UNC-WRRI-83-197

ENZYMATIC PRETREATMENT OF PULP MILL EFFLUENTS PRIOR TO DECOLORIZATION BY LIME PRECIPITATION

by

J. C. Campbell and T. W. Joyce

Department of Wood and Paper Science
North Carolina State University
Raleigh, North Carolina 27650

The work upon which this publication is based was supported in part by funds provided by the U. S. Department of the Interior, Washington, D. C., through the Water Resources Research Institute of The University of North Carolina as authorized by the Water Research and Development Act of 1978.

Project No. A-123-NC

Agreement No. 14-34-0001-2135

May, 1983

ACKNOWLEDGMENTS

We appreciate the kind gift of the culture of *Coriolus versicolor* from Dr. T. Kent Kirk of the U.S. Forest Products Laboratory, Madison, Wisconsin.

Also, we express appreciation to Drs. Elisabeth Wheeler and Chen-Loung Chen for review of this manuscript.

DISCLAIMER STATEMENT

ABSTRACT

The phenol oxidase laccase was examined for its potential use in a process for removing color from pulp and paper mill wastewaters. The precipitation of color from pulp mill effluents by lime is known to be a function of the molecular weight of the color molecules. It has been postulated that any treatment capable of increasing the proportion of high molecular weight color molecules might be able to increase the efficiency of the lime precipitation process. Because laccase can initiate the free radical condensation of phenolic compounds, and color has some phenolic components, this enzyme was used to pretreat various effluents prior to the addition of lime for color removal.

Laccase was produced in laboratory cultures of the white-rot fungus *Coriolus versicolor*. The concentrated culture medium was used for pretreatment of three types of effluent: biologically-treated, whole-mill effluent (from pulping, bleaching, and papermaking), chlorination stage effluent, and caustic extraction stage effluent. The latter two wastewaters were from bleaching of softwood kraft pulp. The 8-hour pretreatments were conducted over a range of pH levels to determine if there was a pH optimum for any potential enzymatic reactions with color bodies.

Results from pretreatments of biologically-treated effluent (BTE) showed only a limited improvement in color removal by lime. For example, 500 mg/L lime removed 82% from pretreated BTE and 78% from BTE without laccase at a pretreatment pH of 5. BTE was not consistent in its response to laccase pretreatment with mill samples obtained at a later date; a pretreatment pH of

7.5 was required for even slight improvements (25% versus 15% from controls using a low, more sensitive lime dose of 250 mg/L). In both cases, however, pretreatments with laccase that had been inactivated were also able to increase the color removal by lime (e.g., 20% in the previous example).

Attempts were made to improve the pretreatment by spiking BTE with o-cresol (2-methylphenol) during pretreatment with laccase. This compound is a substrate for enzymatic reactions using laccase. The o-cresol was able to increase color removal by 250 mg/L lime from about 20% with laccase alone to 63% with cresol and laccase. However, at the pretreatment pH required for cresol reaction (pH 5), color removal from the control (43%) was greater than from laccase-pretreated samples; therefore, the increase using cresol was not considered to be great enough for commercial significance.

Laccase increased color removal from chlorination stage effluent by a maximum of 69%, compared to 57% for the control, at a lime dosage of 1,000 mg/L and a pretreatment pH of 6. The proportion of this increase that might be possible using inactived laccase was not determined. Pretreatment of E-stage effluent with laccase or laccase and cresol did not increase color removal by lime.

None of the increases in color removal was considered to be of the magnitude required for industrial importance. It was concluded that a possible reason that laccase caused only limited improvements in color removal efficiency could be an insufficient phenolic hydroxyl content in the various effluents tested. Enzymatic reactions would, therefore, not be able to substantially increase the molecular weight of color molecules.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY AND CONCLUSIONS

Pretreatment of three types of pulp mill effluents with the enzyme laccase prior to lime addition gave rather limited improvements in color removal efficiency. None of the increases was of the magnitude required for industrial importance.

In the work with biologically-treated, whole-mill effluent (BTE), color removals were only a few percentage points greater than that achieved by lime alone (e.g., 82% versus 78%). Much of this increase could be accomplished by pretreatment with laccase that had been inactivated by boiling, implying that the slight enhancement in color removal observed with active laccase may be largely attributable to non-enzymatic mechanisms. Attempts to improve the pretreatment by application of either greater levels of enzyme, longer pretreatment time, or pretreatment at several pH values were not successful.

The variable nature of effluent from pulp mill operations became apparent in the enzyme pretreatment work with BTE. Mill samples obtained during the initial stages of the study were slightly more responsive to laccase pretreatment than those obtained later. These later samples were best pretreated at pH 7.5, rather than pH 5 as used earlier. These findings emphasize the need for evaluation of any effluent treatment method over a sufficient time period to encounter any effluent variations.

Addition of the phenolic compound o-cresol to BTE during laccase pretreatment was able to increase color removal by 250 mg/L lime from about 20% with laccase alone to 63% with cresol and laccase. However, because color

removal from the control was about 43%, the increase using cresol was not considered great enough for commercial importance.

Pretreatments of caustic extraction stage effluent with laccase or laccase and cresol did not increase color removal by lime. Laccase increased color removal from chlorination stage effluent by a maximum of 69% compared to 57% from the control at a lime dose of 1,000 mg/L and a pretreatment pH of 6. The proportion of this increase that might be possible using inactived laccase was not determined.

Assays of laccase activity in the effluents, at pH 5 using syringaldazine as the substrate, indicated that enzyme activity could be sustained in these wastewaters, given the proper substrate. A possible reason that laccase caused only limited improvements in color removal efficiency could be an insufficient phenolic hydroxyl content in the effluents. The phenolic content of bleachery effluents is known to be especially low (Bennett, et al., 1971). The "very weakly acidic" groups measured by conductometric titration of these effluents can be attributed to enolic hydroxyls as well as phenolic hydroxyls. Enzymatic reactions would, therefore, not be able to substantially increase the molecular weight of color bodies.

1. INTRODUCTION

1.1 General Background

Color has been designated as a "nonconventional" water pollutant, one of three categories established by the Clean Water Act of 1977. Color, along with other nonconvention pollutants such as ammonia, chemical oxygen demand (COD), and certain industry - specific substances, is so classified since it is not included in either the conventional or toxic pollutant categories. Government regulations can limit discharge of any of these nonconventional pollutants where warranted.

As a water pollutant, color is of concern to the pulp and paper industry, the nation's third largest industrial consumer of water (The Kline Guide to the Paper Industry, 1980). An estimated two trillion gallons of wastewater are discharged annually by the pulp and paper industry, much of which is highly colored. This color is mainly attributable to lignin degradation products formed during various pulping and bleaching operations and has some similarity to natural organic, aquatic color (Christman and Ghassemi, 1966). Although no uniform national standard exists for limiting color discharge, each mill is considered on a case-by-case basis for regulation. There is presently no economically practical color removal process.

There are several potential effects of color on receiving waters, but none are completely understood (Rush and Shannon, 1976). Perhaps the most obvious concern, particularly from the public's viewpoint, is that of aesthetics. Colored waters are associated with polluted and possibly harmful conditions. The general expectation is that properly treated wastewaters should have a clean appearance as well as having been chemically purified.

The removal of color from pulp and paper mill effluents is desirable from two particular standpoints related to water use. Discharge of highly colored effluents can downgrade water resources for use by downstream municipalities and industries. Secondly, color removal from process waters increases the possibility for recycle within the mill itself, thereby encouraging conservation of limited water supplies. These motivations are especially applicable in North Carolina, where in many cases pulp mills are located along waterways of low volume or high natural color. Approximately 60 billion gallons of water per year are discharged by the paper industry in North Carolina. Imposition of color restrictions by state and local governments nationwide and growing public concern have given impetus to industrial development of cost effective color removal technologies.

One approach to color removal which has received considerable attention is the lime precipitation process. Mill-scale evaluations have shown that up to 90% of the color in bleachery or whole mill effluents can be removed by applications of lime. The process is a chemical one in which insoluble calcium salts are formed with the molecules responsible for color. Color bodies that fail to precipitate are mainly those of low molecular weight (Bennett, et al, 1971). If the efficiency of precipitation could be extended, the economics of the lime process might become more attractive.

It has been postulated that any treatment that can increase the proportion of high molecular weight color bodies should markedly improve the performance of the lime precipitation process. One study (Schmidt, 1979) attempted to increase the molecular weight by enzymatic polymerization of color molecules. This approach was inspired by the resemblance of certain

color body structures to that of lignin precursor molecules which condense in the biosynthesis of lignin. This polymerization occurs via enzyme - catalyzed free radical formation. Two enzymes known to mediate such condensations in nature, as well as in vitro, are peroxidase and laccase. In experiments using a commercial source of horseradish peroxidase, it was found that the enzyme was able to increase the average molecular weight of color bodies in pulping and bleaching wastewaters. When lime was then applied to the enzyme - pretreated effluents, an increase in the efficiency of color removal appeared possible. Due to difficulties encountered in the use of hydrogen peroxide as the required electron acceptor, and its expense for a mill-scale operation, the recommendation was made that further investigation should utilize laccase. The catalytic actions of the two enzymes are quite similar, but laccase requires only oxygen as the electron acceptor.

The present study, then, sought to determine if laccase can increase the effectiveness of lime precipitation of color from several pulp and paper mill effluent types, and to evaluate the potential industrial feasibility of such treatments.

1.2 Description of Pulping and Bleaching

A general description of the processes used in the production of pulp can aid in the understanding of the color discharge problem faced by the industry. More detailed discussions are presented elsewhere (Casey, 1980; McDonald, 1969). The three basic operations in paper manufacture are pulping, pulp bleaching, and sheet or paperboard formation. A facility may carry out all three or only part, as, for example, pulping followed by paper production with unbleached fibers.

In the pulping of wood, the chief objective is to liberate cellulose fibers for use in fabricating paper or similar products. In the architecture of woody tissue, fibers of cellulose are bound together by lignin, a complex, highly cross-linked polymer of aromatic subunits (Figure 1). The separation can be achieved either mechanically or chemically, with some techniques utilizing both. In mechanical processes, fibers are simply torn apart by pressing a log or wood chips against rotating metal disks or grindstones. Water carries the sheared fibers away for screening, but, since most of the lignin is retained within the fiber bundles, color problems in these wastewaters are not significant.

Chemical pulping processes, on the other hand, attempt to remove a larger portion of the lignin. This removal allows more fiber-to-fiber bonding in paper and, thus, a stronger final product. More than 80% of the wood pulp production in the U.S. is by some chemical means. The sulfate, or kraft, process is overwhelmingly the most common, producing some 95% of the total (Bureau of the Census, 1979). Chips of wood are "cooked" in large pressurized vessels, or digestors, at high temperatures. In the kraft process, the pulping chemicals are sodium hydroxide and sodium sulfide in a solution termed "white liquor". Cooking for 2 to 4 hours under highly alkaline conditions causes extensive lignin degradation, liberating lignin fragments into solution. The cooked chips are blown from the digestor by the force of the released pressure, therby further separating cellulosic fibers. Washing stages remove most of the pulping liquor, now referred to as "black liquor" because of its dark color due primarily to lignin. This lignin has a phenolic hydroxyl content of about 60% (60 hydroxyls per 100 C-9 aromatic subunits),

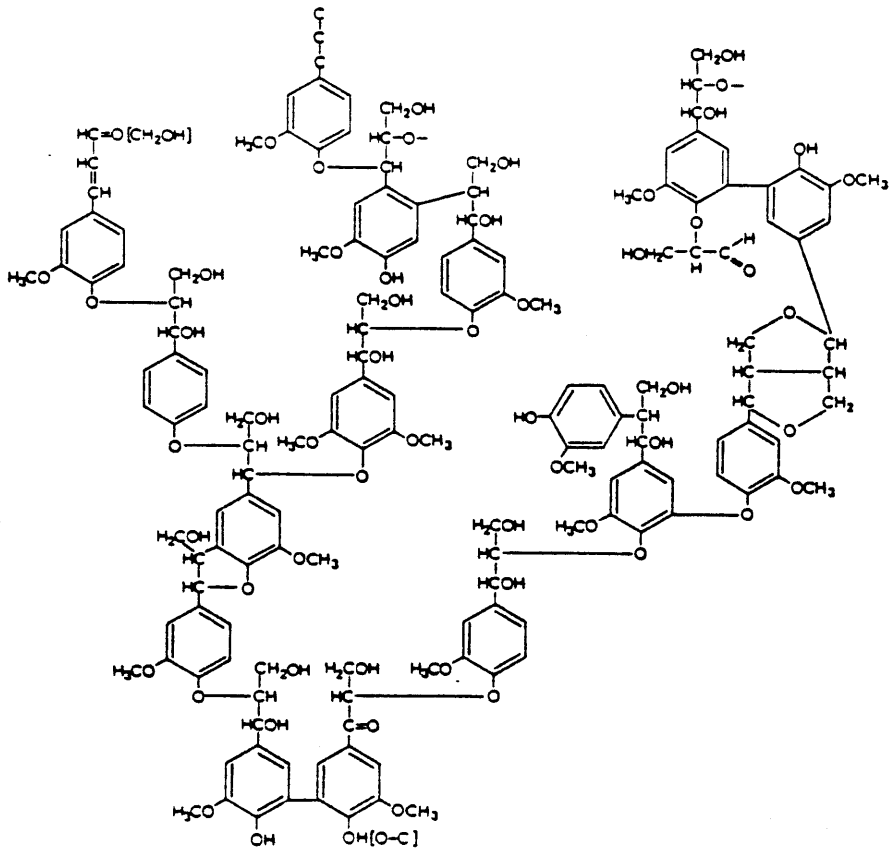


Figure 1. PORTION OF LIGNIN MOLECULE, SHOWING MAJOR LINKAGE TYPES BETWEEN AROMATIC UNITS [after (Sjöström, 1981)].

compared to about 20% in the original polymers (Sjöström, 1981; Ishihara, 1980). After screening, which eliminates undissociated fibers, the pulp contains about 5% lignin. Wastewater sources from pulping can include black liquor spills, condensates from digestor relief and blow gases, and wash and screening waters.

In current practice, it is possible to recover about 90% of the kraft pulping chemicals for reuse (Casey, 1980). The chemical recovery system is, therefore, a major part of the pulping facilities (Figure 2). Inorganic constituents must be reclaimed and converted to their original form. Chemical recovery begins with the evaporation of black liquor from a solids content of 15-20% to a concentration that can support self-combustion (about 50-70%). In the recovery furnace organics are destroyed, leaving an inorganic smelt comprised mainly of sodium carbonate and sodium sulfide. This smelt is next dissolved in water to form the "green liquor". Sodium carbonate is then converted to sodium hydroxide by addition of slaked lime (calcium hydroxide) in the causticizing step. The resulting calcium carbonate precipitate is separated from the liquid, which is now fresh white liquor for use in pulping. The final step in the recovery scheme is the burning of the lime sludge in a kiln for conversion back to calcium oxide.

The sulfite pulping processes should also be mentioned. Although they account for less than 4% of total U.S. pulp production, the waste streams from such operations, especially the neutral sulfite semi-chemical (NSSC), are frequently combined with that of kraft at certain facilities. The major lignin reaction during pulping is sulfonation, yielding more readily dissolvable fragments upon partial hydrolysis. Generally, only about half of

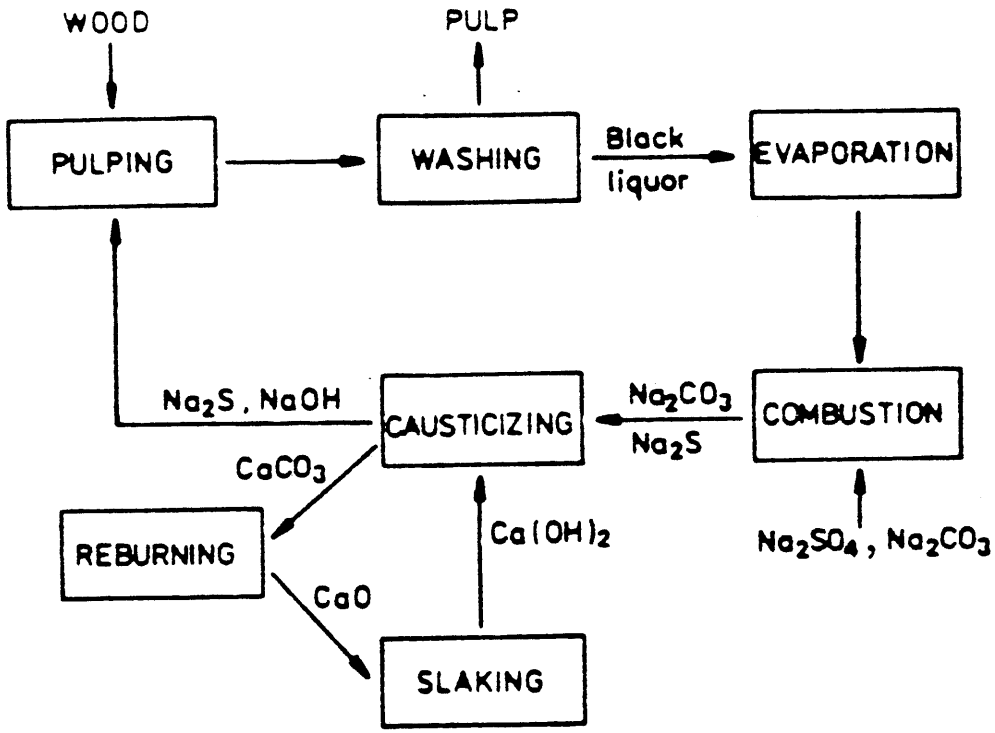


Figure 2. CHEMICAL RECOVERY IN THE KRAFT PULPING SYSTEM [after (Sjöström, 1981)].

the wood lignin is removed in NSSC pulping. The pulp is then defiberized further by mechanical means.

Wood fibers have a brown color after pulping, due mainly to residual lignin. The kraft process is not allowed to proceed past a pulp of about 5% lignin content so as to avoid excessive carbohydrate degradation. Pulps of various degrees of brightness (down to about 0.5 to 1% lignin) are desired for a wide range of final products such as tissue and fine writing papers. Further lignin removal is achieved by multistage bleaching, commonly by chlorine followed by more selective agents such as chlorine dioxide, hypochlorite, and sodium peroxide. Brightening of pulp by these chemicals occurs through two mechanisms, actual lignin removal and chromophore destruction.

