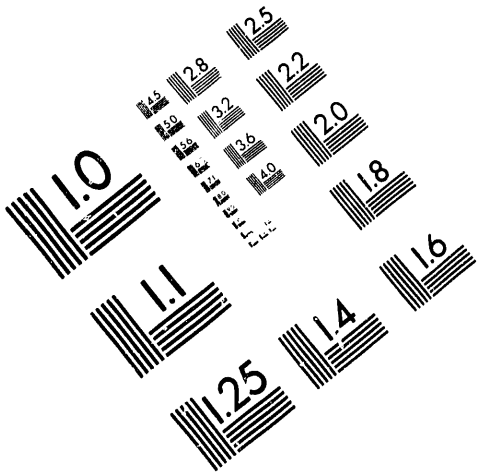

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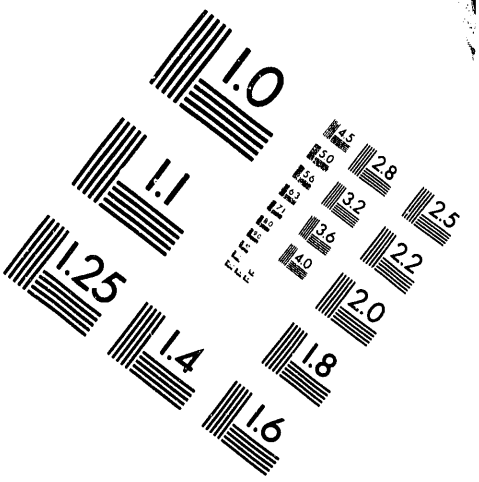

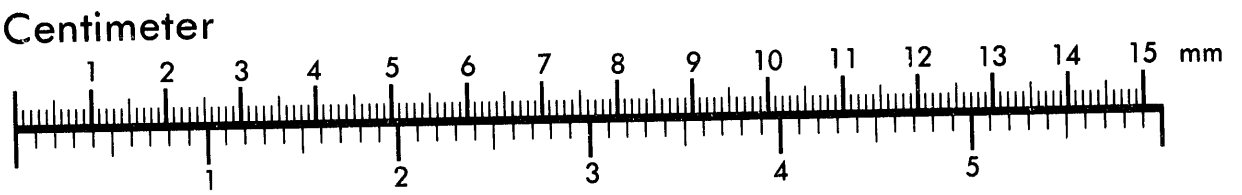

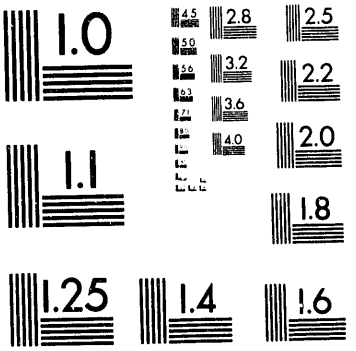

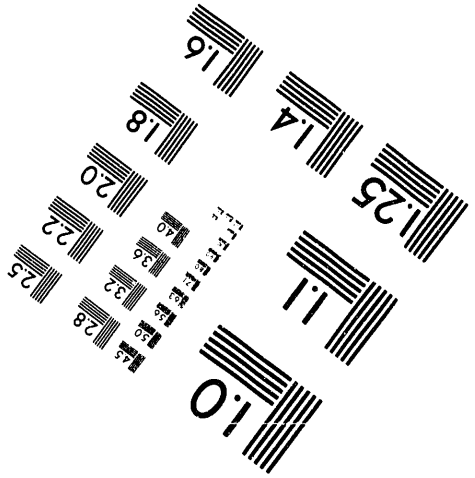

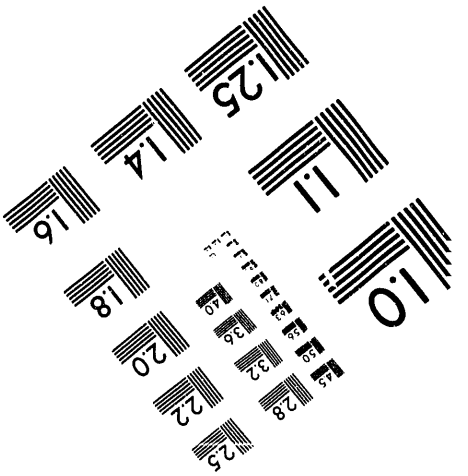

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Phanerochaete Mutants with Enhanced Ligninolytic Activity

