risk (if any) of escape and how this will be mitigated:

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 12.0

11.2 If YES, please give the name of the species.

11.3 What is the origin of the plant?

11.4 What is the form of the plant (seed, seedling, plant, tree…)?

11.5 What is your intention? ☐ Grow and maintain a crop ☐ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☐ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:

11.8 Is the CFIA permit attached? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 13.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☐
Enter Your Name _____ ***Date:*** _____

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☒ 1 ☐ 2 ☐ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☒ YES, location and date of most recent biosafety inspection: **TEB 313a, May 2012.**
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **BIO-UWO-0254 (Rehmann, TEB 313, 313a**

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO

If **YES** please describe:

Make sure that to work in the safety cabinet wearing safety glasses, mask, gloves, lab coat, and clean with ethanol , bleach and disinfectant before and after working carefully and autoclave all the waste.

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
Tell the supervisor, go to the nearest health care clinic with the MSDS of the biological agent or any information help.

15.3 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/workplacehealth.html

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Madhumita Ray** ***Date:*** **7 August, 2012.**

15.4 Additional Comments: _____

16.0 Approvals

1) UWO Biohazards Subcommittee: SIGNATURE: _____
Date: _____

2) Safety Officer for the University of Western Ontario
SIGNATURE: _____
Date: _____

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: _____
Date: _____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

Risk Group Classification for Infectious Agents

Leave feedback about this database (questions, comments or suggestions) ›

‹ Main Riskgroup Page and Definitions
SEARCH: Bacteria › Viruses › Fungi › Parasites ›

Bacteria Search Results

Genus: Salmonella **Species:** typhimurium		
	Risk Group Level	**Notes**
Australia/New Zealand 2002:		
Belgium 2004:	2	
Switzerland 2003:	2	Exceptions are derivates of strain LT2 with stable mutations in the genes aroA, galE oder cya und crp (e.g. strains for Ames Test TA 98, TA 100, TA 1535, TA1530, TA 2631)
United Kingdom 2004:	2	
Germany 2001:	2	AR
NIH 2002	2	
European Community 2000:	2	
Singapore 2004:	2	Singapore Schedule:
Japan:		
Human Pathogen: Yes **Animal Pathogen:** Yes **Plant Pathogen:** No		**Select Agent CDC:** No **Select Agent USDA:** No
MSDS:		

American Biological Safety Association, 1200 Allanson Road, Mundelein, IL 60060-3808
Phone: 1-866-425-1385 (toll free), 847-949-1517 **Fax:** 847-566-4580 **E-mail:** info@absa.org

471
Adopted:
21st July 1997

OECD GUIDELINE FOR TESTING OF CHEMICALS

Bacterial Reverse Mutation Test

INTRODUCTION

1. The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (1)(2)(3). The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.

2. Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds.

3. Definitions used are set out in the Annex.

INITIAL CONSIDERATIONS

4. The bacterial reverse mutation test utilises prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

5. The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents which are not detected by this test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity.

6. The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

7. Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute. It is dependent on chemical class and there are carcinogens that are not detected by this test because they act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells.

PRINCIPLE OF THE TEST METHOD

8. Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

9. Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method (1)(2)(3)(4), the preincubation method (2)(3)(5)(6)(7)(8), the fluctuation method (9)(10), and the suspension method (11). Modifications for the testing of gases or vapours have been described (12).

10. The procedures described in this guideline pertain primarily to the plate incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some compounds may be detected more efficiently using the preincubation method. These compounds belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds (3). It is also recognised that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as "special cases" and it is strongly recommended that alternative procedures should be used for their detection. The following "special cases" could be identified (together with examples of procedures that could be used for their detection): azo-dyes and diazo compounds (3)(5)(6)(13), gases and volatile chemicals (12)(14)(15)(16), and glycosides (17)(18). A deviation from the standard procedure needs to be scientifically justified.

DESCRIPTION OF THE METHOD

Preparations

Bacteria

11. Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used. It is essential that the cultures used in the experiment contain a high titre of viable bacteria. The titre may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

12. The recommended culture temperature is 37°C.

13. At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) that have been shown to be reliable and reproducibly responsive between laboratories. These four *S. typhimurium* strains have GC base pairs at the primary reversion site and it is known that they may not detect certain oxidising mutagens, cross-linking agents and hydrazines. Such substances may be detected by *E.coli* WP2 strains or *S. typhimurium* TA102 (19) which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

1. *S. typhimurium* TA1535, and
2. *S. typhimurium* TA1537 or TA97 or TA97a, and
3. *S.* typhimurium TA98, and
4. *S.* typhimurium TA100, and
5. *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101), or *S. typhimurium* TA102.