In the chlorination stage, frequently the first in bleaching operations, Cl_2 attacks lignin under highly acidic conditions (about pH 2) to form substituted and oxidized fragments with a high carboxyl content (Sjöström, 1981), and aromaticity is destroyed to a large extent (Bennett, et al, 1971). These fragments from lignin depolymerization are more soluble due to their lower molecular weight and increased hydrophilicity. Some degree of carbohydrate degradation occurs as well, releasing the products into the bleachery effluent. A portion of the degraded lignin dissolves in the chlorination stage, but the greater amount is removed from the pulp in the alkaline extraction stage which follows. The high alkalinity ionizes phenolic hydroxyl and carboxyl groups, thereby increasing the solubility of lignin fragments. From 70 to 80% of the residual lignin is removed during chlorination and extraction. Regardless of the subsequent types of bleaching

stages used, the principal color sources in the entire bleachery operation are these two initial stages, accounting for about 95% of the color discharged from bleaching. Likewise, 70-80% of the BOD_5 and most of the toxicity of bleaching waste waters originate here.

The color loadings of the various waste streams from pulping and papermaking can differ from each other dramatically (Figure 3), as well as from the same source at other mills (Table 1). Over the past 20-30 years, the volume of pulping wastewaters has decreased due to improved reuse schemes. The color contribution from bleachery effluent has thereby increasingly become a more significant portion of the total. The color from the bleach plant can be 80-90% of the total from pulping and bleaching combined (Rush and Shannon, 1976). Of the bleachery color, as much as 90% is from the alkaline extraction stage, meaning that up to 70% of the total mill color load in contributed by a source that is only about one-fifth of the total volume.

1.3 Nature of Color Bodies and Effects on Receiving Waters

Although the structures of color-bearing compounds in pulping and bleaching effluents have not been fully characterized, lignin and its degradation products are generally considered to be the major contributors. Specific chromophores involved depend upon the pulping and bleaching processes and wood species used. Investigations into the identity of chromophoric structures have revealed the important ones to be quinones, quinonemethides, and vinyl groups conjugated with an aromatic ring (Bennett, *et al*, 1971). Examples of possible structures are presented in Figure 4. These structures can be incorporated into larger, complex molecular species. Those present in bleachery spent liquors are generally thought to be acidic chlorine-

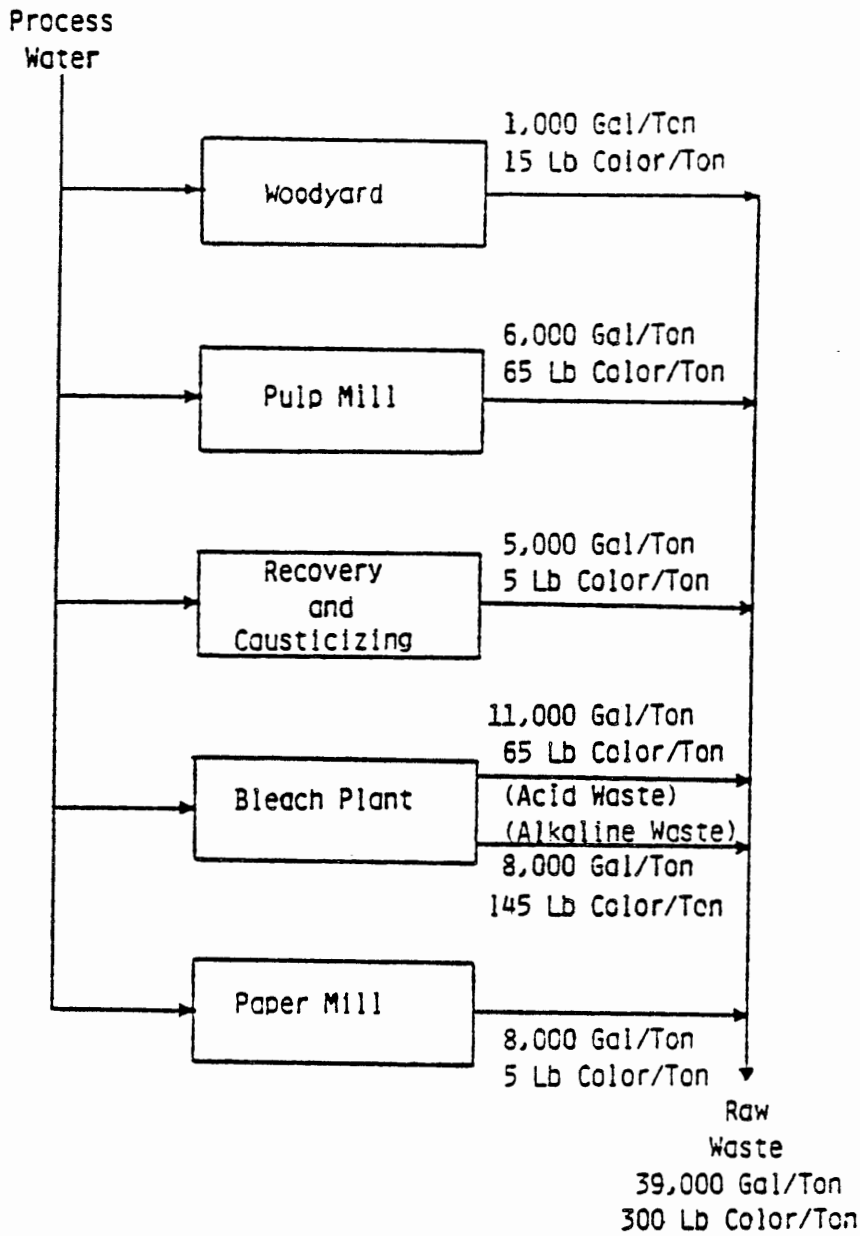


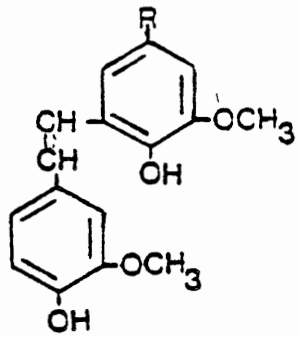
Figure 3. COLOR LOADINGS OF WASTE STREAMS FROM A BLEACHED KRAFT MILL, PER TON OF PULP [after (EPA, 1976)].

Table 1. COLOR LEVELS IN VARIOUS WASTE STREAMS FROM BLEACHED KRAFT PULP PRODUCTION
(Rush and Shannon, 1976)

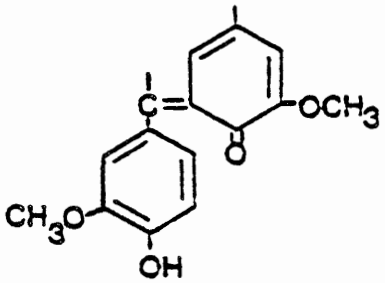
SOURCE		COLOR UNITS, (Pt-Co)*
Pulping		700 - 3,700
Bleaching Stages		
	Acid	600 - 2,300
	Alkaline	4,400 - 23,000
Whole Mill		1,200 - 3,700
Other Liquids		
	Coffee	10,000 - 15,000
	Beer	1,000

*Platinum - Cobalt Standard (NCASI, 1971)

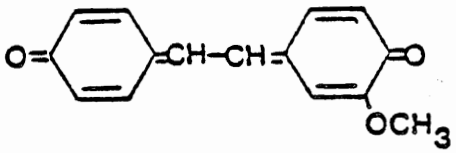
CHROMOPHORES



DIHYDROXYSTILBENE TYPE



QUINONEMETHIDE TYPE



STILBENEQUINONE TYPE

Figure 4. PROPOSED CHROMOPHORIC STRUCTURES IN PULPING AND BLEACHING EFFLUENTS [after (Wong, 1976)].

substituted, oxidized lignin fragments of relatively low molecular weight and low aromatic content (Bennett, et al, 1971; Hardell and de Sousa, 1977).

The lignin-derived compounds in pulping and bleaching effluents have been shown to have molecular weights ranging from less than 400 up to 150,000 in a typical combined mill effluent (Obiaga and Ganczarczyk, 1974). This polydispersity, illustrated in Figure 5, creates an inherent difficulty in devising color removal technology. Color bodies in bleachery wastes are of lower molecular weight than those from pulping due to further degradation, and those in alkaline extraction waste liquors are usually somewhat larger than in the acidic chlorination wastewater.

It has been quite apparent that various molecular weight fractions are associated with different amounts of color. In kraft lignin the greatest color contribution arises from molecules of intermediate molecular weight (avg. MW = 5,600), while very little is from the smallest weight fraction (MW <4,000), and almost none from the largest constituents (MW > 150,000) (Rankin and Benedek, 1973). The relationship between color and molecular weight in spent bleaching liquors has previously been demonstrated (Hardell and de Sousa, 1977). The solids of chlorination and alkaline extraction stages were separated by ultrafiltration and subjected to organic carbon and color analyses. In spent chlorination liquor, the lowest molecular weight fraction (MW <1,000) contained 60% of the organic carbon, yet contributed only 20% of the total color. The fraction in the extract liquor accounted for 20% of organic carbon but only 1.5% of color. The single largest color contribution in chlorination liquor was from those constituents of the molecular weight range 10,000-25,000 (about one-third of total color). In

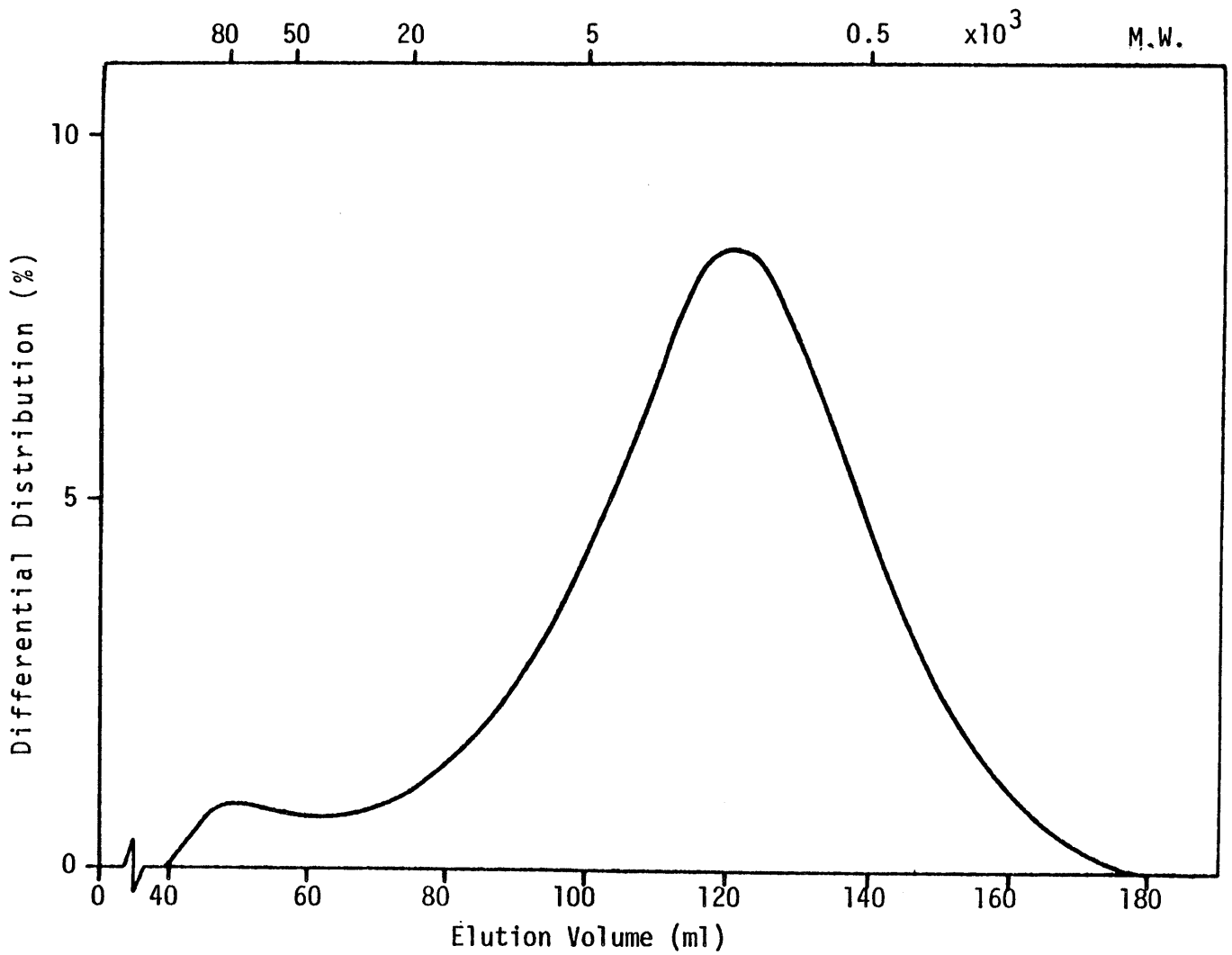


Figure 5. MOLECULAR WEIGHT DISTRIBUTION OF COLOR BODIES IN PULPING EFFLUENT [after (Ganczarczyk and Obiaga, 1974)].

extraction liquor this fraction and the fraction with the largest molecular weight components (MW> 25,000) were equally colored, for a combined contribution of about three-fourths of the total color.

The most frequently given justification for removing color from pulp and paper mill wastewaters is that of aesthetic objections. Numerous effects of color on the environment and water use have been suggested, but not all have been substantiated (Rush and Shannon, 1976). Although direct evidence relating to mill color sources is lacking in many instances, several effects of color, particularly from natural sources ,on water use are known (American Water Works Association, 1970). Color can increase chlorine demand, foul ion exchange resins, and interfere with coagulation in municipal and industrial water treatment systems. Contributions to taste problems in potable water have been suspected. Color can cause difficulties in colorimetric analyses needed for treatment plant control. Many industries require low-color process waters; reuse of pulp and paper industry wastewaters would be facilitated by removal of color. Among the possible environmental effects of color are reduction of primary productivity (photosynthesis) in waters due to inhibition of light penetration, interference with behavior or survival of aquatic organisms that are dependent upon light perception, and chelation of potentially toxic metals to create forms more readily available to organisms (Rush and Shannon, 1976).

The long term stability of color from pulping and bleaching wastes has been demonstrated (Bouveng and Solyom, 1973) using a model aquatic system. Biologically-treated effluents from kraft pulping, chlorination, and alkaline extraction were added to vessels containing organisms from trophic levels

representative of those in natural aquatic systems. After 40 weeks at room temperature and 12-hour daily illumination, about two-thirds of the original color remained in each of the effluent types. These removal rates were considered to be greater than that expected in the natural environment because of the enhanced biological activity in laboratory situations.

1.4 Color Removal Technology

1.4.1 Overview

Although the pulp and paper industry has been generally quite successful in removing conventional pollutants such as BOD_5 and total suspended solids (TSS) from its waste streams, color reduction has proven to be a more difficult task in both technical and economic terms. The ultimate solution to the color problem most likely lies in various process modifications in pulping and bleaching technology, yet some external effluent treatment will probably be necessary in many situations to reach the required reductions (Rush and Shannon, 1976). Much research has been directed recently to such internal measures as the use of non-chlorine bleaching chemicals (e.g., O_2), substitution of large portions of Cl_2 with ClO_2 in the first stage of chlorination, more efficient spill control, and countercurrent washing in bleachery stages (Sjöström, 1981). These operations have as their goal the reduction of all pollutants, including BOD_5, COD, toxicity and color, with emphasis on water reuse. At existing facilities, however, economics may dictate the installation of further external treatments in preferance to process modifications.

The current practice of primary clarification of wastewater followed by biological treatment, commonly aerated lagoons or activated sludge units, can eliminate conventional pollutants at an efficiency up to 95%. In contrast, these treatment systems are capable of color reductions of only 0-30% (Rush and Shannon, 1976). This resistance of color bodies to biological treatment is largely due to the inability of the microbial population to metabolize lignin - derived chromophores. A direct approach to improving the color reduction ability of biological processes has been recently reported (Dogherty, 1982). In the use of the first U.S. patent to be granted with an applications claim to a microorganism, the Sybron Corp. augmented the resident microbial population of an aerated lagoon with a mutant strain of the bacterium, Pseudomonas aeruginosa. This organism, mutated from normal inhabitants of the treatment system, is able to metabolize "color lignins", and is claimed to remove 40% of effluent color and considerable BOD_5 in laboratory studies. Mill-scale evaluation is being conducted at a Canadian bleached kraft mill. Another biological process under investigation for color removal potential is one utilizing the white-rot fungus, Phanerochaete chrysosporium, a species capable of lignin degradation in nature (Eaton, et al, 1980; Campbell, et al, 1982). In this process kraft bleaching effluent is treated in a rotating biological contactor (RBC) unit. The RBC discs, covered with fungal growth, rotate through the effluent in a cylindrical vessel so as to expose the fungal surface to both the wastewater and an oxygen-enriched atmosphere. Lab-scale work demonstrated a maximum of 50-60% color removal in 24-hour batch treatments.

Several physical and chemical processes have been examined for color

removal ability, focusing on either whole-mill, chlorination stage, or alkaline extraction stage effluents. An excellent, comprehensive discussion of color reduction technology for the paper industry can be found in a previous review of the problem (Rush and Shannon, 1976). Several methods have reached mill-scale evaluation, among them: coagulation with metals such as calcium, aluminum, iron, and magnesium (Rush and Shannon, 1976); the Uddeholm-Kamyr (Andersson, 1977) and Rohm and Haas (Rock, et al, 1974) resin adsorption processes; ultrafiltration (Lundahl and Mansson, 1980); ion exchange (Lindberg, et al, 1980); reverse osmosis (Cruner, 1973); and activated carbon (Wong, et al, 1977). In a comparison of metal ion precipitation processes, activated carbon, ion exchange, and reverse osmosis (Puiu, 1978) it was concluded that only lime or alum present economic feasibility for full scale use. Difficulties in the use of aluminum or iron salts have necessitated careful monitoring and control of pH and sludge dewatering (Rush and Shannon, 1976). Although alum, lime, or magnesium have all been shown to be effective in precipitating color from kraft and NSSC effluents, application of lime to kraft wastes may be the most attractive, since separate incineration equipment is not required for recovery as would be the case for the other two metals (NCASI, 1977).

The vast number of reports concerning color removal processes makes it apparent that the method of choice for a given mill depends greatly upon site-specific economics. The variety of effluent types and mill situations that exist will probably preclude application of any one decolorization process at all locations. For this reason, further study and innovation is warranted for several of the above technologies.