by

Satya N. Kakar,*
Aleida Perez, and Jose Gonzales
Argonne National Laboratory
Argonne, Illinois 60439

DISCLAIMER

Key words: Ligninolytic Mutants, Lignin Peroxidases, White Rot Fungus, *Phanerochaete chrysosporium*, Bioremediation

*Address of the corresponding author:
Environmental Research Division
Building 202, Room BE-107
Argonne National Laboratory
9700 South Cass Avenue, Argonne, IL 60439-4833

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Summary

In addition to lignin, the white rot fungus *Phanerochaete chrysosporium* has the ability to degrade a wide spectrum of recalcitrant organopollutants in soils and aqueous media. Although some of the organic compounds are degraded under nonligninolytic conditions, most are degraded under ligninolytic conditions with the involvement of the extracellular enzymes, lignin peroxidases, and manganese-dependent peroxidases, which are produced as secondary metabolites triggered by conditions of nutrient starvation (e.g., nitrogen limitation). The fungus and its enzymes can thus provide alternative technologies for bioremediation, biopulping, biobleaching, and other industrial applications. The efficiency and effectiveness of the fungus can be enhanced by increasing production and secretion of the important enzymes in large quantities and as primary metabolites under enriched conditions. One way this can be achieved is through isolation of mutants that are deregulated or are hyperproducers or supersecretors of key enzymes under enriched conditions. Through ultraviolet-light and gamma-rays mutagenesis we have isolated a variety of mutants, some of which produce key enzymes of the ligninolytic system under high-nitrogen growth conditions. One of the mutants, 76UV, produced 272 units (U) of lignin peroxidases enzyme activity per liter (L) after nine days under high nitrogen (although the parent strain does not produce this enzyme under these conditions). The mutant and the parent strains produced up to 54 U/L and 62 U/L, respectively, of the enzyme activity under low-nitrogen growth conditions during this period. In some experiments the mutant showed 281 U/L of enzyme activity under high nitrogen after 17 days.

Introduction

Only a few groups of microorganisms can degrade a complex, heterogeneous, branched aromatic polymer like lignin, which is composed of diverse organic structural units linked randomly by a variety of different chemical bonds (1). Of these organisms, the white rot fungus *Phanerochaete chrysosporium* has been used extensively to study lignin biodegradation (ligninolysis) because it degrades lignin efficiently and completely to carbon dioxide and water. Since the random lignin structure requires nonspecific lignin degradation, other compounds with structural similarities to the aromatic moieties of lignin (e.g., many xenobiotic compounds such as pentachlorophenol, dioxin, benzo[α]pyrene, certain polymeric dyes, trinitrotoluene, and pesticides) are also degraded by the fungus (2-5). Active degradation of lignin and hazardous organic compounds by *P. chrysosporium* has been shown to take place in nutrient-limited cultures and to be dependent on the ligninolytic enzyme system (6,7). The key enzymes of the ligninolytic system that seem to be involved are the extracellularly secreted peroxidases, lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs), which have been shown to catalyze the initial oxidation in the overall degradation process (7). Purified preparations of both LiPs and MnPs have also been shown to oxidize a variety of xenobiotic organic compounds. Purified LiPs have been shown to oxidize polycyclic aromatic hydrocarbons (PAHs) to quinones, to oxidize chlorinated phenols to benzoquinone, and to cleave dioxin molecules, while MnPs have been shown to oxidize PAH compounds to quinones or acetoxylated PAHs and dichlorophenol to chloro-p-benzoquinone (6-9).