In order to detect cross-linking mutagens it may be preferable to include TA102 or to add a DNA repair-proficient strain of *E.coli* [e.g. *E.coli* WP2 or *E.coli* WP2 (pKM101).]

14. Established procedures for stock culture preparation, marker verification and storage should be used. The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains, and tryptophan for *E. coli* strains). Other phenotypic characteristics should be similarly checked, namely: the presence or absence of R-factor plasmids where appropriate [i.e. ampicillin resistance in strains TA98, TA100 and TA97a or TA97, WP2 uvrA and WP2 uvrA (pKM101), and ampicillin + tetracycline resistance in strain TA102]; the presence of characteristic mutations (i.e. rfa mutation in *S. typhimurium* through sensitivity to crystal violet, and uvrA mutation in *E. coli* or uvrB mutation in *S. typhimurium*, through sensitivity to ultra-violet light) (2)(3). The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

Medium

15. An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin or tryptophan, to allow for a few cell divisions, is used (1)(2)(9).

Metabolic activation

16. Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (1)(2) or a combination of phenobarbitone and ß-naphthoflavone (18)(20)(21). The post-mitochondrial fraction is usually used at concentrations in the range from 5 to 30% v/v in the S9-mix. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. For azo-dyes and diazo-compounds, using a reductive metabolic activation system may be more appropriate (6)(13).

Test substance/Preparation

17. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the bacteria. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

Test conditions

Solvent/vehicle

18. The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the bacteria and the S9 activity (22). If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water.

Exposure concentrations

19. Amongst the criteria to be taken into consideration when determining the highest amount of test substance to be used are cytotoxicity and solubility in the final treatment mixture. It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a substance may be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5 µl/plate. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture. Test substances that are cytotoxic already below 5 mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

20. At least five different analysable concentrations of the test substance should be used with approximately half log (i.e. $\sqrt{10}$) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a concentration-response is being investigated.

21. Testing above the concentration of 5 mg/plate or 5 µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities.

Controls

22. Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each assay. Positive control concentrations that demonstrate the effective performance of each assay should be selected.

23. For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used. The following chemicals are examples of suitable positive controls for assays with metabolic activation:

Specializing in Biomolecular Testing kits for Toxicity, Mutagenicity, and Genotoxicity.

96 Well Reverse Mutation Ames Test

Ames Reverse Mutation

The Muta-ChromoPlate kit is a rapid cost effective reverse mutation 96 well Ames Test which can be conducted within 3-5 days.

- Frame-Shift Mutation
- Insertion Mutation
- Missense Mutation
- Nonsense Mutation
- Deletion Mutation
- Duplication Mutation

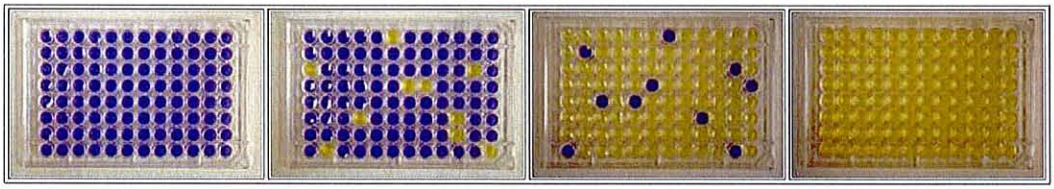
MUTA-CHROMOPLATE™

5051 - Basic Kit

B5051 - Bacterial Strain Kit

The Muta-ChromoPlate kit is a 96 well microplate version of the salmonella typhimurium Ames Test, used for detection of mutagenic activity (Follows the OECD 471 Guidelines for Testing Of Chemicals - Bacterial Reverse Mutation Test).