1.4.2 Lime Precipitation of Color

One of the more promising color removal processes that has been examined on a mill scale is lime precipitation. Early studies on reduction of color from pulp and paper mill wastes utilized the existing chemical treatment technology of municipal water supply decolorization. Screening of over thirty coagulants and adsorbants demonstrated varying degrees of effectiveness (Moggio, 1955). Lack of recovery methods, or other technological difficulties, such as corrosion problems, prevented many from further consideration. Lime was considered to have the greatest potential for application in the industry. Among the factors recognized as favoring use of lime were:

1. Lime is comparatively low in cost and is readily available (especially in treatment of kraft mill effluent, where lime is produced as part of the chemical reovery system, i.e., causticizing of green liquor),
2. Recovery methods are already well-developed, and,
3. Personnel at kraft mills have experience with lime production and recovery.

In short, the fact that lime is an integral part of the kraft pulping process offered an opportunity for both recovery of lime used for decolorization and for destruction of the removed color by combustion in the lime kiln. Since this early study, lime precipitation has been one of the most widely investigated color removal processes in the pulp and paper industry. Although problems remain to be solved, it has been considered by some to be the most readily applicable of the various physical and chemical

methods studied (Rush and Shannon, 1976).

The mechanism of lime precipitation of color has been examined in a study supported by the National Council of the Paper Industry for Air and Stream Imporvement (Bennett, et al, 1971). Color removal was found to be a chemical reaction in which insoluble calcium-organic salts formed rather than a physical adsorption or absorption of color onto lime particles. The process was found to be a function of two main factors: the enolic and phenolic hydroxyl content of the effluent and molecular weight distribution of the color bodies. The reaction demonstrated a pH dependence, requiring a pH of about 12 for complete ionization of the hydroxyl groups which reacts with the calcium ion.

Effluents studied by the NCASI projects were those from chlorination and extraction stages. The organic solids of each effluent were subjected to elemental and functional group analyses and molecular weight determination. The solids in the spent liquors were mainly characterized as acidic chlorine-substituted lignin oxidation products of relatively low molecular weight. Spectroscopic analysis revealed very little aromaticity. Attention was directed to carboxyl, phenolic, and enolic groups in developing a theory to explain the mechanism of color removal by lime. Experiments were performed utilizing selective chemical blocking of specific functional groups, followed by lime addition. It was concluded that for chlorination and extraction effluents, enolic hydroxyls are most likely the predominant groups involved in precipitation by lime.

However, in a solution of commercially-available lignin ("Indulin") obtained from kraft pulping, phenolic hydroxyl groups appeared to have the

major role in the lime reaction. Selective modification of functional groups was utilized here also. Furthermore, the phenolic hydroxyl content of kraft lignin was considerably greater than in spent bleaching liquors.

The scheme outlined in Figure 6 represents the reaction sequences proposed in the NCASI reports for color removal by lime. The formation of keto-enols in molecules containing chromophoric groups is shown, with alkaline conditions shifting the equilibrium to the enolic form. Alkali is also required for ionization of enolic or phenolic hydroxyls. The resulting anion then forms an insoluble salt with the calcium of added lime. It was noted that, in the study of lime precipitation of acidic chlorination stage color, it could not be determined whether lime reacted with original functional groups or with ones produced by hydrolysis of chloro-substituents by alkali of the lime solution (as indicated in Figure 6).

The apparent influence of molecular weight in the lime decolorization process was seen when attempts were made to understand why approximately 15% of the original color could not be precipitated. This problem was approached by treating liquor from the caustic extraction stage with lime and recovering the precipitated and non-precipitated organic solids by ion exchange techniques. The compositions of the two fractions were similar, each containing the functional groups that had been established as capable of producing insoluble salts with calcium. The average molecular weights, however, were significantly different. The value for solids which precipitated was estimated to be about 500, whereas it was less than one half, or 210, for the non-precipitated solids. Later work (Dugal, et al, 1975) substantiated these implications. It was determined that lime will

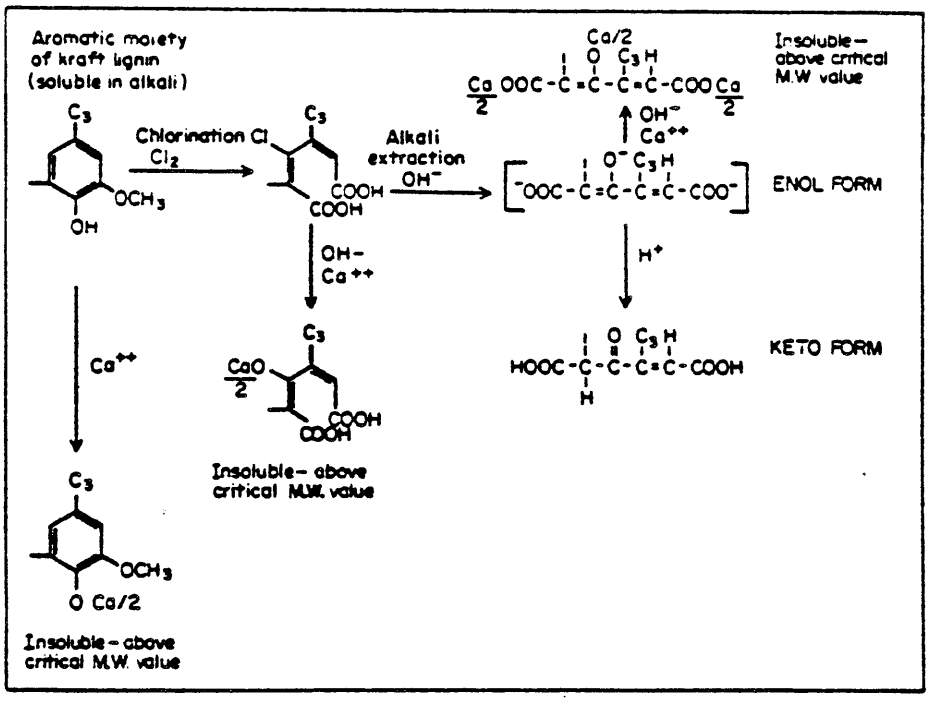


Figure 6. PROPOSED REACTIONS IN THE LIME PRECIPITATION OF COLOR FROM PULPING AND BLEACHING EFFLUENTS [after (Bennett, et al, 1971)].

precipitate almost all high molecular weight color bodies (MW> 5,000), partially removes those from 400 to 5,000, but generally cannot precipitate species below 400.

A basic scheme is common to all lime precipitation processes for color removal from pulp and paper mill effluents. Effluent and slaked lime are mixed and sent to a clarification basin where the lime-organic sludge settles. The various processes developed for lime treatment generally differ according to lime dosage, effluent type, and methods of handling the lime-organic sludge and decolorized effluent.

Processes have been developed along two directions, termed massive lime and minimum ("mini") lime processes. The first employs lime application rates much greater than that required for stoichiometric reaction with color bodies. In early work, problems were encountered in dewatering the lime-organic sludge when a minimum lime dose was used (Moggio, 1955), leading attention to trials with greater lime doses. In laboratory tests, levels of 10,000 to 30,000 mg/L lime produced the most dense and most easily dewatered sludge because of the great excess of calcium hydroxide particles (Rush and Shannon, 1976). A one-year study by International Paper Company evaluated the incorporation of a massive lime system for color removal at a demonstration mill in Springhill, La. (Wright, et al, 1974). The facility produced bleached kraft pulp at about one-fifth of usual full scale. A dosage of 20,000 mg/L lime was applied to alkaline extraction stage and unbleached decker (pulp thickener) effluents. In this design, the dewatered lime-organic sludge was used for recausticizing the mill's green liquor to produce white liquor, since most of the lime was still available as calcium hydroxide. All of the

alkaline extraction stage and most of the unbleached decker effluent could be treated without exceeding the lime kiln capacity for lime production in the quantity required for causticizing. The total mill color load was thus lowered by about 70%. Further work with this process was not pursued, however, due to several serious difficulties. Foaming in causticizers and lime mud washers was a constant problem, and the white liquor concentration was about 15% lower than when produced in the conventional manner with fresh lime. Implications for full scale use included requirements for a larger volume of white liquor for wood digestion and, consequently, increased capacity in the chemical preparation and recovery systems.

There have been several versions of the mini-lime process (Davis, 1971; Gould, 1973; Spruill, 1973); these have been able to overcome the problem of lime sludge dewatering to a large degree. Perhaps the most successful is the full-scle system operated by Continental Can Company at Hodge, La. (Spruill, 1973). This mill had been under pressure from the state of Louisiana to lower the color in wastewaters discharged into a low volume stream. Most of the 15 million gallons produced per day by combined kraft and NSSC boardmill effluents could be treated. In this scheme (Figure 7) a small dose of about 1,000 mg/L lime is slaked and mixed with waste liquor. After primary clarification, the decolorized overflow is sent to a recarbonater for removal of remaining dissolved calcium. In the recarbonator, lime kiln stack gas is introduced as a source of CO_2 for formation of insoluble calcium carbonate. After settling in a clarifier, the sludge is routed to the primary clarifier to aid in the formation of a dewaterable precipitate. This combined, somewhat fibrous sludge, is dewatered on a solid bowl centrifuge to approximately 35%

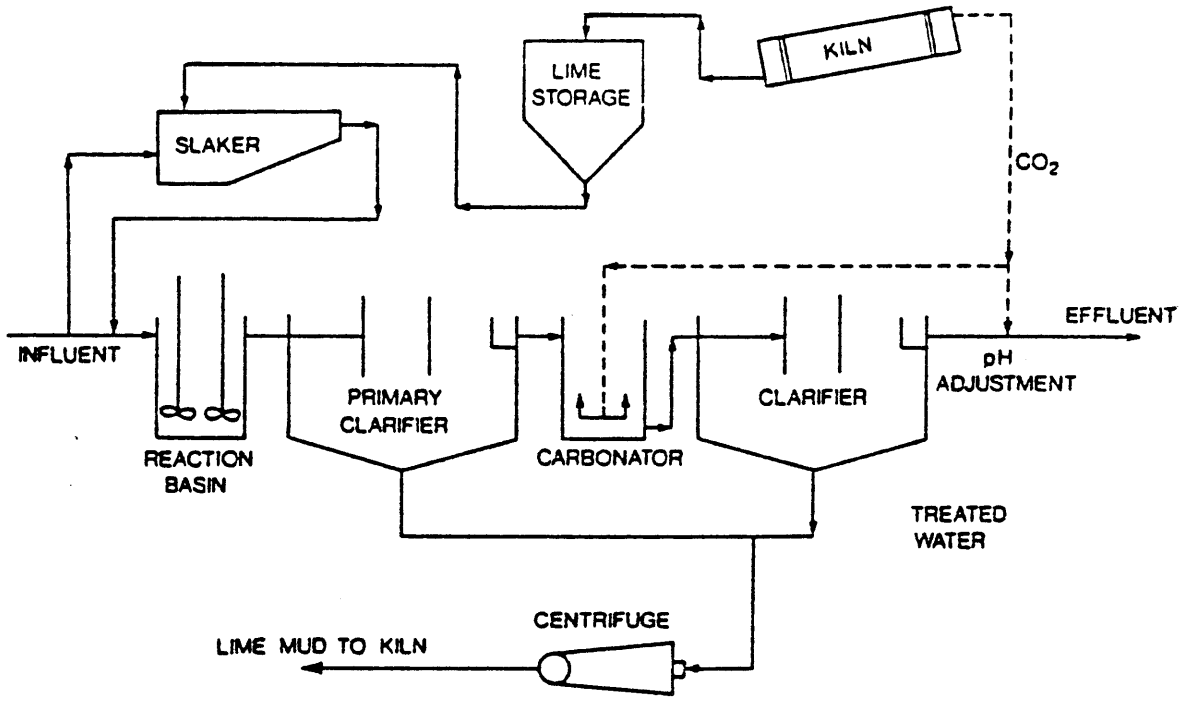


Figure 7. MINIMUM LIME PROCESS FOR COLOR REMOVAL FROM PULPING WASTEWATER [after (Rush and Shannon, 1976)].

solids before entering the kiln for lime recovery and incineration. Color removal efficiency depended upon the proportion of NSSC effluent. The original color of 1,200 Pt-Co units was reduced to 300-400 (70% average removal) when NSSC effluent was included; 80 to 90% reduction was obtained on kraft waste alone. A major problem occasionally developed in the recarbonation system, probably related to lack of pH control. Although removal was usually about 85% after primary clarification, sometimes color increased during recarbonation to around 35 to 40% of the original, so that modifications have been necessary (Rush and Shannon, 1976). A brief report on the system (Moll, 1976) stated that decolorization had averaged 80% in the preceding year, and operating costs were estimated at 50 cents per ton of pulp (1976 dollars) at 1,500 tons pulp production per day.

A process somewhat similar to that at Hodge, Louisiana, has been described for two Georgia-Pacific bleached kraft mills at Woodland, Maine, and Crossett, Arkansas (Gould, 1973). Alkaline extraction stage is treated with slaked lime at 2,000 to 3,000 mg/L In a two-year period at the Woodland mill, initial color of 10,000-15,000 Pt-Co units was generally lowered by 85-90%, and a BOD_5 reduction of 45% was possible. About 80% of spent lime was reclaimed. The system at the Crossett mill was designed for use during the summer months, when mill effluent is nearly the entire flow of the small receiving stream (Rush and Shannon, 1976).

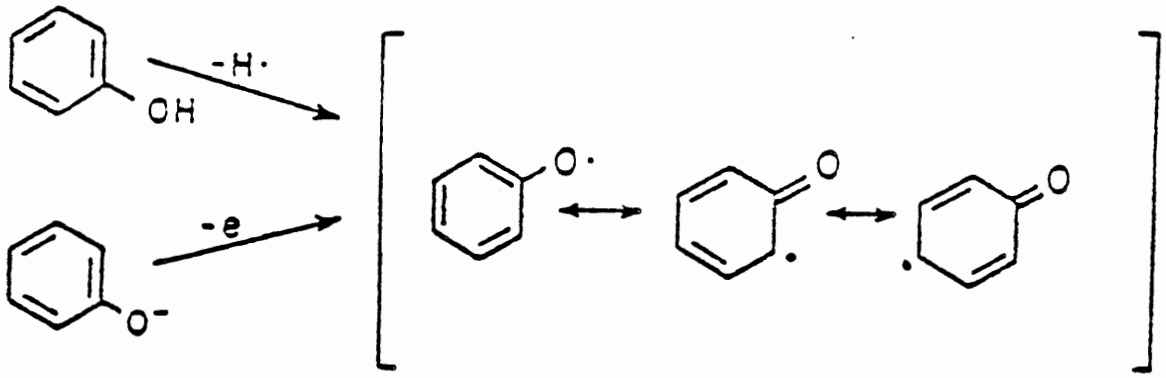
Lime coagulation has been investigated as a general treatment for producing an effluent which could meet Canadian regulations on toxicity, COD, BOD, and TOC, as well as color (Naish and Sandilands, 1977). The lab scale study, supported by the Canadian Government's CPAR (Cooperative Pollution

Abatement Research) Program resulted in a design proposal and cost estimate for a softwood bleached kraft mill. The combined bleach plant seal tank overflows would be treated with 2,000 mg/L lime, the optimal level for pollutant removal, in a scheme similar to that at the Hodge plant. Bench scale results showed various parameters to be reduced as follows: toxicity, 86%; color, 85%; and COD, BOD, and TOC, approximately 60%. The capital expenditure estimate for full scale was $4.25 million with operating costs of $6.76 per ton of pulp (1977 dollars), assuming a single lime kiln was used for both pulping needs and waste treatment. The authors felt that consideration of lime for pollution control at a given mill would depend upon comparison with other possible methods for that particular site.

1.5 Laccase and Technological Use of Phenol Oxidases

Laccase (Enzyme Commission 1.14.18.1) is one of the most studied phenol oxidases present in wood-rotting fungi. In nature it is especially found in the fungi, particularly the Basidiomycetes, with fewer representatives among the higher plants (Mayer and Harel, 1979). Laccase catalyzes the oxidation of phenols to phenoxy radicals, using O_2 as the electron acceptor with the concurrent formation of water. It is not certain whether electron abstraction involves unionized phenol or the phenoxylate ion (Sarkanen, 1971). Nevertheless, the resulting phenoxy-free radical has several resonance stabilized forms, as illustrated in Figure 8. Two such radicals can condense, as has been shown in cell-free laccase experiments (Freudenberg, 1968), in which coniferyl alcohol formed dimers, trimers, and oligomers with bonds similar to those in lignin. Higher molecular weight products are created when the original phenols are part of a larger structure, as, for example, when the

A.



B.

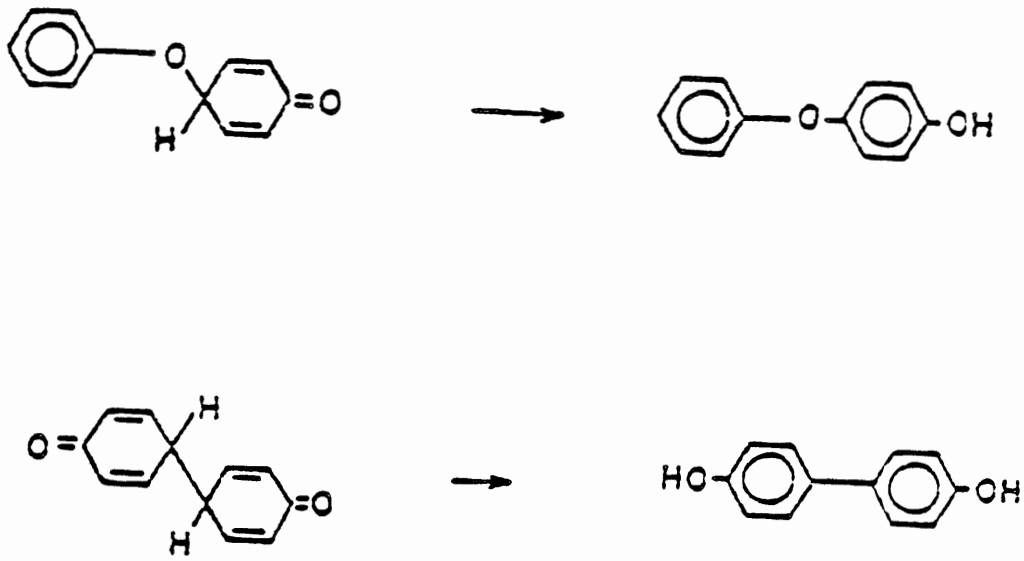


Figure 8. MESOMERIC FORMS OF PHENOXY FREE RADICALS FORMED BY LACCASE ACTIVITY. (A) AND EXAMPLES OF DIMERS RESULTING FROM CONDENSATION OF RADICALS (B).

incubation of laccase with milled wood lignin increases the molecular weight of the material (Ishihara, 1980). The actual involvement of laccase in lignin biosynthesis in plants, however, does not appear to be widespread. There is more evidence supporting the enzyme peroxidase, also a phenol oxidase, as the natural catalyst (Gierer and Opara, 1973). Regardless, the substrates and products of these two enzymes are essentially the same, although peroxidase utilizes hydrogen peroxide (H_2O_2) as the electron acceptor (Sarkanen, 1971).