Because of their unique ability to nonspecifically degrade hazardous compounds, *P. chrysosporium* and its peroxidases have strong potential for bioremediation and other industrial applications, either as a single detoxification step or as part of an integrated treatment process. Several promising scenarios for application of the fungus or direct use of its secreted enzymes have been suggested. However, efforts to exploit the full potential of the fungus or an enzyme-based system in bioremediation and other applications have been limited because of the slow growth of the fungus, the specific physiologic conditions required, the low levels of ligninolytic enzyme production, and the synthesis of the ligninolytic enzymes as secondary metabolites triggered by nutrient starvation. Thus, improved methods for large-scale production are essential for progress in applications and for eventual implementation of technologies using the fungus or its enzymes.

We describe here a genetic approach for overcoming these problems. We have isolated and partially characterized mutants that overproduce and secrete the ligninolytic enzymes, particularly LiPs, under nitrogen-rich conditions. In fact, one of the mutants requires high levels of nitrogen to regulate the increased and sustained activity of LiPs characterized in this study. Our strains can be further developed for potential industrial applications.

Methods

Fungus: The present experiments were carried out by using the *Phanerochaete chrysosporium* strain BKM-F-1767, obtained from the U.S. Department of Agriculture Forest

Products Laboratory, Madison, Wisconsin. The fungus was maintained on malt extract agar slants (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar, pH 4.5) or was stored as a stock conidial suspension in 15% glycerol at -70°C .

Conidiation: For production of conidia, the fungus was inoculated on malt extract agar plates. Conidia were profusely produced after four days of growth at 37°C. Conidia harvested from plates by washing with sterile distilled water were filtered through sterile glass wool to remove pieces of mycelia. The concentration of conidia was determined by cell count in a hemocytometer. Mutants were isolated by using ultraviolet (UV) light or gamma (γ) ray irradiation.

Isolation of Mutants: For UV light treatment, ten milliliters (mL) of conidial suspension (10^6/mL) was treated with UV light from a germicidal lamp in a petri dish. The suspension was gently agitated during treatment. On the basis of a mortality curve, the treatment time was adjusted to give 0.2% survival. For γ-ray irradiation, a conidial suspension (10^6/mL) was treated with a ^{60}Co γ-ray source at Argonne at a total dose of 35,000 rads. This treatment gave approximately 2% survival. Both treatments were carried out at room temperature.

After irradiation, the treated conidial suspensions were appropriately diluted and plated on colony-forming medium containing the indicator dye Poly R-478 [composed of a poly(vinylamine)sulfonate backbone with anthrapyridone chromatophore] or Remazol Brilliant

Blue R. (Both dyes were purchased from Sigma Chemical Company.) The colony-forming synthetic medium contained 2% agar and either low (2.4 mM, LN) or high (24 mM, HN) nitrogen as described by Tien and Kirk (10). This medium was supplemented with 4% sorbose (in place of glucose), 0.01% deoxycholate, and 0.02% of the dye solution. The plates were incubated at 37°C. Sorbose and deoxycholate in the medium restrict fungus growth to form colonies (11). Since dyes are polymeric, their initial degradation depends on extracellular enzyme activity, and the efficiency of decolorization seems to be correlated with the ability to degrade several lignin model compounds. Thus, the dyes provide a useful screening method for ligninolytic activity (12). The wild-type parent strain decolorizes the dyes under LN and not under HN conditions. Deregulated mutants are expected to decolorize the dyes on HN plates, and mutants that hyperproduce or supersecrete the ligninolytic enzymes are expected to produce larger zones of clearing on LN plates. Colonies that do not decolorize the dyes on LN plates are expected to be mutants deficient for or lacking ligninolytic activity (Fig.1). For our purpose, colonies or segments of colonies that decolorized the dyes within 3-4 days on HN dye plates were selected. These putative mutant colonies were repeatedly subcultured by using mycelial transfers and were screened on the selection medium through at least six transfers. Colonies that consistently decolorized the dyes on HN medium were finally transferred to malt extract plates for conidiation. Final mutant selection was made by plating conidia on HN dye plates. Single-colony isolates were then picked. Glycerol stocks of conidia from single-colony mutant isolates were stored at -70°C.