Developed to test mutagenic materials in water soluble extracts of , sediment, air, chemicals, food components, cosmetics, waste waters, potable waters and any other material that can be solubilized or placed into micro suspension in water such that the material being tested can be taken up by the test strain. The Muta-ChromoPlate provides a clear colour endpoint. Reagents, cultures and other consumable components are supplied ready-to-use in a non-specialized laboratory. Te



The Muta-ChromoPlate provides a clear colour endpoint. Reagents, cultures and other consumable components are supplied ready-to-use in a non-specialized laboratory. The Muta-ChromoPlate kit is based on the most generally used and bacterial reverse mutation test, known as the Ames Test (Ames et al., 1975 Mutation Research 31:347)

The test employs a mutant strain, or several strains, of Salmonella typhimurium, carrying mutation(s) in the operon coding for histidine biosynthesis. When these bacteria are exposed to mutagenic agents, under certain conditions, reverse mutation from amino acid (histidine) auxotrophy to prototrophy occurs.

Traditionally, reverse-mutation assays have been performed using agar plates. An alternate assay performed entirely in liquid culture is the 'Fluctuation Test' based on multiple yes/no colour endpoints (Hubbard, S.A *et al.*, 1994, pp. 141-160, in Kilbey *et al.* (Eds.), Handbook of Mutagenicity Testing (2nd Ed,.) Elsevier Sciences, NY. This test principle is being applied in the Muta-ChromoPlate test kit.

The Muta-ChromoPlate Kit is generally more sensitive (up to 10 times) than the pour-plate assay, because it allows testing of higher concentrations of sample (up to 75% v/v). The assay procedure is simple and requires minimal training. Consumable components are provided with ready-to-use and step-by-step instructions. "Instructions for Use" are provided with the basic kit. The only equipment required are a 37 degree Celsius incubator and a single and a multi-channel micropipettor.

S9 Activation Enzymes

S9 is a crude liver enzyme extract that can, under certain conditions, convert materials without any genotoxic activity to active genotoxic entities. The chemical process involved is probably different for different materials. In addition, the lifetime of the activated moieties is

extremely variable: some may be extremely short-lived. This is the reason for incubating the S-9 with the bacteria and the tested material at the same time.

Applications:

Testing of pharmaceuticals for mutagenic activity.

Testing of industrial effluents for presence of possible mutagenic compounds.

Screening of municipal discharges for possible routine presence or spills of mutagenic compounds.

Screening of surface and ground water for mutagenic residues.

Screening of potable water supplies for the presence of chemicals with mutagenic potential.

Screening of water soluble air pollutants for mutagenic agents.

Evaluation of pure or complexed raw mixtures for potential mutagenicity.

A convenient and easy to use teaching look for university and college laboratories.

The Muta-ChromoPlate kit (Ames Test Kit) is designed to be user friendly by removing potential contamination concerns.

Strains Currently Available to be shipped are: TA97a, TA98, TA100, TA102 and the TA1535.

Reverse Mutations in Various Bacterial Strains

Base-Pair Substitutions TA100; TA1535; TA102 (Site A-T)	**What is a Base Pair Substitution?** Insertion Mutation Missense Mutation Nonsense Mutation Deletion Mutation Duplication Mutation
Frame Shift Mutations TA98; TA97a	**What is a Frame shift Mutation?** Frame Shift Mutation

Click Here for a visual understanding on how the Muta-ChromoPlate kit is preformed

2011 Pricing

Chemical and CAS No.
9,10-Dimethylanthracene [CAS no. 781-43-1]
7,12-Dimethylbenzanthracene [CAS no. 57-97-6]
Congo Red [CAS no. 573-58-0] (for the reductive metabolic activation method)
Benzo(a)pyrene [CAS no. 50-32-8]
Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)]
2-Aminoanthracene [CAS no. 613-13-8]

2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterised with a mutagen that requires metabolic activation by microsomal enzymes, e.g., benzo(a)pyrene, dimethylbenzanthracene.

24. For assays performed without metabolic activation system, examples of strain-specific positive controls are:

	Chemical and CAS No.	Strain
(a)	Sodium azide [CAS no. 26628-22-8]	TA1535 and TA100
(b)	2-Nitrofluorene [CAS no. 607-57-8]	TA98
(c)	9-Aminoacridine [CAS no. 90-45-9] or ICR191 [CAS no. 17070-45-0]	TA1537, TA97 and TA97a
(d)	Cumene hydroperoxide [CAS no. 80-15-9]	TA102
(e)	Mitomycin C [CAS no. 50-07-7]	WP2 uvrA and TA102
(f)	N-Ethyl-N-nitro-N-nitrosoguanidine [CAS no. 70-25-7] or 4-nitroquinoline 1-oxide [CAS no. 56-57-5]	WP2, WP2 uvrA and WP2 uvrA (pKM101)
(g)	Furylfuramide (AF-2) [CAS no. 3688-53-7]	plasmid-containing strains

25. Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals may be considered, when available.

26. Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

PROCEDURE

Treatment with test substance

27. For the plate incorporation method (1)(2)(3)(4), without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions, 0.1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) and 0.5 ml of sterile buffer are mixed with 2.0 ml of overlay agar. For the assay with metabolic activation, usually 0.5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 to 30% v/v in the metabolic activation mixture) are mixed with the overlay agar (2.0 ml), together with the bacteria and test substance/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

28. For the preincubation method (2)(3)(5)(6) the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0.5 ml) usually for 20 min. or more at 30°-37°C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0.05 or 0.1 ml of test substance/test solution, 0.1 ml of bacteria, and 0.5 ml of S9-mix or sterile buffer, are mixed with 2.0 ml of overlay agar. Tubes should be aerated during pre-incubation by using a shaker.

29. For an adequate estimate of variation, triplicate plating should be used at each dose level. The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

30. Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (12)(14)(15)(16).

Incubation

31. All plates in a given assay should be incubated at 37°C for 48-72 hours. After the incubation period, the number of revertant colonies per plate is counted.

DATA AND REPORTING

Treatment of results

32. Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and untreated control if used) and positive control plates should also be given.

33. Individual plate counts, the mean number of revertant colonies per plate and the standard deviation should be presented for the test substance and positive and negative (untreated and/or solvent) controls.

34. There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing, the method of treatment (plate incorporation or liquid preincubation), and metabolic activation conditions.

Evaluation and interpretation of results

35. There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system (23). Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (24). However, statistical significance should not be the only determining factor for a positive response.

36. A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test

37. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

38. Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

Test report

39. The test report must include the following information:

Test substance:

- identification data and CAS no., if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance, if known.

Solvent/Vehicle:

- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in solvent/vehicle, if known.

Strains:

- strains used;
- number of cells per culture;
- strain characteristics.

Test conditions:

- amount of test substance per plate (mg/plate or μg/plate) with rationale for selection of dose and number of plates per concentration;
- media used;
- type and composition of metabolic activation system, including acceptability criteria;
- treatment procedures.

Results:

- signs of toxicity;
- signs of precipitation;
- individual plate counts;
- the mean number of revertant colonies per plate and standard deviation;
- dose-response relationship, where possible;
- statistical analyses, if any;
- concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations;
- historical negative (solvent/vehicle) and positive control data, with e.g. ranges, means and standard deviations.

Discussion of the results.

Conclusion.

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ANNEX

DEFINITIONS

A reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino-acid requiring strain (histidine or tryptophan, respectively) to produce a strain independent of an outside supply of amino-acid.

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome.

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA

Saccharomyces cerevisiae Final Risk Assessment

ATTACHMENT I--FINAL RISK ASSESSMENT OF

SACCHAROMYCES CEREVISIAE

(February 1997)

I. INTRODUCTION

Saccharomyces cerevisiae has an extensive history of use in the area of food processing. Also known as Baker's Yeast or Brewer's Yeast, this organism has been used for centuries as leavening for bread and as a fermenter of alcoholic beverages. With a prolonged history of industrial applications, this yeast has been either the subject of or model for various studies in the principles of microbiology. Jacob Henle based his theories of disease transmission on studies of strains of Brewer's Yeast. Currently, S. cerevisiae is the subject of a major international effort to characterize a eucaryotic genome (Anderson, 1992).

History of Commercial Use and Products Subject to TSCA Jurisdiction

Saccharomyces cerevisiae, in addition to its use in food processing, is widely used for the production of macromolecular cellular components such as lipids, proteins including enzymes, and vitamins (Bigelis, 1985; Stewart and Russell, 1985).

The Food and Drug Administration rates Brewer's Yeast extract as Generally Recognized as Safe (FDA, 1986). Furthermore, the National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules (DHHS, 1986) considers S. cerevisiae a safe organism. Most experiments involving S. cerevisiae have been exempted from the NIH Guidelines based on an analysis of safety (see Appendix C-II of the NIH Guidelines). While alcoholic beverages, vitamins, and bread leavening are covered under the Federal Food, Drug and Cosmetic Act, the production of enzymes and other macromolecules may be subject to TSCA regulation. The abundance of information on S. cerevisiae, derived from its role in industry, has positioned it as a primary model for genetic studies and, by extension, as a strong candidate for genetic manipulation for TSCA applications (Dynamac, 1990).