Some controversy exists concerning the functions of laccase, and it appears that its role can vary according to species and developmental stage. Several roles have been postulated, among them: a role in lignin biodegradation, perhaps regulatory (Ander and Eriksson, 1976); detoxification of small molecular weight phenolics produced by host plants or released during lignin degradation (Mayer and Harel, 1979; Gierer and Opara, 1973); and pigmentation and increased structural strength in fruiting bodies of certain fungi (Leatham and Stahmann, 1981). An investigation of the effect of laccase on lignin degradation in cultures of a wood-rotting fungus (Haars and Huttermann, 1980) suggested that laccase can cause repolymerization of lignin cleavage products.

The growing field of technological applications for biological systems and enzymes has not overlooked the phenol oxidases. The removal of phenolics and aromatic amines from industrial wastewaters by peroxidase has been the subject of studies by Klibanov and co-workers (Klibanov, et al, 1980; Klibanov and Morris, 1981; Alberti and Klibanov, 1981). Especially targeted for investigation were toxic and carcinogenic compounds. Currently employed treatment processes were claimed to be unsatisfactory, for either economic or

efficiency reasons. In studies of over 40 different chemicals, peroxidase from horseradish and H_2O_2 were applied to solutions of pure compounds present at 100 mg/L. The insoluble polymerization products were removed after 3 hours treatment time by centrifugation in laboratory experiments. Removal efficiencies ranged from 50 to 99%, depending upon the compound treated. In actual practice, removal would be accomplished by sedimentation or filtration. Treatment pH for maximum efficiency varied according to the compound treated, but effective removal covered a broad pH range for most. The efficiency was postulated to depend upon the reactivity of the compound toward peroxidase, the insolubility of the polymerization product, or a combination of these factors (Klibanov, et al, 1980). Enzyme purity was not found to be critical (Alberti and Klibanov, 1981). An aqueous extract from horseradish roots proved to be as effective as commercially purified, purchased peroxidase.

It was discovered that the precipitation by peroxidase of certain less easily removed compounds could be enhanced when one of high removal efficiency was also added. For example, enzyme treatment left more than 50% of o-aminophenol in solution; yet, when 2,3-dimethylphenol (almost 100% removable) was present, 95% of the aminophenol could be removed. Two possible explanations for this phenomenon were suggested (Klibanov, et al, 1980), based upon the two removal factors stated above. A compound that is a less reactive substrate for peroxidase produces relatively few free radicals; addition of a more highly reactive compound increases the concentration of free radicals, thus raising the probability for the first compound to participate in polymer formation. Alternately, the situation might exist where a compound readily

reacts enzymatically, yet forms products of insufficient hydrophobicity or molecular weight to precipitate. The presence of an easily removed compound whose polymers are insoluble could result in mixed polymers which would also be insoluble.

The enzyme treatment was also able to remove certain non-phenolics and non-aromatic amines, compounds that are not substrates of peroxidase, when reactive compounds were in solution as well (Alberti and Klibanov, 1981). More than 60% of naphthalene could be removed from a solution treated with peroxidase, H_2O_2, and 100 mg/L 2,3-dimethylphenol. These results have significance considering that the types of industrial wastewaters concerned are usually mixtures of several compounds.

Wastewater from the industrial production of triarylphosphates (flame retardants) were next treated with peroxidase (Alberti and Klibanov, 1981). The effluent contained over 150 various chemicals, including phenols, cresols, xylenols, isopropyl alcohol, as well as nonphenolics such as triarylphosphates, with a total concentration of 105 mg/L phenols. Treatment with peroxidase, either purified or crude, and H_2O_2 for 40 hours accomplished about a 96% reduction in phenol content.

Similar attempts to remove phenols from industrial effluents have involved fungal phenol oxidase (Vedralova, et al, 1980). The wastewater in this case was from coal coking operations and contained 1,500 mg/L phenol and 200 mg/L resorcinol. An unspecified mushroom species was the source of a crude phenol oxidase preparation. After 28 hours of enzyme treatment, the phenolic content of the wastewater had been reduced by 85%, and after 48 hours, 98%. It was suggested that the "melanin" product, which did not

flocculate in the treatments, could be removed by acid precipitation or adsorption on bentonite. It was necessary to dilute the effluent by a factor of almost 2.5 in order to obtain a reasonable reaction rate. To avoid prohibitively large enzyme requirements for such increased volumes, the authors realized that some measure such as enzyme immobilization on a support material would probably be essential.

Schmidt and Joyce investigated the feasibility of utilizing peroxidase to improve precipitation of color by lime from pulping and bleaching effluents (Schmidt, 1979; Schmidt and Joyce, 1980). Because one of the major factors influencing the precipitability of color bodies by lime is molecular weight, the authors reasoned that any process capable of increasing the average molecular weight should be able to effect corresponding increases in color removal with lime. The ability of peroxidase to polymerize phenolics made this enzyme a candidate for treating color bodies, molecules derived from lignin with some phenolic groups retained. Enzyme use was, therefore, envisioned as a pretreatment prior to lime addition.

Two types of wastewater were studied. One was pulping effluent that had undergone biological treatment. The other consisted of color bodies that had survived lime precipitation from combined alkaline extraction and decker effluents. This last material represented molecules resistant to removal, due, in part, to their having a low molecular weight.

The effect of enzyme treatment on molecular weight distribution in effluents was first examined. Treatment with 25 mg/L horseradish peroxidase and 9 mM H_2O_2 for 12 hours at pH 6 caused a slight but significant shift toward the higher molecular weight range (MW >5,000), as determined by gel

permeation chromatography, for both effluent types. Lime can almost completely remove color bodies with molecular weights greater than 5,000 (Dugal, et al, 1975). This shift was 5.5% of the total peak area for biologically-treated material, and 8% in lime-treated samples. For the latter, the transfer was mainly from the middle molecular weight range (1,000 <MW <5,000). Lime precipitation was conducted on samples of biologically-treated pulping effluent, pretreated with 2.5 mg/L peroxidase and 2.5 mM H_2O_2. This pretreatment allowed 94% color removal at a lime dosage that gave only about 10% removal from non-pretreated samples.

The importance of careful selection of H_2O_2 dosage was quite apparent. Enzyme pretreatments over a range of H_2O_2 application levels (0 to 15 mM) were followed by lime precipitation. Peroxide at 2.5 mM allowed the greatest color removal, with less effectiveness at values higher or lower. This observation seemed to be due to peroxide participating in competitive reactions relative to color removal, namely enzymatic polymerization on the one hand, and degradation of color bodies on the other. Degradation, which would interfere with lime precipitation, was evident from the molecular weight distribution in effluents treated with high levels of H_2O_2 (180 mM) without enzyme. It was speculated that further interference with polymerization might occur by destruction of phenolic groups by H_2O_2. The optimal H_2O_2 concentration appeared to be one that maximized polymerization while minimizing degradative attack on color bodies.

The authors concluded that this work was encouraging in demonstrating that the lime precipitability of color bodies of pulp mill effluents can be enhanced by a treatment that increases the proportion of higher molecular

weight species. Such a treatment might be designed either for achieving greater color removal or for reducing the amount of lime required for removal. The study did not attempt to evaluate the technical or economic feasibility of a mill-scale process. Because of the difficulties encountered in the use of H_2O_2 and the added expense of the chemical, the recommendation was made that further work utilize the similar enzyme laccase.

2. MATERIALS AND METHODS

2.1 Production of Laccase

Because the enzyme laccase is not commerically available, it was produced in laboratory cultures of a white-rot fungus. The species selected was *Coriolus versicolor* (L. ex. Fr.) Quel. (synonyms *Polyporus*, *Polystictus* and *Trametes versicolor*). The isolate Madison 697-R was obtained from the U.S. Forest Products Laboratory, Madison, Wisconsin. This fungus has several features recommending it as an excellent source of laccase. Enzyme production is high compared to many species and is secreted into the medium surrounding the mycelia. Tissue extraction for laccase recovery is, therefore, not necessary (Fahraeus and Reinhammar, 1967).

Enzyme production was carried out essentially according to previous reports (Fahraeus and Reinhammar, 1967). Fungus cultivation was in three stages: fungal mat formation, mycelial pellet formation, and liquid suspension culture for laccase induction (Figure 9). For mat formation, mycelia were transferred from meal extract-agar slants to 25 ml of synthetic growth medium (Fahraeus and Reinhammar, 1967) in 125-ml flasks. After 7-10 days, the mat-like growths from two such flasks were transferred to a flask containing 100 ml of sterile water and several glass beads. Vigorous shaking produced a mycelial suspension to be used in the second stage, pellet formation. Ten milliliters of suspension (about 85 mg of fungal mass, dry weight) were added to each of 6 flasks continaing 100 ml of growth medium. These flasks were placed in a reciprocating shaker-water bath at 100

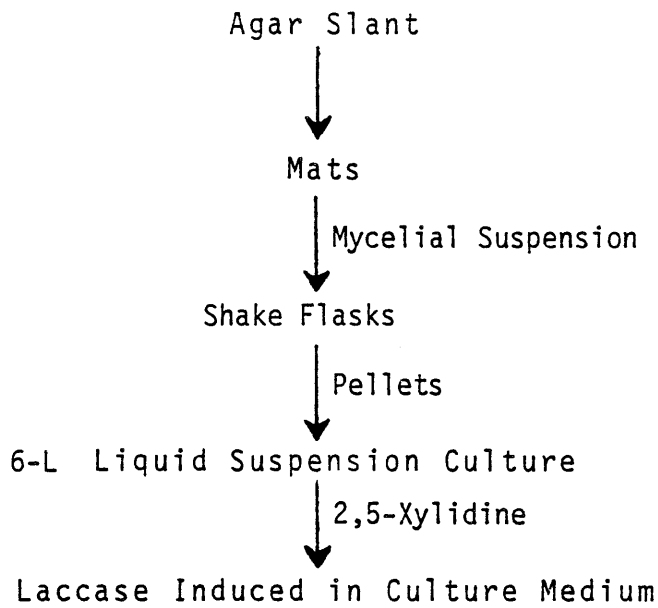


Figure 9. CULTURE OF CORIOLUS VERSICOLOR FOR PRODUCTION OF LACCASE.

oscillations per minute for 4 days at 28°C. Round or oblong pellets, about 2-3 mm in diameter, were formed.

For the last stage, the contents of 6 shake flasks were transferred to a 12-L round bottom flask, which had four interior baffles and was equipped with an impeller, sparge tube, and ports for sampling and additions (Figure 10). Growth medium was added to make 6 L total liquid volume. All equipment and medium were sterilized by autoclaving at 121°C for 20 minutes prior to use. Air was supplied by an oil-free compresser and passed through two glass tubes (3 cm x 10 cm) packed with glass wool for removal of microorganisms (Evans, et al, 1970) before being sparged into the sealed vessel at a rate of 4 L/minute. The glass impeller stirred the pellet suspension at approximately 100 rpm, and the culture was maintained at 28°C by a water bath surrounding the flask.

Laccase concentration in the culture medium is almost neglibible unless chemically induced (Table 2). Xylidine (2,5-dimethylaniline; Eastman Kodak Co.) in 50% ethanol was added for this purpose after 4 days of growth to give a concentration of 2×10^{-4} M in the medium, and again 2 days later in half that amount. Preliminary work using shake flask cultures established this particular addition schedule as the most satisfactory for obtaining high laccase production. On the eighth day of fungal culture, the entire vessel contents were filtered through several layers of cheesecloth followed by filter paper to separate mycelia from the growth medium. The filtered volume was approximately 4 L.

Because a very pure preparation of laccase would probably be prohibitively expensive for industrial application, further processing of the

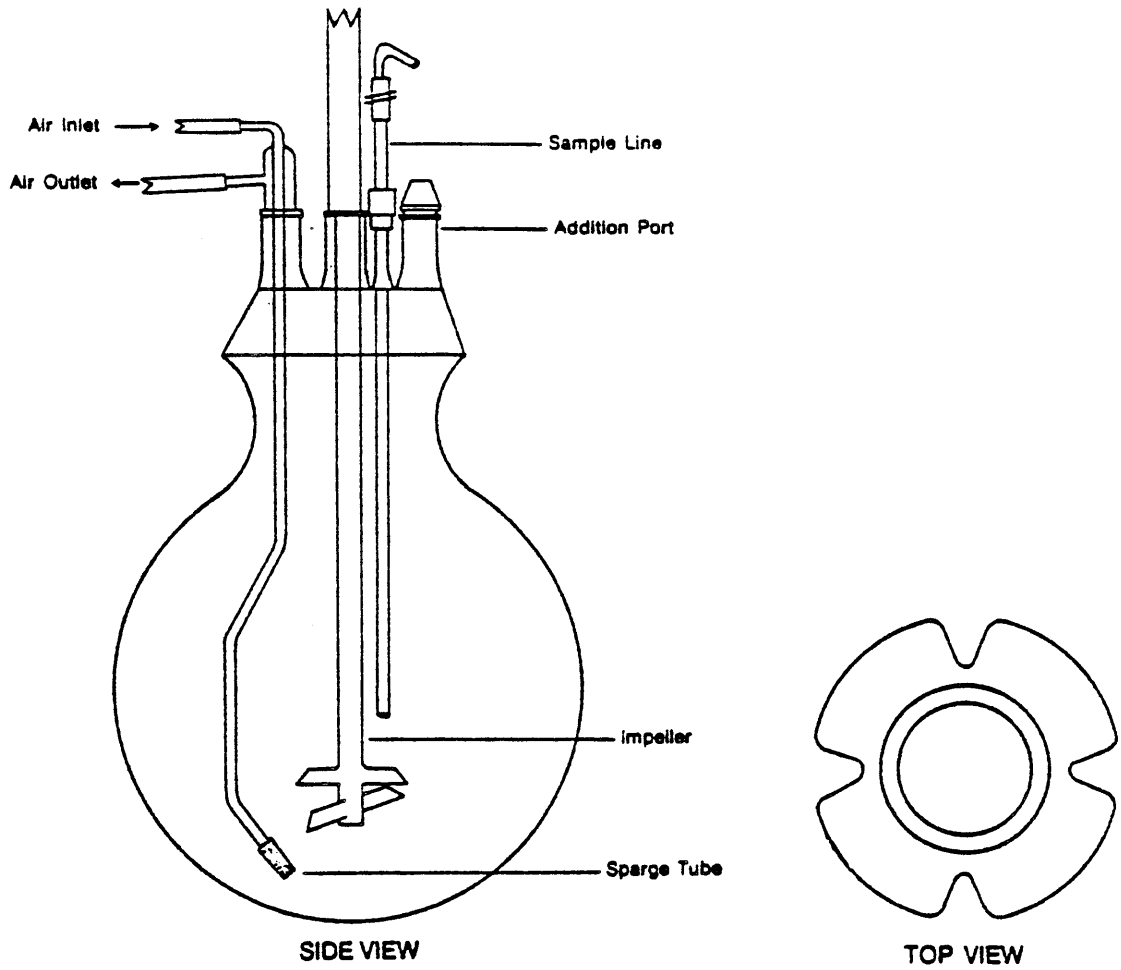


Figure10. DIAGRAM OF CULTURE FLASK AND ASSOCIATED EQUIPMENT FOR PRODUCTION OF LACCASE. (A) Side view of equipment. (B) Top view of culture flask.

Table 2. PRODUCTION OF LACCASE FROM *CORIOLUS VERSICOLOR* AND CONCENTRATION BY HOLLOW-FIBER ULTRAFILTRATION.

Step	Activity*			Yield	Concentration of Activity (-fold)
	Total Units	Units / L	Units / g protein	(%)	
Suspension Culture: Day 1		1			
2		5			
3		30			
4		30			
5		390			
6		590			
7		930			
8	4,840	1,240	9,690	100	-
Ultrafiltration: Retentate	5,080	8,650	14,840	105	7
Filtrate	0	0	0		

* Using syringaldazine as substrate.

medium was intended primarily for concentration of the enzyme activity rather than extensive pruification. Several methods were examined, including ultrafiltration (UF) by a thin channel system (Amicon Model TCF 10) using several membrane types, ion exchange on DEAE - cellulose followed by UF by a hollow-fiber system (Amicon Model CH4, Diaflow H1P10-20 fibers, molecular weight cut-off: 10,000), and hollow-fiber ultrafiltration alone. The latter approach was selected due to both its simplicity and the fact that no enzyme activity loss occurred. Losses were quite significant using the other methods, ranging up to 75%.

The hollow-fiber UF column was operated with an inlet pressure of about 15 psi. When the desired concentration was reached, the column was back-flushed with 0.2 M phosphate buffer, pH 5.0, to remove remaining concentrated medium from the fibers. Ultrafiltration of a typical fungal culture reduced the initial volume of approximately 4 L to about 600 ml, while laccase activity was concentrated about 7-fold (Table 2). Essentially all the protein in the culture medium remained in the concentrated solution. All concentration steps were carried out at 4°C; the final enzyme solution was also stored at 4°C until used.

A test was conducted to verify that the activity measured by reaction with syringaldagine was not attributable to peroxidase, an enzyme produced by many fungi, and which is capable of oxidizing substrates similar to those of lacccase (Harkin and Obst, 1973). Peroxidase requires peroxide as the electron acceptor; the presence of endogenous H_2O_2 in a culture medium assayed with syringaldazine would give a positive result, even when no H_2O_2 is added. Because the enzyme catalase can destroy H_2O_2 (with the formation of

water and O_2), it was added to concentrated medium (Harkin and Obst, 1973) in order to observe any effect on the assay with syringaldazine. The assay response was unchanged by medium which had been incubated with 75 ppm catalase for periods up to 2.5 hours.