Mutant Characterization: The mutant and the parent strain were grown at 37°C in 25 mL of defined medium in 250-mL Erlenmeyer flasks as stationary cultures containing either low (2.4 mM) or high (24 mM) nitrogen by using conidial inoculation and periodic oxygenation as described by Tien and Kirk (10). The experiments were run in triplicate each time and repeated 3-4 times. Extracellular samples (1 mL) of the growth fluid carefully withdrawn periodically before oxygenation (without disturbing the fungus mat) were analyzed for lignin peroxidase activity. The lignin peroxidase activity in each culture fluid aliquot was determined in a 1-mL assay reaction mixture by measuring the rate of oxidation of veratryl alcohol to veratryl aldehyde. The activity was expressed as units per liter (U/L) as described by Tien and Kirk (10).

Results

After UV and γ-ray irradiation, more than 28,000 surviving colonies were screened for their ability to decolorize polymeric dyes. Of the 186 putative mutants initially selected, only 12 were eventually found to consistently decolorize the dyes after serial subculturing for six or more transfers, 6 from Poly R-containing plates (3 from UV and 3 from γ-ray irradiation), and 6 from Remazol Blue-containing plates (3 each from UV and γ-ray irradiation) (Table 1). Only one of the mutants, 76UV, obtained after UV irradiation and selected from a Poly R plate, was partially characterized further. Among the surviving colonies, the rate of mutant generation was 0.02% after UV irradiation and 0.1% after γ-ray irradiation. These values are

a lower estimate because many mutants could have been missed during screening due to the heterokaryotic, multinucleate nature of the conidia and subsequently the mycelium.

The growth patterns of the parent strain and the mutant 76UV stationary cultures under LN and HN are compared in Table 2. Both the wild-type and the parent strain apparently achieved maximum growth within three days. The parent strain grew slightly better under both nitrogen conditions.

The ability of the mutants selected from HN Poly R dye plates to decolorize Remazol Blue and vice versa is compared in Table 3. Although most of the mutants selected on Poly R plates also decolorized Remazol Blue under HN conditions, one of the mutants did not. The same was true for one of the mutants selected for decolorizing Remazol Blue; it did not decolorize Poly R under HN conditions. The parent strain did not decolorize Poly R or Remazol Blue under HN conditions. Interestingly, the mutants selected on Poly R decolorized Remazol Blue within three days, while the mutants selected on Remazol Blue took nearly six days to decolorize Poly R (data not shown).

The lignin peroxidase activities of the mutant and parent strains over a period of 17 days under LN and HN conditions are presented in Table 4. Both batch-to-batch variations and variations among replicates occurred. Table 4 shows the range of activities noticed for the mutant strain and compares the best results obtained for the mutant and parent strains. Under LN conditions, both the parent and mutant strains produced low levels of lignin peroxidase

activity on day 6. The activity peaked on day 9. The parent strain does not produce lignin peroxidase activity under HN conditions. The mutant, on the other hand, had four to five times more lignin peroxidase activity on day 9 under HN conditions, and this high level of activity was maintained through day 17 (281 U/L). In this experiment, 183 U/L of enzyme activity was noticed after 21 days (data not shown). The earliest activity seen in the mutant under HN conditions (40 U/L) occurred on day 3 (results not shown). The mutant thus not only can produce more of the enzyme under enriched conditions but also can sustain a high level of enzyme activity for a much longer time than the parent strain under LN growth conditions.