II. IDENTIFICATION AND CLASSIFICATION

A. Taxonomy and Characterization

Saccharomyces cerevisiae is a yeast. The organism can exist either as a singlecelled organism or as pseudomycelia. The cells reproduce by multilateral budding. It produces from one to four ellipsoidal, smoothwalled ascospores. S. cerevisiae can be differentiated from other yeasts based on growth characteristics and physiological traits: principally the ability to ferment individual sugars. Clinical

identification of yeast is conducted using commercially available diagnostic kits which classify the organism through analysis of the ability of the yeast to utilize distinct carbohydrates as sole sources of carbon (Buesching et al., 1979; Rosini et al., 1982). More recently, developments in systematics have led to the design of sophisticated techniques for classification, including gasliquid chromatography of lysed whole cells (Brondz and Olsen, 1979).

As a result of the application of newer techniques arising from innovative approaches, the taxonomy of Saccharomyces is subject to greater scrutiny. The initial classification was based principally on morphological characteristics with specific physiological and biochemical traits used to differentiate between isolates with similar morphological traits. Using these criteria, there are as many as 18 species listed in the literature. In addition, what had been classified as one large heterogeneous species, S. cerevisiae, may, in the future, be divided into four distinct species based on DNA homology studies. The four species are S. cerevisiae, S. bayanus (also known as S. uvarum), S. pasteurianus (also known as S. carlsbergensis), and S. paradoxus. All four represent industrially important species. None of these organisms or other closely related species has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment.

Any assessment of Saccharomyces must take into consideration the malleability of the current classification. For this assessment of S. cerevisiae the reviews of the organism are based on the classification proposed by Van der Walt (1971).

B. Related Species of Concern

None of the above strains or other closely related species has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment.

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Colonization and Pathogenicity

S. cerevisiae is a commonly used industrial microorganism and is ubiquitous in nature, being present on fruits and vegetables. Industrial workers and the general public come into contact with S. cerevisiae on a daily basis through bothinhalation and ingestion (see section IV). Saccharomyces spp. are frequently recovered from the stools and throats of normally healthy individuals. This indicates that humans are in constant contact with these yeasts.

There are individuals who may ingest large quantities of S. cerevisiae every day, for example, people who take the yeast as part of a "health food" regimen. Therefore, studies were conducted to ascertain whether the ingestion of large numbers of these yeasts might result in either colonization, or colonization and secondary spread to other organs of the body. It was found that the installation of very large numbers of S. cerevisiae into the colons of animals would result in both colonization and passage of the yeasts to draining lymph nodes. It required up to 10^{10} S. cerevisiae in a single oral treatment to rats to achieve a detectable passage from the intestine to the lymph nodes (Wolochow et al., 1961). The concentrations of S. cerevisiae required were well beyond those that would be encountered through normal human daily exposure.

S. cerevisiae is not considered a pathogenic microorganism, but has been reported rarely as a cause of opportunistic infections. Eng et al. (1984) described five cases of such infections and reviewed the

literature on eight other S. cerevisiae infections (also briefly reviewed by Walsh and Pizzo, 1988). All of the patients in the cases had underlying disease. Some of them had also received antibiotic therapy, thereby suppressing normal bacterial flora and allowing mycotic organisms to become established.

A low concern for the pathogenicity of S. cerevisiae is also illustrated by a series of surveys conducted at hospitals over the last several years. S. cerevisiae accounted for less than 1% of all yeast infections isolated at a cancer hospital and in most of the cases the organism was isolated from the respiratory system (Kiehn et al., 1980). At YaleNew Haven Hospital over the past five years, there have been 50 isolates of S. cerevisiae recovered from patients; however, most of the isolates were considered contaminants (Dynamac, 1991).

2. Toxin Production

There have been no reports of isolates of S. cerevisiae that produce toxins against either humans or animals. However, S. cerevisiae has been shown to produce toxins against other yeasts. These toxins, termed "killer toxins", are proteins or glycoproteins produced by a range of yeasts. The yeasts have been genetically modified to alter activity and are used in industrial settings as a means of controlling contamination of fermentation systems by other yeasts (Sid et al., 1988).