2.2 Laccase Assay

Laccase activity was measured using syringaldazine N-N - bis - (3,5 - dimethoxy - 4 - hydroxybenzylidene) hydrazine (Aldrich Chem. Co., 99+% purity) as substrate (Petroski, et al, 1980). Growth medium filtrate (1.0 mL), concentrated laccase solution (0.01 mL), or laccase-treated effluent was mixed with 0.2 M potassium phosphate buffer (pH 5.0) for a total volume of 3.0 ml. Syringaldazine was added (0.02 mL of a 1.6 mg/mL solution in methanol, 4.47 mM) and after mixing, the absorbance at 525 nm was immediately measured in a Pye - Unicam UV - Visible recording spectrophotometer until the absorbance was no longer linear. The rate of absorbancy increase per minute in the linear range allows the calculation of enzyme activity as follows:

$$\frac{Abs_{525}/min}{65{,}000\ L\cdot mole^{-1}\cdot cm^{-1}} \times \frac{1}{cm} \times \frac{10^6 \mu moles}{mole} \times \frac{3\ mL}{mL\ laccase\ solution}$$

$$= \frac{\mu moles/min}{L}$$

where 65,000 is the molar extinction coefficient of the oxidized form of syringaldazine, and the light path is 1 cm in length.

One activity unit (μmoles/min) thus represents the amount of enzyme which will oxidize 1 μmole of syringaldazine per minute.

2.3 Protein Determination

A protein - dye binding method of (Bradford, 1976) was used for determination of protein in enzyme solutions. Sample (0.5 mL) was added to 5.0 mL of the dye Coomassie Brilliant Blue G-250 (aqueous solution of 0.01% dye, 4.7% ethanol, and 8.5% phosphoric acid). The solutions were mixed on a vortex mixer, and the absorbance at 595 nm was read after 10 minutes. Solutions of bovine serum albumin, from 5 to 50 μg per 0.5 ml, served as protein standards.

2.4 Effluents

Three effluent types were obtained from Weyerhaeuser's pulp and paper operations at Plymouth, N.C. Over 2,200 tons/day of materials such as fine papers, corrugating medium, liner board, brown sack paper, and fluff pulp are produced from bleached and unbleached kraft and NSSC pulps at this mill.

Biologically-treated, whole-mill effluent (BTE) was collected at the final discharge point. Plant treatment consists of primary clarification followed by biological treatment in a 72 - acre aerated lagoon (about 5 days residence time) and settling in retention ponds (10 - 14 days). The color of discharged wastewater is usually 1,400-1,500 Pt-Co units.

The chlorination and alkaline extraction stages from the bleaching of kraft pulp were the bleachery effluents studied .

All effluents were stored at 4°C and used within 6 weeks of acquisition from the mill.

2.5 Pretreatment of Effluent with Laccase

Laccase treatments were conducted in 500-mL graduated cylinders. Effluent pH was adjusted to a desired value using HCl or NaOH. Laccase solution was added to 300 mL of effluent and mixed by inversion of the cylinder. In order to ensure a sufficient supply of O_2, required by laccase for reaction, O_2 was introduced from a gas cylinder by a sparge tube immersed to the bottom of the treated solution. Treatments were carried out at room temperature for 8 or 12 hours.

The volume of concentrated laccase solution added in various treatments was based upon the activity level in the particular enzyme preparation used and the activity desired in the effluent volume (activity units/L).

Controls were prepared, consisting of effluent plus water in the same volume as the laccase solutions added in enzyme-treated samples. Other samples received heat-inactivated laccase, prepared by boiling in a water bath for 30 minutes and assayed prior to use to verify inactivation; these treatments were conducted in order to observe any effect of protein itself on color removal.

2.6 Lime Precipitation of Color

A modified jar test (Schmidt, 1979) was used for treating effluent samples with lime for color removal. Lime slurry (10% wt/wt) was added by pipette to 200 mL of effluent in a 16-oz. glass jar while the sample was stirred at half speed on a magnetic stirrer. The pH was adjusted to 12 when necessary, to provide the alkalinity for the precipitation process. Samples were then stirred at full speed for 15 seconds, followed by gentle agitation on a reciprocating shaker table at 100 oscillations/minute for 20 minutes.

After a 2-hour settling period, supernatant was withdrawn by pipette for color measurement.

2.7 Color Measurement

Color determinations were performed according to NCASI (NCASI Tech. Bull. No. 253, 1971). Samples, or suitable dilutions, were adjusted to pH 7.6 $\pm$ 0.1 with NaOH or HCl and filtered on 0.8 μm membrane filters (Metricel, Gelman Sciences) to remove suspended solids. Absorbance was measured at 465 nm and compared to standard solutions of potassium chloroplatinate (K_2PtCl_6) - cobaltous chloride ($CoCl_2 \cdot 6\ H_2O$). Color is thus expressed in "Pt-Co units".

2.8 Conductometric Titration

Effluent samples were filtered on 0.8 μm membrane filters prior to titration. During the titration of 200-mL samples will 0.1 N NaOH or 0.1 N HCl, conductivity (Barnstead conductivity bridge) and pH were measured. In a plot of conductivity versus milliequivalents of titrant, a break-point at approximately pH 10 can be interpreted as representing the presence of "very weakly acidic" groups such as phenolic and enolic hydroxyls (Bennett, et al, 1971).

3. RESULTS AND DISCUSSION

3.1 Biologically-Treated, Whole-Mill Effluent

3.1.1 Lime Precipitation of Color from Non-Pretreated BTE

Biologically-treated, whole-mill effluent was selected as a source of color bodies for laccase-lime studies because they cannot be removed by conventional secondary treatment. They, therefore, require another method for removal. Additionally, color reversion has been reported in kraft pulping and bleaching wastewaters when treated with lime prior to biological treatment (Lang and Miller, 1977).

Because laccase application is intended as a pretreatment of effluent before lime precipitation of color, it was necessary to first determine the color removal efficiency of lime from non-pretreated BTE. Lime dosages from 50 to 7,500 mg/L were applied in a modified jar test, and the color of each supernatant after settling was measured (Figure 11). A maximum of 93% color removal, at 5,000 mg/L lime, was obtained. Lime levels that remove less than the maximum amount of color should be more sensitive to any effects of a pretreatment. Dosages of 500 mg/L or less were used in trials where such sensitivity was desired.

3.1.2 Color Removal from Laccase-Pretreated BTE at Several Lime Dosages

Before investigating the effect of laccase pretreatment on lime precipitation of color, it was necessary to establish that enzyme activity is in fact sustained in the environment of the efluent. Effluent pH was adjusted to 5, and concentrated laccase was added at a level of 150 units/L. The solution was sparged with O_2 for 8 hours; aliquots were then withdrawn each

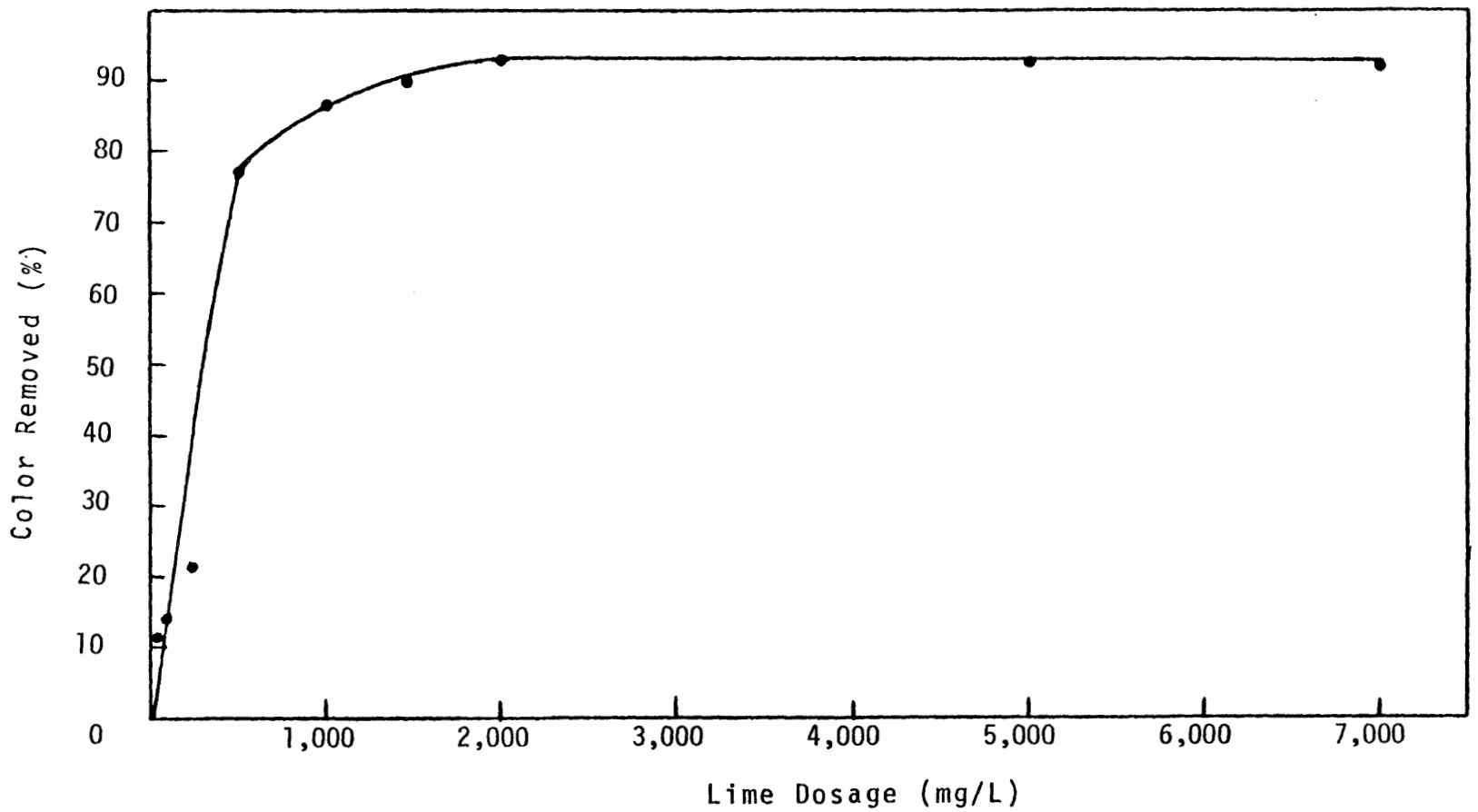


Figure 11. COLOR REMOVAL FROM BTE BY LIME.

hour for enzyme assay. The mean activity over the 8-hour period was 118 $\pm$ 9 units/L (Table 3), or about 80% of the applied activity. Fluctuations in activity were probably due to sampling variability, as the enzyme assay itself was quite reproducible on a given aliquot. It is apparent that, although laccase activity in the effluent was less than the amount calculated from that in the concentrated enzyme solution, activity was essentially maintained during an 8-hour period. Enzyme activity in all subsequent pretreatments was measured immediately after laccase addition and at the end to confirm that enzyme viability was maintained. In all cases, values were found to be similar at the two sample times.

The effect of laccase pretreatment on color removal by lime was next examined. A laccase level of 150 units/L was used in these experiments. This addition is twice that used in similar studies of horseradish peroxidase pretreatments of biologically-treated pulping effluent (Schmidt, 1979). BTE was pretreated for 8 hours at a pH of 5, a value frequently found to be optimal for reaction of fungal laccase with a variety of substrates (Leonowicz and Grzywnowicz, 1981; Fahraeus and Reinhammer, 1967). While the pretreatment conditions were not all necessarily optimal, they appeared to be reasonable for initial studies. A range of lime dosages was used in order to determine if pretreatment might extend the removal efficiency beyond that achievable at high lime levels and also whether high removals could be obtained using less lime. A set of samples was pretreated for each lime dosage; pretreatments were with both laccase and inactivated laccase. At three of the dosage levels (100, 500, and 1,000 mg/L), samples were also pretreated with an enzymatically inactive protein, bovine serum albumin (BSA) at the same protein concentration

TABLE 3. LACCASE ACTIVITY IN BTE DURING 8-HOUR PRETREATMENT PERIOD AT pH 5. Laccase applied at 150 units/L.

PRETREATMENT TIME (Hr)	LACCASE ACTIVITY (Units/L)
0	115
1	125
2	135
3	110
4	115
5	125
6	110
7	110
8	120

in the BTE as for enzyme additions (4 mg/L). This protein served as an additional control for observing the effect of such protein polymers on lime precipitation of color.

Color removals from these treatments are shown in Figure 12. At all lime dosages, laccase pretreatment improved color reduction as compared to controls which contained no enzyme. At 100 mg/L lime, 10% of the inital color (1,500 Pt-Co units) was removed from the control and 28% from laccase-pretreated BTE. Pretreatment with inactived laccase, however, also resulted in an increase in color removal, at 23%. Assays of the samples containing inactived enzyme revealed no activity, at both inital and end points of the pretreatment. At 500 mg/L lime, laccase again increased the removal of color, 82% compared to 78% from the control, but this higher level of lime masked the more evident effect seen at 100 mg/L. Color removal from BTE pretreated with inactived laccase again fell between that of the control and laccase samples, at 80%. When 1,000 mg/L lime was applied, laccase pretreatment allowed 94% removal compared to 87% from the control, regardless of whether the enzyme was active or inactive. Finally, using 150 mg/L lime, the relative results were similar, with color reductions of 90% for the control, 97% for laccase-pretreated effluent, and 95% from BTE containing inactive laccase.

An effect similar to that seen with inactived laccase has been previously reported in investigations using peroxidase (Schmidt, 1979). When biologically-treated pulping effluent was pretreated with peroxidase and H_2O_2, lime precipitation removed 94% of color at a lime dosage that allowed only 10% removal from controls. Effluent pretreated with peroxidase alone, which is enzymatically inactive in the absence of peroxide, or with boiled peroxidase,

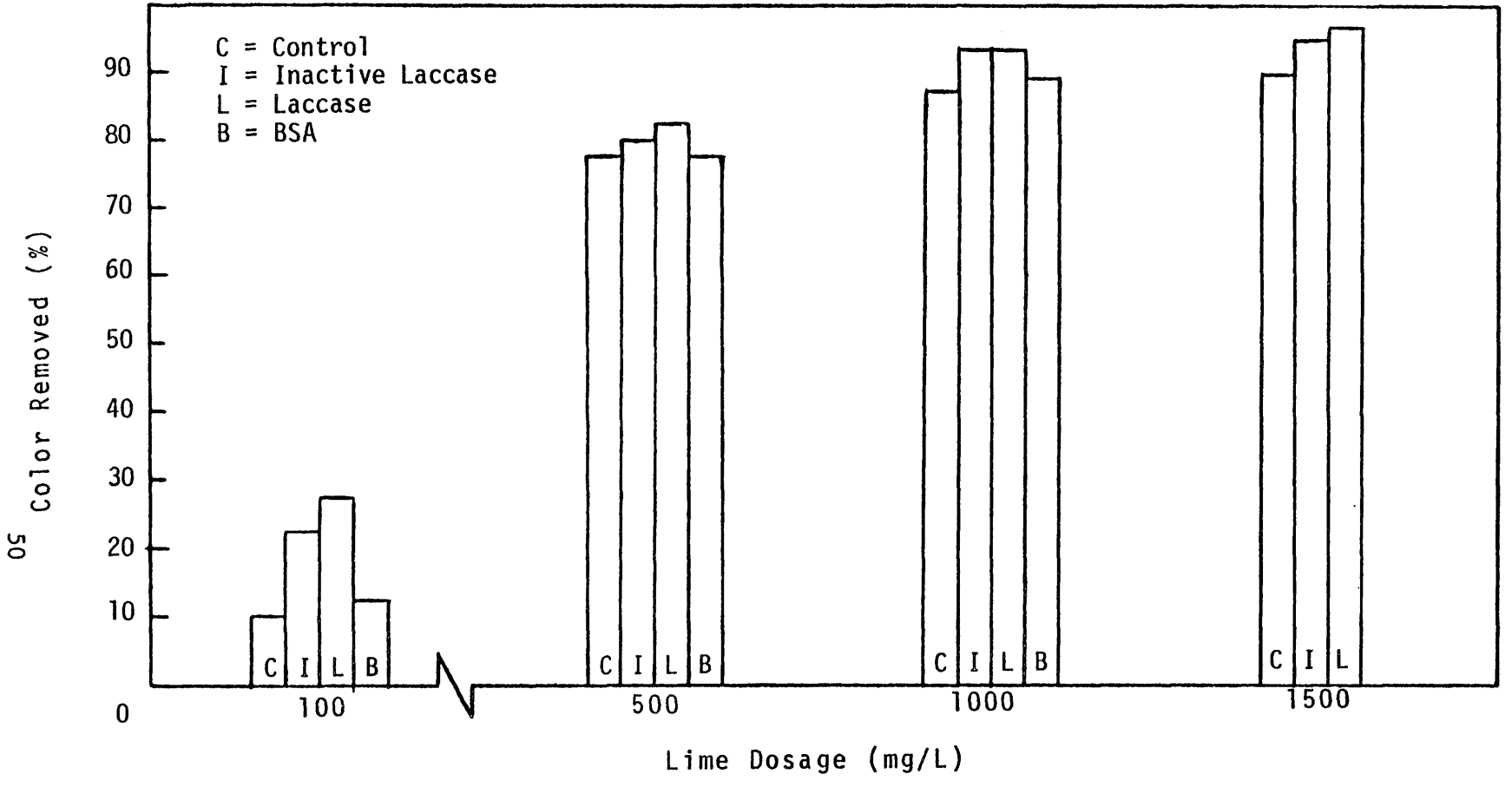


Figure 12. COLOR REMOVAL BY LIME FROM BTE PRETREATED FOR 8 HOURS AT PH 5. Laccase = 150 units/L; Protein = 4 mg/L.

was also greatly decolorized by lime, with an 85% reduction. An explanation suggested by Schmidt (1979) for this effect by protein involves the calcium ion as a bridge between protein and color molecules. At the high pH of lime treatement (pH 12) protein molecules are negatively charged, mainly due to ionization of carbonyl groups. It was hypothesized that positively-charged calcium ions bridge protein to the color bodies, which are also negatively charged, thereby forming a higher molecular weight complex more susceptible to precipitation than in the absence of the protein. Laccase, with a molecular weight of about 64,400, is a relatively acidic protein (Fahraeus and Reinhammar, 1967), i.e., it contains a relatively large proportion of acidic amino acids, such as aspartic and glutamic acids. Furthermore, the protein content of the laccase solution could include others beside laccase, although the culture procedure used has been claimed to yield a medium in which the enzyme is essentially the only protein present (Fahraeus and Reinhammar, 1967). Other proteins in the impure solution could contribute to the behavior observed.

Pretreatment of BTE with the serum albumin, however, affected color removal very little or not at all at lime dosages of 100, 500, and 1,00 mg/L (Figure 12). The molecular weights of laccase and BSA are similar (64,400 and 66,000, respectively). The difference in response using these two proteins may lie in their different physical structures. The configuration of some laccases is reported to be elongated (Mayer and Harel, 1979), whereas, the globular structure of BSA would be expected to expose fewer of its ionizable groups for interaction with the solution components.