Discussion

The white rot fungus *Phanerochaete chrysosporium* can rapidly and completely degrade lignin to carbon dioxide and water (1). In addition, the fungus has been shown to be a nonspecific degrader of many structurally diverse, recalcitrant, hazardous xenobiotic organic compounds (2-5). Active degradation of lignin and most of the hazardous organics by *P. chrysosporium* occurs under nutrient-limiting conditions and depends on the ligninolytic enzyme system, which comprises the LiPs and MnPs as well as hydrogen-peroxide-producing enzymes (6,7). The key enzymes of the ligninolytic system, LiPs and MnPs, have been shown to catalyze the initial oxidation of the overall degradation process (7). The fungus and its peroxidases thus have great potential in bioremediation, pulp and paper, and other industries. However, the industrial exploitation of the fungus and its key ligninolytic enzymes is hampered by both the

slow growth of the fungus and the low yields of peroxidase enzymes produced only as secondary metabolites.

Until recently most of the effort aimed at industrial production has involved manipulating the physiological requirements or developing different bioreactor systems. Classical genetic manipulation techniques have always been useful for developing a comprehensive bank of mutants. Mutants of *Phanerochaete* with altered ligninolytic capabilities can be isolated by using various screening tests (13-15). Our results indicate that the genetic approach offers a viable alternative for producing fungal strains that overproduce and secrete the ligninolytic enzymes under nutrient-rich conditions. Enzyme production can start early (within 3 days), and the activity can be sustained for a longer (more than 17 days) period of growth. This is evident for LiPs in our studies. We are testing for activities of other ligninolytic enzymes. The mutants are thus likely to be superior to the wild type for enzyme production and for degradation of pollutants. They may be able to handle higher concentrations of pollutants and at higher rates.

More recently it has been demonstrated that ligninolytic enzymes are not always necessary for mineralization of organopollutants. Degradation of the BTEX (benzene, toluene, ethylbenzene, and xylenes) group of compounds, individually or as a composite mixture, is favored under nonligninolytic culture conditions, in which extracellular peroxidases are not produced (16). The fungus thus has the added versatility to degrade a wide spectrum of

recalcitrant organopollutants both under nonligninolytic and ligninolytic (using extracellularly produced enzymes) culture conditions.

Analysis of mutants is also useful for understanding regulatory processes. Limited reports of isolation of mutants that produce ligninolytic enzymes under nitrogen-rich conditions have appeared. Orth et al.(14) have described a mutant that produces higher lignin peroxidase activity both under nitrogen-rich and nitrogen-limited conditions. Our mutant is different in that it produces higher amounts of lignin peroxidase activity than the wild-type parent only under high-nitrogen conditions and has the same amount of activity as the parent strain under nitrogen-limiting conditions. Our mutant is also different from those described by Boominathon et al.(15), which had higher lignin peroxidase activity than the wild type under low-nitrogen conditions. Under high-nitrogen conditions, their mutants produced low levels of lignin peroxidase activity, much less than that produced by the wild type under low-nitrogen conditions. In the mutant described in this study, enhanced and sustained lignin peroxidase production seems to be regulated by the presence of high nitrogen in the medium. [We used 24 mM nitrogen compared to the 11 mM used by Orth et al. (14)]. All the results for the mutant indicate that the production of ligninolytic enzymes may have multiple regulatory mechanisms. We are using our mutants to further study regulation in *P. chrysosporium*. The ability of some of the mutants to decolorize only one or the other dye and of other mutants to decolorize both dyes is also suggestive of differential regulation mechanisms. Further analysis of these mutants is likely to elucidate the regulation of ligninolytic enzyme production during secondary metabolism.

Phanerochaete chrysosporium and perhaps other white rot fungi thus are a promising group of microorganisms for industrial applications including the bioremediation of recalcitrant xenobiotic compounds. The results indicate a potential for both direct use of the fungus and use of the purified enzymes. The usefulness of the fungus can be enhanced by isolating mutants that are deregulated or are hyperproducers and supersecretors of key enzymes as primary metabolites and under nonrestrictive growth conditions. Our studies indicate that a variety of such mutants can be obtained. Cloning and heterologous expression of key enzymes and understanding and manipulating their regulation at the molecular level are other key approaches for improving the potential capabilities of this fungus.