3. Measure of the Degree of Virulence

A number of individual virulence factors have been identified as being associated with the ability of yeasts to cause disease. The principal virulence factors associated with yeasts appear to be phospholipase A and lysophospholipase. It is believed that these enzymes enhance the ability of the yeast to adhere to the cellwall surface and result in colonization as a first step in the infectious process. Nonpathogenic yeast had considerably lower phospholipase activities. Of a wide range of fungi assayed for phospholipase production, S. cerevisiae was found to have the lowest level of activity (BarrettBee et al., 1985). Therefore, based on the phospholipase virulence factor S. cerevisiae is considered a nonpathogenic yeast.

A second factor associated with virulence in yeast is the ability of a fungus to impair the host's immune capabilities. The cell walls of most fungi have the capacity to impede the immune response of the host. In a study to determine the overall pathogenicity of a number of yeasts used in industrial processes, animals exposed to both high levels of S. cerevisiae and cortisone demonstrated a greater ability of the fungus to colonize compared with those animals treated with only the yeast. However, the animals suffered no illeffects from exposure to S. cerevisiae (Holzschu et al., 1979). Therefore, this study suggests that even with the addition of high levels of an immunosuppressant agent, S. cerevisiae appears to be nonpathogenic.

4. Ability to Transfer Virulence Factor Genes

S. cerevisiae does not carry virulence factors to humans or animals. However, the species does carry linear, doublestranded plasmids which can be transmitted to other Saccharomyces. These plasmids carry genes that encode the "killer toxins" discussed above can be transferred from one Saccharomyces to another. Therefore, gene constructs involving the incorporation of traits using these linear plasmids should be considered to be nonstable.

5. Summary

In conclusion, S. cerevisiae is a organism which has an extensive history of safe use. Despite

considerable use of the organism in research and the presence of S. cerevisiae in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the human had a debilitating condition. Factors associated with the virulence of yeasts (i.e., phospholipases) indicate that this organism is nonpathogenic. The organism has not been shown to produce toxins to humans.

B. Environmental Hazards

S. cerevisiae is ubiquitous in nature. It has been recovered from a variety of sites under varying ecological conditions. The organism is used in a variety of industrial scenarios. S. cerevisiae is commonly recovered from a variety of fresh fruits and vegetables, generally those fruits with high levels of fermentable sugars. However, it is not listed as the causative agent of food spoilage for fruits and vegetables (Phaff et al., 1966). The only adverse effect to the environment noted in the literature is the presence of the "killer toxins" which is active against other strains of Saccharomyces.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

S. cerevisiae is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986).

No data were available for assessing the release and survival specifically for fermentation facilities using S. cerevisiae. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, i.e., near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use areamonitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

S. cerevisiae is a normal inhabitant of soils and is widespread in nature. S. cerevisiae is able to take up a wide variety of sugars and amino acids. These traits enhance the organism's ability for long term survival. S. cerevisiae can be isolated from fruits and grains and other materials with a high concentration of carbohydrates (LaVeck, 1991).

2. Releases

Estimates of the number of S. cerevisiae organisms released during production are tabulated in Table 1 (Reilly, 1991). The uncontrolled/untreated scenario assumes no control features for the fermentor offgases, and no inactivation of the fermentation broth for the liquid and solid waste releases. The containment criteria required for the full exemption scenario assume the use of features or equipment that minimize the number of viable cells in the fermentor off-gases. They also assume inactivation procedures resulting in a validated 6log reduction of the number of viable microorganisms in the liquid and solid wastes relative to the maximum cell density of the fermentation broth.

TABLE 1. Estimated Number of Viable Saccharomyces cerevisiae

Organisms Released During Production

Uncontrolled/ Full

Release Media Untreated Exemption Release

(cfu/day) (cfu/day) (days/year)

Air Vents $2x10^8$ - 1x1011 $<2x10^8$ - 1x1011 350

Rotary Drum Filter 250 250 350

Surface Water $7x10^{12}$ $7x10^6$ 90

Soil/Landfill $7x10^{14}$ $7x10^8$ 90

Source: Reilly, 1991

These are "worstcase" estimates which assume that the maximum cell density in the fermentation broth for fungi is 10^7 cfu/ml, with a fermentor size of 70,000 liters, and the separation efficiency for the rotary drum filter is 99 percent.