Another explanation for the increased color removal from samples pretreated with boiled enzyme might lie in the possible agglomeration of the denatured material, such that a surface is provided for adsorption of color. Inactivation by boiling is, therefore, not a true control for the effect of protein.

Regardless of the mechanism of removal enhancement by inactived enzyme, this series of treatments demonstrated that at the laccase application level used here, the enzyme pretreatment does not, in a substantial manner, either extend the maximum color removal possible by lime precipitation or allow the use of significantly less lime to achieve large color reductions. Further pretreatments of BTE with laccase attempted to discover if color removal could be improved by increased levels of enzyme, longer pretreatment time, or a different pretreatment pH.

3.1.3 Effects of Laccase Application Level

Color removal from BTE pretreated at laccase application levels of 300 and 1,000 units/L were compared to that at 150 units/L. Pretreatment pH was 5 and the lime dosage was 500 mg/L. The mean control removal was 77%; all pretreatment removal values were scaled based upon this value (Figure 13). Comparison of the removal percentage for the three laccase levels shows very little effect with increased application. Doubling the level gave essentially no improvement, while addition of almost 7 times as much laccase only raised the efficiency by 4 percentage points (81% versus 85%). This increase over controls (85% versus 77%) is probably not sufficient to be of commercial significance. Extention of the 8-hour pretreatment time by one-half, to 12 hours, had no effect using 300 units/L laccase.

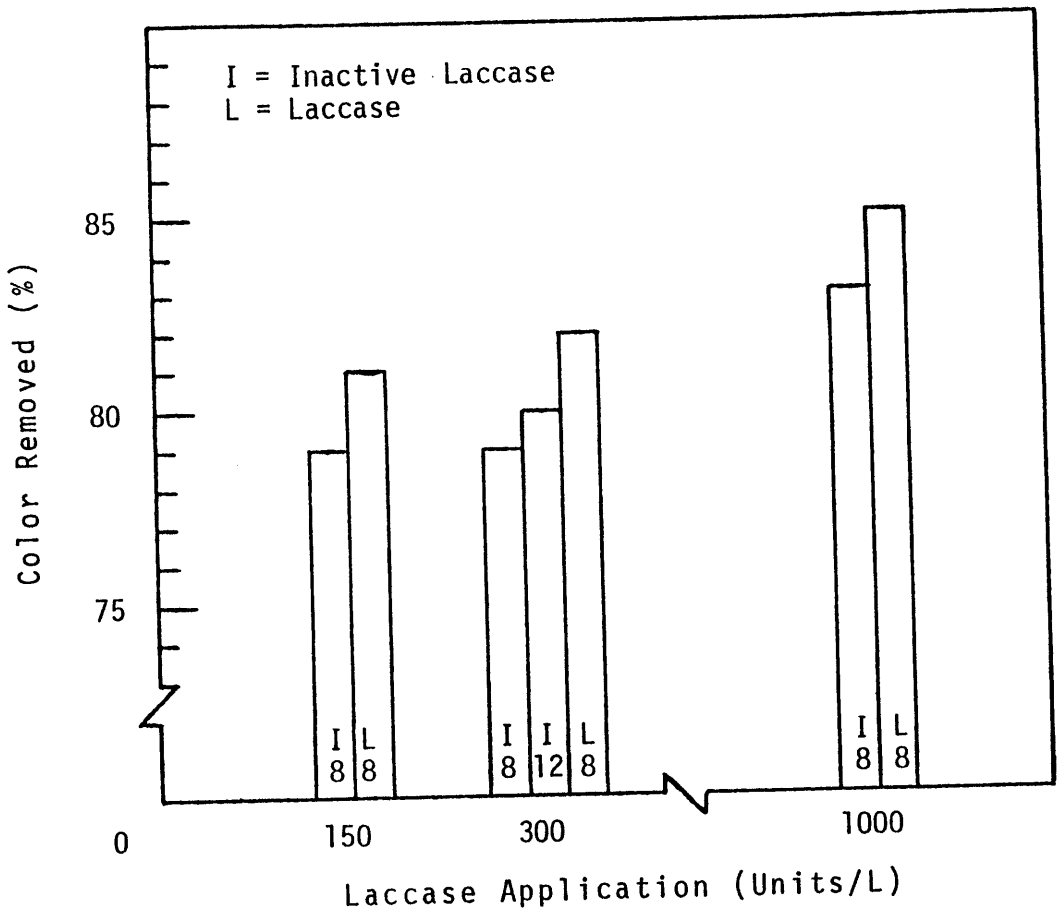


Figure 13. EFFECT OF LACCASE APPLICATION LEVEL AT PH 5 ON COLOR REMOVAL FROM BTE BY 500 MG/L LIME. Pre-treatments for 8 or 12 hours. Values scaled based upon mean control removal of 77%.

At all laccase levels, color removal from effluent containing inactived laccase was only 2-3 percentage points less than with active laccase. Color removal with inactive enzyme does not appear to be greatly influenced by protein concentration in the effluent within the range investigated (3.5, 7, and 22 mg/L).

3.1.4 Effect of Pretreatment pH

In an attempt to increase the small color removal enhancement seen at pH 5, samples of BTE were next pretreated with 150 units/L at several other pH values. It has been demonstrated by other workers (Klibanov, *et al*, 1980) that the extent of polymerization of various phenolic compounds, as catalyzed by peroxidase, was a function of treatment pH, although the maximum efficiency covered a broad pH range for most compounds examined. For this series of laccase treatments, a lime dosage of 250 mg/L was selected. This dosage should be low enough to be sensitve to any differing effects of pretreatment, and also indicates the potential for a given treatment to achieve a high removal with less lime than that required without lacase. The results for controls and pretreated samples are shown in Figure 14. Unexpectedly, color removal from laccase-pretreated effluents was less than that from controls for every pretreatment pH except 7.5, the unadjusted pH of BTE. Replications of treatments at several of the pH values verified these results. The effluent from which samples were taken was obtained from the same mill, but at a later date than the previous effluent. Effluent color was approximately the same at all dates of mill sampling. Storage time was not a factor in this change in effluent response to pretreatment, since samples taken in the first week of storage gave similar results to samples taken after four weeks. All

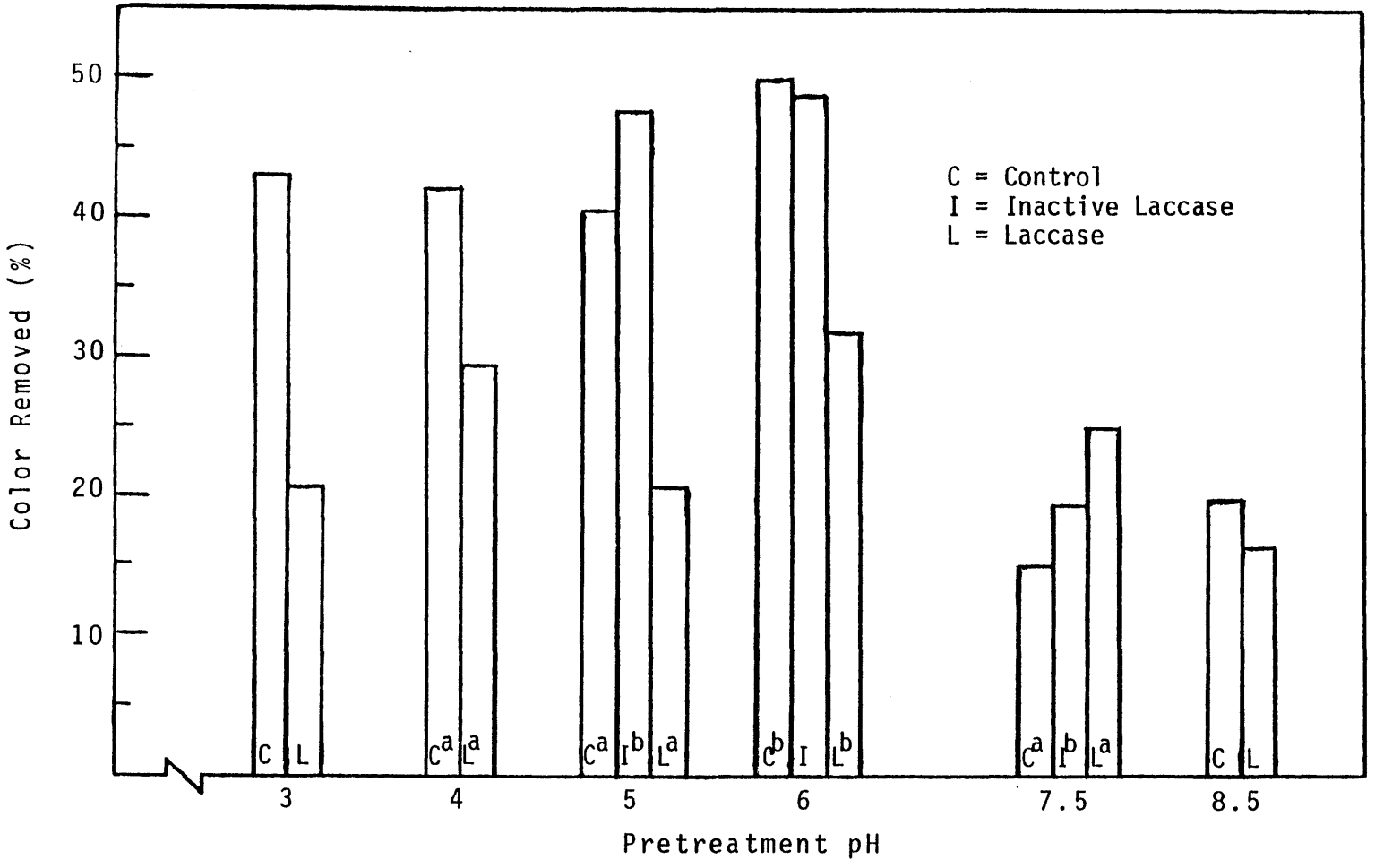


Figure 14. EFFECT OF PRETREATMENT PH ON COLOR REMOVAL FROM BTE BY 250 MG/L LIME. Laccase = 150 units/L; pretreatment time = 8 hours. ([a]Mean of 3 trials; [b]Mean of 2 trials.)

experimental conditions were identical to those earlier described, including use of the same solution of concentrated enzyme which was unchanged in activity. Assays of laccase activity in effluents treated at pH 5 were of the same magnitude as in past experiments. Confirmation that the different effluent behavior was not due to experimental differences was seen by the simultaneous treatment at pH 5 of BTE from the most recent sampling date and from BTE obtained about 5 weeks earlier. Color removals were equivalent to those found before for each sample; removal from older samples was increased by laccase pretreatment, but, for the most recent BTE, removal from samples containing laccase was again less than that for the control at pH 5. Effluent from all subsequent sampling dates from the mill exhibited this same response to pretreatment.

Conductometric titration of BTE from both types of sample showed little difference in "very weakly acidic" functional groups (primarily phenolic and enolic hydroxyls). Effluent that had responded to laccase pretreatment with increased color removal contained 1.9 milliequivalents per gram of organic solids (2.6 milliequivalents/L), while the value for BTE from the later sample date was 2.8 (5.3 milliequivalents/L). Although this determination is a somewhat approximate analysis, these results suggest that the difference in effluent behavior may not be due to a difference in the amount of phenolic substrate available for laccase attack.

The series of pretreatments at several pH levels demonstrated an effect of pH on color removal from the control. At a pH of 3, 4, 5, or 6, about 40% to 50% removal was seen, while at pH 7.5 or 8.5, this value was about 20%. Adjustment of pH below the normal BTE pH of 7.5 could reduce the solubility of color bodies during the pretreatment, but samples were always readjusted to pH 12 during the lime addition procedure. This last step would, presumably, cause resolubilization.

For pretreatments with inactived enzyme (pH 5, 6, and 7.5), color removal was either the same as controls or slighter greater, as seen before.

It is not clear why active laccase caused less removal than either the controls or inactive enzyme for all pretreatment pH levels except 7.5. More detailed information, such as the effect of the various pretreatments on the molecular weight distribution of color bodies, would be required for a better understanding of the results seen.

3.1.5 Effect of Spiking with Phenolic Compounds

In an investigation of peroxidase treatment of wastewaters for the removal of phenolic compounds by polymerization, Klibanov and coworkers (1980) found that compounds which were not very effectively removed by precipitation could be almost entirely removed in the presence of a phenol that has a high reactivity with peroxidase. Based upon these findings, the compound o-cresol (2-methylphenol) was chosen for spiking BTE during pretreatments with laccase to determine if improvements in color removal could be obtained. An 86% removal of o-cresol had been reported by treating a 1 mM solution with peroxidase at an application level of 150 units/L. A pH of 4 was optimal for removal using peroxidase.

Because the pH optimum using laccase was not known, 1 mM solutions of o-cresol were prepared in phosphate buffers of pH 4,5,6, and 7 and treated with 150 units/L laccase. Visual assessment of the amber color that was produced over an 8-hour period was made and taken as an approximate indication of the extent of reaction. A pH of 5 or 6 gave the darkest response after 8 hours, although at pH 6 color was slower to develop. The solution at pH 4 was almost as dark but even slower in developing. Only a very slight color was produced at pH 7. Adjustment of these solutions to a higher or lower pH after the treatment period did not immediately alter the color intensities, suggesting that the different colors observed were not solely a function of the degree of ionization of the reaction product.

Three pH levels, 5, 6, and 7.5, were selected for laccase pretreatments of BTE spiked with o-cresol. Although the cresol reaction was low at pH 7, pH 7.5 was included because laccase was shown to have some effectiveness (Figure 14). The treatments and controls for the spike studies are presented in Table 4. Laccase pretreatments received 150 units/L. Cresol was added at either 0.05 mM or 1 mM for samples at pH 5 and 1 mM for those at pH 6 and 7.5. After an 8-hour pretreatment period with O_2 sparging, the lime test was performed.

Cresol alone was colorless and remained so at all pretreatment pH levels and after lime addition. The same was true when inactive laccase was added to cresol. BTE, containing cresol but no enzyme, gave essentially the same results after lime as for BTE alone. Therefore, no color contributions arose as a consequence of treatment with cresol in the absence of active laccase. However, when active laccase was added to solutions of cresol at a pH of 5 or 6, an amber color and a cloudy suspension immediately appeared, as was seen

Table 4. BTE - CRESOL SPIKE STUDY: TREATMENTS AND CONTROLS

CONTROL	TREATMENT
BTE	BTE + Laccase
	BTE + Inactive Laccase
BTE + Cresol	BTE + Cresol + Laccase
	BTE + Cresol + Inactive Laccase
Cresol	Cresol + Laccase
	Cresol + Inactive Laccase

previously. These changes were not observed at pH 7.5. After pretreatment at pH 5 or 6, color was approximately 250 Pt-Co units. Lime addition at 250 mg/L removed about one-half of this color.

Figure 15 shows the effect of 1 mM cresol spiking on color removal by 250 mg/L lime for pH 5 pretreatments. A level of 0.05 mM cresol had no effect. Removals are shown after the 8-hour pretreatment as well as after lime. The greatest effect of cresol on removal was seen for BTE containing laccase. Without cresol, removal by lime was less than for the control, 20% compared to 43%. Pretreatment with laccase and cresol caused the effluent to become visibly darker with suspended material, presumably a colored product from enzymatic polymerizatin of cresol and possibly of phenolics present in the effluent. Not only was removal by lime increased to 63%, but a 40% removal occurred after the pretreatment itself. Suspended material and color were removed by the filtration step of the color measurement procedure performed on the sample after pretreatment with laccase and cresol before the addition of lime. Another portion of this BTE pretreated with laccase and cresol was centrifuged at 18,000 rpm for 30 minutes; a small, dark pellet formed. Color determination on the supernatant showed a 37% removal of color. Centrifugation or filtration of the controls and pretreated samples having no cresol removed very little (7%) or no color.

The increased color removal caused by cresol addition to BTE containing laccase does not necessarily confirm that color bodies were participating in enzymatic formation of polymers with cresol. The possiblity exists that color was adsorbed onto the suspended material and removed during subsequent filtration, centrifugation, or lime precipitation. Regardless of the

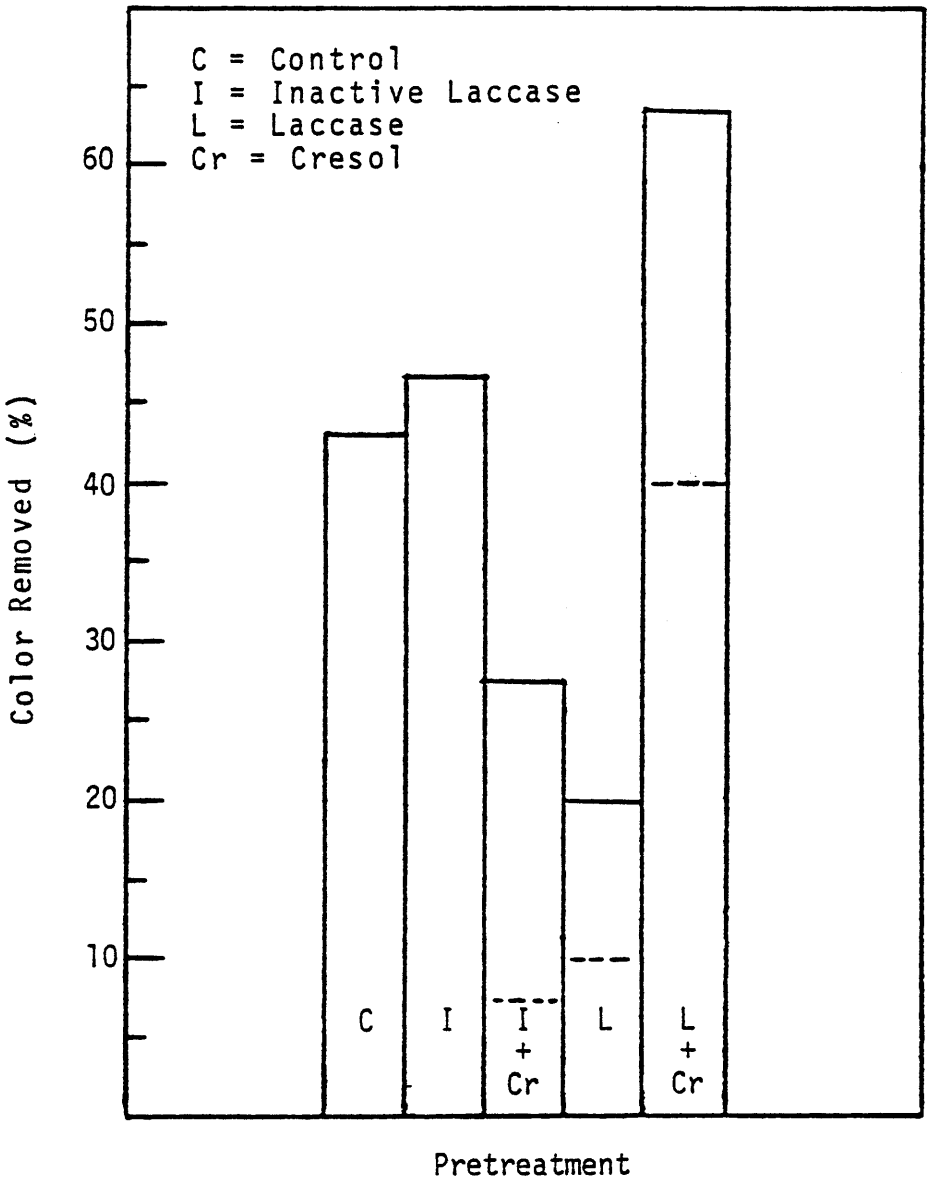


Figure 15. EFFECT OF O-CRESOL (1 mM) ON COLOR REMOVAL BY 250 MG/L LIME FROM BTE PRETREATED FOR 8 HOURS AT PH 5. Laccase = 150 units/L. Dashed lines represent color removed after pretreatment (before lime).

mechanism of this increased removal, the improvement is not of the magnitude required for commercial application.