The fact that the key enzymes are produced and secreted extracellularly gives the fungus and its enzymes added advantages. Secretion simplifies enzyme purification, allows the target compound to be easily accessed because it need not be soluble to enter the cell, and allows very low levels of pollutants to be treated. Recent demonstrations that the fungus can degrade environmentally persistent organopollutants in soils by using as nutrient supplements several inexpensive plant residues including ground corn cobs, wood chips, peat, and wheat straw make this organism even more promising (3,17). Lignin-degrading fungi can accelerate degradation of organopollutants found in soils and covalently bound to lignin or lignin-derived materials (18).

Acknowledgements

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Table 1

ISOLATION OF LIGNIN PEROXIDASE DEREGULATED MUTANTS

	UV Light		^{60}Co Gamma Rays	
	Poly R (HN)[a]	Remazol Blue (HN)	Poly R (HN)	Remazol Blue (HN)
Survival Level (%)	0.2	0.2	2	2
No. Colonies Screened	20335	2350	1656	3799
No. Mutants Purified	3	3	3	3
Mutant Rate (%)	0.015	0.128	0.181	0.074

[a]HN = high-nitrogen medium (24 mM nitrogen).

Table 2

COMPARATIVE GROWTH OF MUTANT AND PARENT STRAINS[a]

		DAY			
		3	6	9	13
Parent	LN	+++	+++	+++	+++
	HN	++++	++++	++++	++++
Mutant 76UV	LN	++	++	++	++
	HN	+++	+++	+++	+++

[a]Symbols: LN = low-nitrogen medium (2.4 mM nitrogen)

HN = high-nitrogen medium (24 mM nitrogen)

++ = good growth

++++ = excellent growth

Table 3

DECOLORIZATION OF POLY R AND REMAZOL BLUE BY MUTANTS[a]

	LN	HN
Poly R Mutants on Remazol Blue		
UV Mutants		
56UV	+	+
76UV	++	++
γ-Ray Mutants		
17γ	-	-
104γ	+	+
183γ	++	++
Parent Strain	+	-
Remazol Blue Mutants on Poly R		
UV Mutants		
6UV	+	-
14UV	+	+
17UV	++	++
γ-Ray Mutants		
2γ	++	++
6γ	++	++
Parent Strain	+	-

[a]Symbols: + = intensity of decolorization
- = no decolorization

LN = low-nitrogen conditions (2.4 mM nitrogen)
HN = high-nitrogen conditions (24 mM nitrogen)

Table 4

LIGNIN PEROXIDASE ACTIVITY OF MUTANT AND PARENT STRAINS (UNITS PER LITER)[a]

		DAY			
		6	9	13	17
Parent	LN	23	62	46	16
	HN	0.4	0.1	0	0.8
Mutant 76UV	LN	37	54	24	17
	HN	8	272	253	281
(Range)			(91-272)	(38-253)	(18-281)

[a]Symbols: LN = low-nitrogen medium (2.4 mM nitrogen)
HN = high-nitrogen medium (24 mM nitrogen)