3. Air

Specific data which indicate the survivability of S. cerevisiae in the atmosphere after release are currently unavailable. Survival of vegetative cells during aerosolization is typically limited due to stresses such as shear forces, desiccation, temperature, and UV light exposure. As with naturally-occurring strains, human exposure may occur via inhalation as the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Air releases from fermentor offgas could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from this source, the sector averaging form of the Gaussian algorithm described in Turner (1970) was used. For purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by controls/equipment for offgases, potential human inhalation dose rates are estimated to range from 3.0×10^3 to 1.5×10^6 cfu/year for the uncontrolled/untreated scenario and less than that for systems with full exemptions. It should be noted that these estimates represent hypothetical exposures under reasonable worst case conditions (Versar, 1992).

4. Water

The concentrations of S. cerevisiae in surface water were estimated using stream flow values for water bodies receivingprocess wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release data (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/l). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers facilities that have a NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10year period. The use of this methodology to estimate concentrations of S. cerevisiae in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process. Estimated concentrations of S. cerevisiae in surface water for the uncontrolled/untreated and the full exemption scenarios are tabulated in Table 2 (Versar, 1992).

TABLE 2. S. cerevisiae Concentrations in Surface Water

Receiving Flow (MLD*)	Stream Flow (cfu/l)	Organisms (cfu/l)
Mean 7Q10	Mean 7Q10	

Uncontrolled/Untreated

10th Percentile 156 5.60 $4.5x10^{4}$ $1.25x10^{6}$

50th Percentile 768 68.13 $9.11x10^{3}$ $1.03x10^{5}$

Full Exemption

10th Percentile 156 5.60 $4.5x10^{-2}$ $1.25x100^{0}$

50th Percentile 768 68.13 $9.11x10^{-3}$ $1.03x10^{-1}$

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*MLD = million liters per day

Source: Versar, 1992

5. Soil

Since soil is a natural habitat for S. cerevisiae, it would be expected to survive well in soil. These releases could result in human and environmental exposure (Versar, 1992). It iscurrently estimated that over one million tons of naturally-occurring yeast are produced annually during brewing and distilling practices (LaVeck, 1991).

6. Summary

Although direct monitoring data are unavailable, worst case estimates do not suggest high levels of exposure of S. cerevisiae to either workers or the public resulting from normal fermentation operations.

V. INTEGRATED RISK ASSESSMENT

A. Discussion

There is an extensive history of use of and exposure to S. cerevisiae with a very limited record of adverse effects to the environment or human health. Yeast has been used for centuries as a leavening for bread and fermenter of beer without records of virulence. S. cerevisiae is currently classified as a class 1 containment organism under the NIH Guidelines based largely on the extensive history of safe use.

Factors associated with the development of disease states in fungi have been reviewed. Data suggests that only with the ingestion of high levels of S. cerevisiae or with the use of immunosuppressants can S. cerevisiae colonize in the body. Even under those conditions, there were no noted adverse effects. In the few cases which S. cerevisiae was found in association with a disease state, the host was a debilitated individual, generally with an impaired immune system. In other cases the organism was recovered from an immunologically privileged site (i.e., respiratory tract). Many scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen. The cases noted in the above Human Health Assessment, where S. cerevisiae was found in association with a

disease state, appear to be classic examples of opportunistic pathogenicity (see III.A.3).

The organism is not a plant or animal pathogen. Despite the fact that S. cerevisiae is ubiquitous in nature, it has not been found to be associated with disease conditions in plants or animals. The only adverse environmental condition that was noted is the production of "killer toxins" by some strains of the yeast. These toxins have a target range that is limited to susceptible yeasts. The toxins, proteins and glycoproteins, are not expected to have a broad environmental effect based largely on the anticipated short persistence of the toxins in soil orwater and by the limited target range. S. cerevisiae "killer toxin" has been used industrially to provide a level of protection against contamination by other yeasts in the fermentation beer.

The current taxonomy of Saccharomyces is under revision based on the development of alternative criteria. However, this should not have a major effect on the risk associated with closely related species. Saccharomyces, as a genus, present low risk to human health or the environment. Criteria used to differentiate between species are based on their ability to utilize specific carbohydrates without relevance to pathogenicity. Nonetheless, this risk assessment applies to those organisms that fall under the classical definition of S. cerevisiae as described by van der Walt (1971).

S. cerevisiae is a ubiquitous organism which, despite its broad exposure, has very limited reported incidence of adverse effects. The extensive history of use, the diversity of products currently produced by the organism, and the attention given this organism as a model for genetic studies collectively makes this organism a prime candidate for full exemption. The increased knowledge derived from the ongoing research should further enhance this organisms' biotechnological uses.

B. Recommendation

Saccharomyces cerevisiae is recommended for the tiered exemption.

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