Results from the cresol spike at pH 6 are presented in Figure 16. Phenol (1 mM) was also used in BTE at this pH; visual observation of buffered phenol solutions containing laccase had shown pH 6 to give the greatest color development. A higher lime dosage, 500 mg/L, was applied to increase the potential for any removal enhancements to approach 90% removal. The control and the inactived enzyme pretreatment removed approximately the same amount of color, 77% and 75%, respectively. The laccase pretreatment gave less color reduction (57.5%) than the control, as seen before. Phenol was ineffective in improving the color reduction in laccase pretreatment, and cresol addition only raised the color removal to the level of the control. The failure to enhance color removal beyond the control level at this lime dosage perhaps implies a limitation to the cresol spike; the cresol spike was apparently only able to compensate for the negative effect of laccase on color removal. As in the spike conducted at pH 5, the sample containing laccase and cresol became visibly darker during the pretreatment period, and during filtration for color measurement underwent a 52% color reduction. Centrifugation removed 68% of the color.

Spiking with cresol at pH 7.5 did not change color removals from any sample (Figure 17). Darkening of test solutions or formation of suspended material was not observed during pretreatment. Filtration and centrifugation of pretreated samples had no effect on color removal. Although laccase is capable of causing a slight improvement in color removal at this pH,

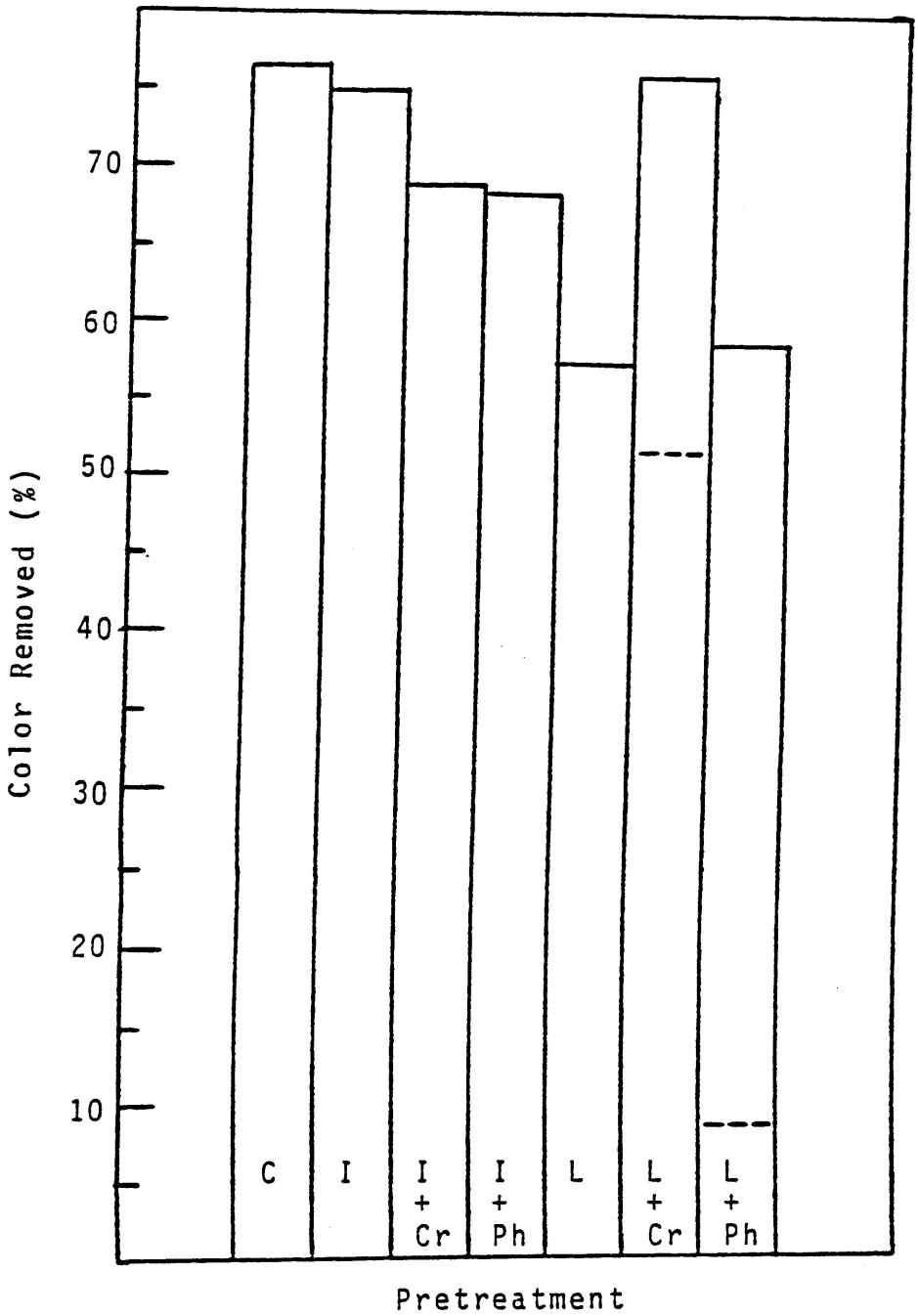


Figure 16. EFFECT OF O-CRESOL (1 mM) OR PHENOL (1 mM) ON COLOR REMOVAL BY 500 MG/L LIME FROM BTE PRETREATED FOR 8 HOURS AT PH 6. Laccase = 150 units/L. Dashed lines represent color removed after pretreatment (before lime). C = Control, I = Inactive Laccase, L = Laccase, Cr = Cresol, and Ph = Phenol.

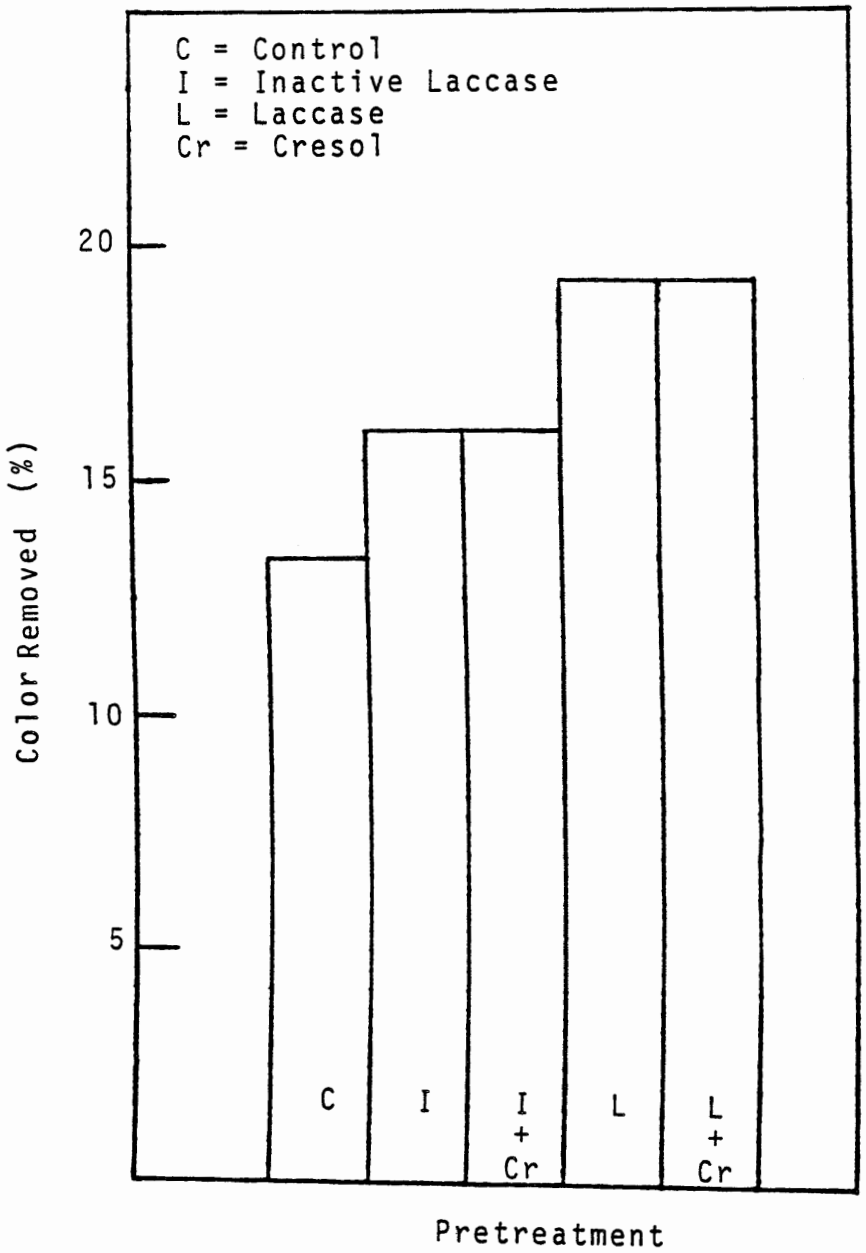


Figure 17. EFFECT OF O-CRESOL (1 mM) ON COLOR REMOVAL BY 250 MG/L LIME FROM BTE PRE-TREATED FOR 8 HOURS AT PH 7.5. Laccase = 150 units/L.

apparently the reaction with cresol is insufficient to produce results similar to those at pH 5 and 6.

3.2 Extraction Stage Effluent

3.2.1 Lime Precipitation of Color from Non-Pretreated E-Stage

It was of interest to examine the effect of laccase on color removal from E-stage effluent by lime, as this wastewater is the single largest contributor to pulp mill color loadings. The color of this effluent, obtained from the bleaching of softwood pulp, was approximately 9,000 Pt-Co units. Phenolic hydroxyl groups, the substrate for laccase, are generally quite low in concentration in E-stage effluents (Bennett, et al, 1971). Conductometric titration showed 5.1 milliequivalents of "very weakly acidic" groups per liter, or 1.8 per gram of solids, a value similar to that found for BTE. Bennett and coworkers (1971) reported 14 milliequivalents per gram of organic solids for E-stage effluent.

Color removal from non-pretreated E-stage effluent as a function of lime dosage is shown in Figure 18. Lime levels from 500 to 15,000 mg/L were used. A maximum color removal of 93% was observed at 15,000 mg/L, although one-half that dosage, 7,500 mg/L, removed 89%.

3.2.2 Effect of Pretreatment pH

Samples of E-stage effluent were adjusted to a pH of 3, 4, 5, 6, and 7 and pretreated with 150 units/L laccase for 8 hours with oxygen sparging. Assay of laccase activity in the pH 5 sample using syringaldazine as substrate showed activity to be essentially unchanged from the beginning to the end of pretreatment. Activity was about 95 units/L.

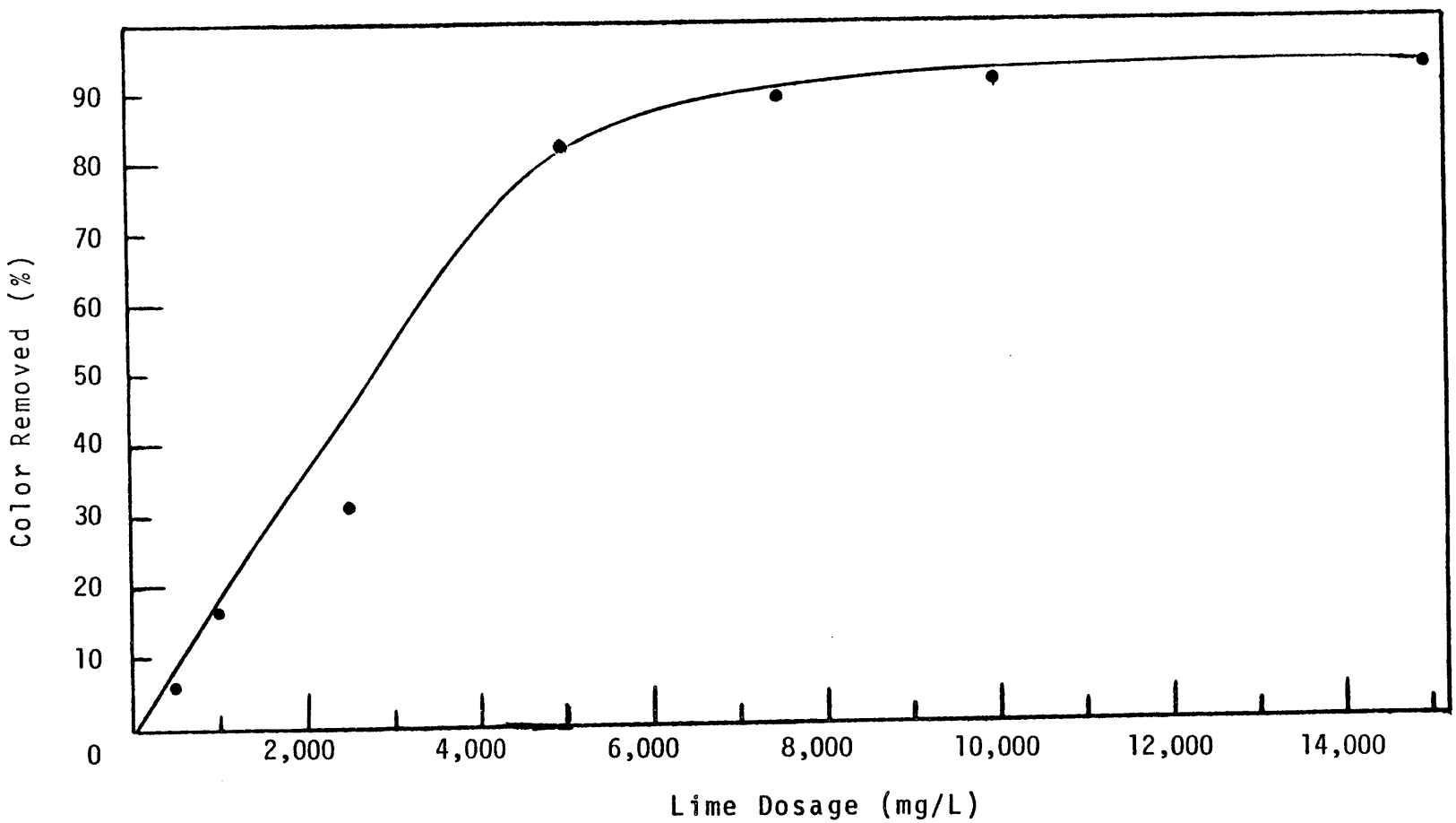


Figure 18. COLOR REMOVAL FROM E-STAGE EFFLUENT BY LIME.

Color removal after addition of 2,500 mg/L lime to the samples is shown in Figure 19. All controls were higher than seen with this lime dosage on non-pretreated E-stage effluent (Figure 18), where removal was about 30%. Apparently, treatment at a lower pH for several hours prior to lime addition causes greater color removal than from unadjusted effluent (about pH 10). A similar phenomenon was observed with BTE, although much less pronounced. None of the laccase pretreatments succeeded in significantly improving color removal. It is probable that the phenolic content of E-stage effluent is too low to produce a substantial effect by reaction with laccase. Another possiblity is that phenolic structures, if present, are chlorine-substituted at critical positions on the ring, such that after the enzymatic formation of a phenoxy free radical, other mesomeric forms are prohibited. Condensation does not occur between two phenoxy free radicals.

3.2.3 Effect of Spiking with Phenolic Compounds

The results of spiking E-stage effluent with 1 mM cresol or phenol during pretreatment at pH 5 are shown in Figure 20. A lower lime dosage, 1,000 mg/L, was used so as not to obscure any effects of the pretreatments. Color removal was 54% from the control, but only about 20% from pretreatments with active or inactived laccase, regardless of the addition of cresol or phenol. Neither filtration nor centrifugation removed a significant amount of color. Darkening of the spiked samples was not apparent, although the highly colored effluent could have prevented a noticable change.