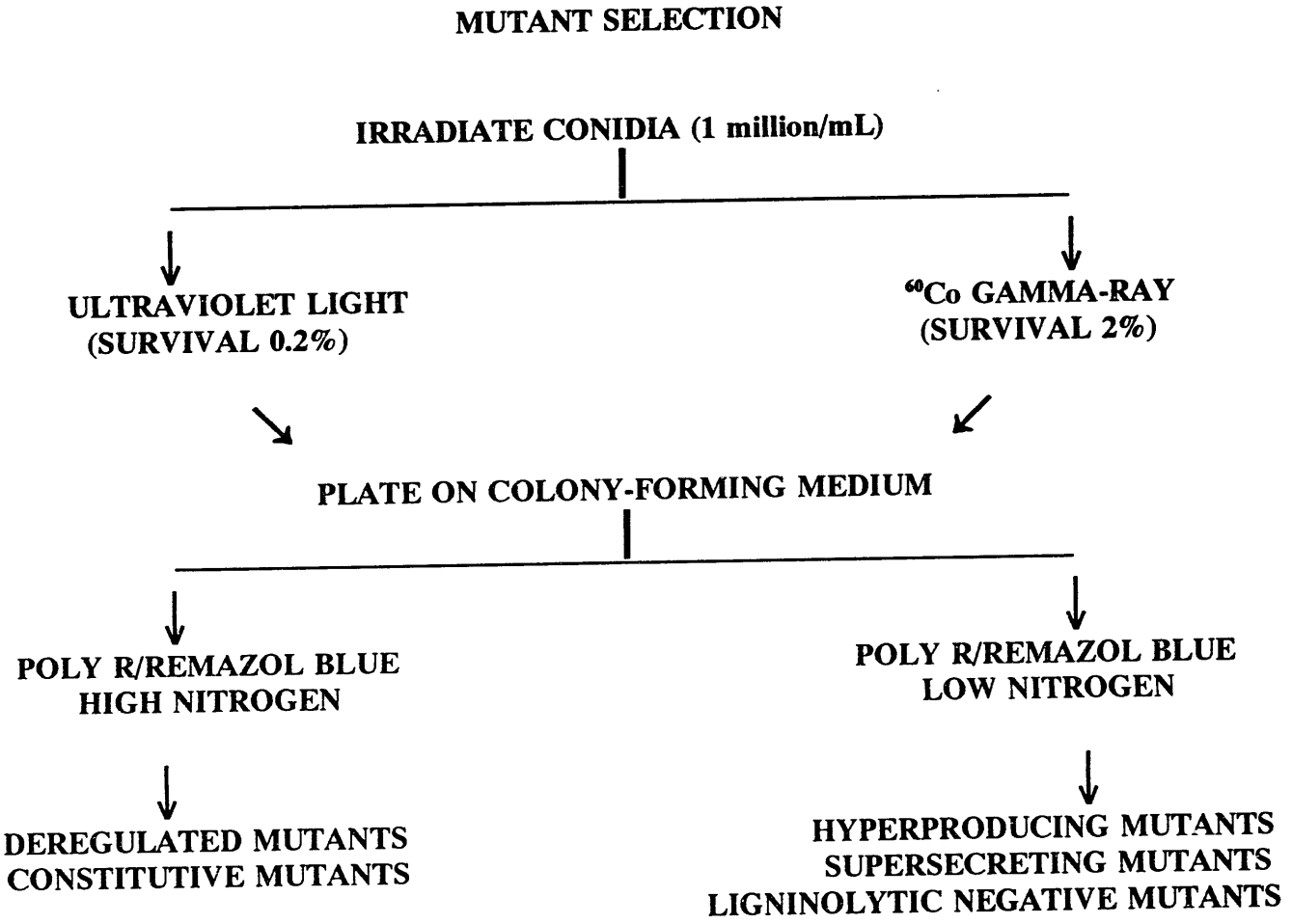
MUTANT SELECTION
IRRADIATE CONIDIA (1 million/mL)
ULTRAVIOLET LIGHT
(SURVIVAL 0.2%)
^{60}Co GAMMA-RAY
(SURVIVAL 2%)
PLATE ON COLONY-FORMING MEDIUM
POLY R/REMAZOL BLUE
HIGH NITROGEN
POLY R/REMAZOL BLUE
LOW NITROGEN
DEREGULATED MUTANTS
CONSTITUTIVE MUTANTS
HYPERPRODUCING MUTANTS
SUPERSECRETING MUTANTS
LIGNINOLYTIC NEGATIVE MUTANTS

Fig. 1

Figure Legend

Figure 1. Scheme for isolating various mutants of ligninolytic system.

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