3.3 Chlorination Stage Effluent

3.3.1 Lime Precipitation of Color from Non-Pretreated C-Stage

Chlorination stage effluent had a color of approximately 900 Pt-Co

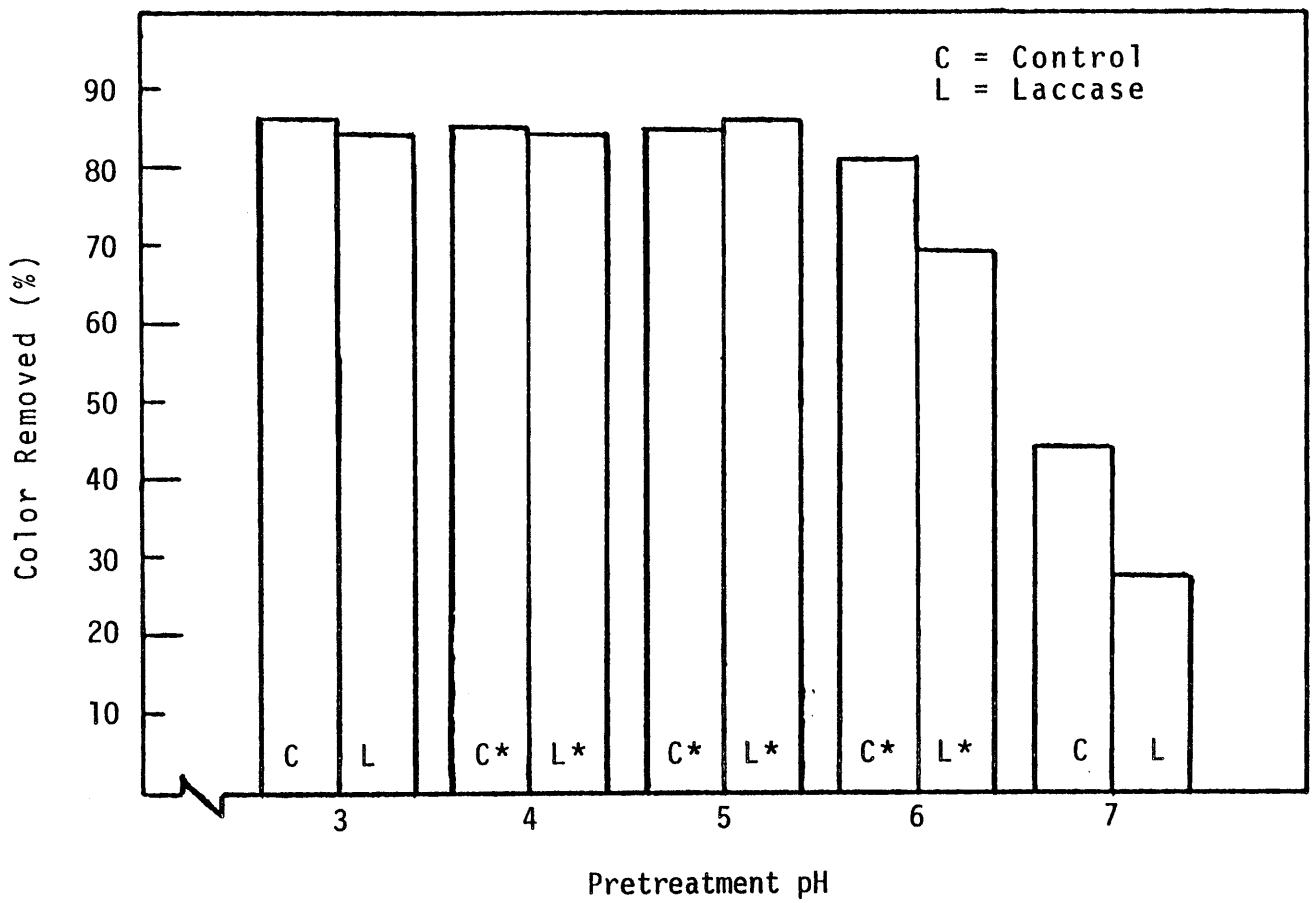


Figure 19. EFFECT OF PRETREATMENT PH ON COLOR REMOVAL FROM E-STAGE EFFLUENT BY 2500 MG/L LIME. Laccase = 150 units/L; pretreatment time = 8 hours. (*Mean of 2 trials).

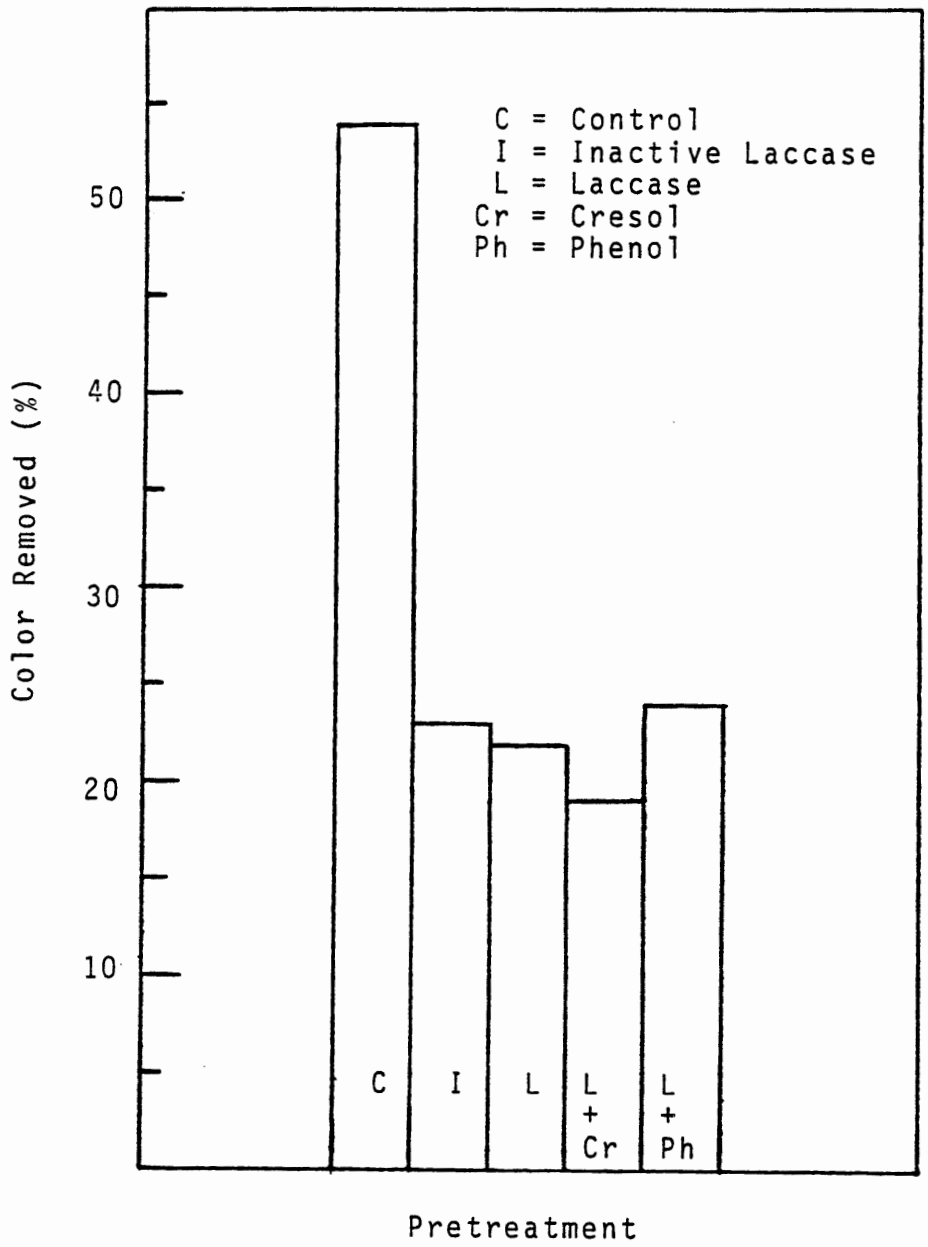


Figure 20. EFFECT OF O-CRESOL (1 mM) OR PHENOL (1 mM) ON COLOR REMOVAL BY 1000 MG/L LIME FROM E-STAGE EFFLUENT PRETREATED FOR 8 HOURS AT PH 5. Laccase = 150 units/L.

units. Conductometric titration gave a value of 3.3 milliequivalents of "very weakly acidic" groups or 1.4 per gram of solids, similar to that for BTE and E-stage effluent. Bennett and coworkers (1971) reported a value of 11.7 milliequivalents per gram of organic solids.

Figure 21 shows color removal from non-pretreated C-stage effluent according to lime dosage. The highest color removal obtained was 78% after the addition of 8,000 mg/L lime.

3.3.2 Effect of Pretreatment pH

Laccase applications of 150 units/L were successful in improving color removal by 1,000 mg/L lime when C-stage was pretreated at a pH of 4, 5, 6, 7, 8, or 9 for 8 hours with oxygen sparging (Figure 22). The greatest increase occurred at pH 6, with 69% removal from effluent containing laccase and 57% from the control. Color removals from samples pretreated with inactive laccase were not examined. In view of the fact that the laccase pretreatment was only moderately successful, no further efforts were directed at color removal from this relatively small color source.

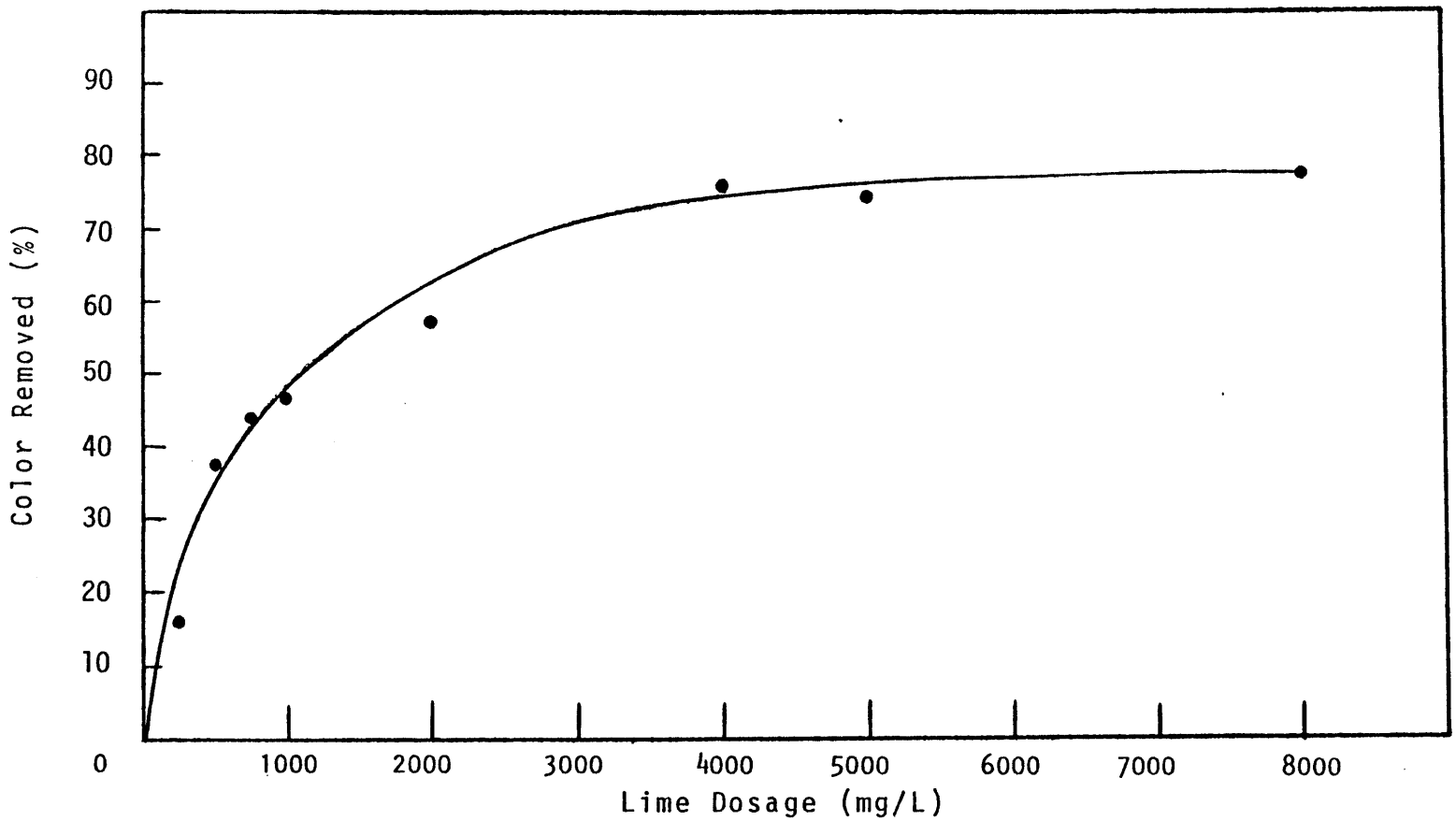


Figure 21. COLOR REMOVAL FROM C-STAGE EFFLUENT BY LIME.

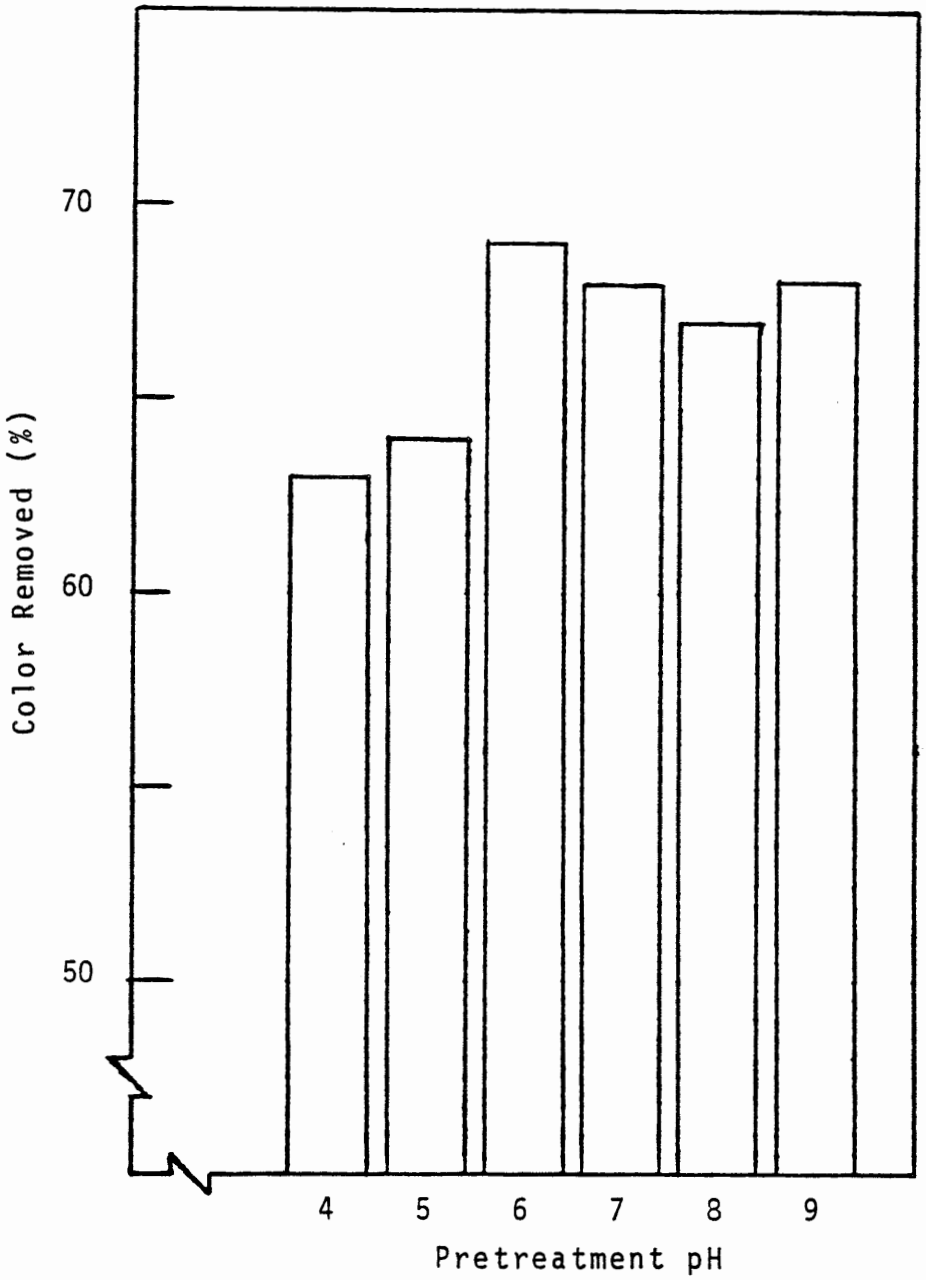


Figure 22. EFFECT OF PRETREATMENT PH ON COLOR REMOVAL FROM C-STAGE EFFLUENT BY 1,000 MG/L LIME. Laccase = 150 units/L; pretreatment time = 8 hours. Values from laccase pretreatments scaled based upon mean control removal of 57%.

References

Alberti, B.N., Klibanov, A.M., "Enzymatic Removal of Dissolved Aromatics from Industrial Aqueous Effluents". Paper presented to American Chemical Society, New York, Aug., 1981.

American Water Works Association, "Coagulation and Color Problems", Journal of the American Water Works Association 62: 311-314, 1970

Amicon Corporation, Amicon Products for Separations Technology, Publication No. 553, Scientific Systems Divison, Danvers, Mass., 1981.

Ander, P., Eriksson, K.-E., "The Importance of Phenol Oxidase Activity in Lignin Degradation by the White-Rot Fungus Sporotrichum pulverulentum", Archives of Microbiology 109: 1-8, 1976.

Andersson, K.A., "The Non-Polluting Bleach Plant", TAPPI 60 (3): 95-96, 1977.

Bennett, D.J., Dence, C.W., Kung, F.-L., Luner, P., Ota, M., "The Mechanism of Color Removal in the Treatment of Spent Bleaching Liquors with Lime", TAPPI 54 (12): 2019-2026, 1971.

Bouveng, H.O., Solyom, P., "Long Term Stability of Waste Lignins in Aquatic Systems", Svenk Papperstidning 76: 26-29, 1973.

Bradford, M.M., "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding", Analytical Biochemistry 72: 248-254, 1976.

Bureau of the Census, U.S. Department of Commerce, Current Industrial Report Series M262A, Pulp, Paper, and Board, 1979.

Campbell, A.G., Gerrard, E.D., Joyce, T.W., Chang, H.-m., and Kirk, T.K., "The MyCoR Process for Color Removal from Bleach Plant Effluent: Bench Scale Studies", Proceedings of the 1982 TAPPI Research and Development Conference, Asheville, N.C., Aug. 29- Sept. 1, 1982.

Casey, J.B. Pulp and Paper Chemistry and Chemical Technology, Vol. I, 3rd edition, John Wiley and Sons, New York, 1980.

Christman, R.F., Ghassemi, M., "Chemical Nature of Organic Color in Water", Journal of the American Water Works Association 58: 723-741, 1966.

Cruver, J.E., "Reverse Osmosis - Where It Stands Today", Water and Sewage Works, pp. 74-78, Oct., 1973.

Davis, C.L., Jr., "Lime Precipitation for Color Removal in Tertiary Treatment of Kraft Mill Effluent at the Interstate Paper Corp., Riceboro, Ga.", Chemical Engineering Progress Symposium Series 67: 458-465, 1971.

Dogherty, E., "Mutant Bacteria Undergo Field Evaluation", Pulp and Paper Canada 83: 32-33, 1982.

Dugal, H.S., Swanson, J.W., Dickey, E.E., Buchanan, M.A., "Effect of Lime Treatment on Molecular Weight Distribution of Color Bodies from Kraft Linerboard Decker Effluents", TAPPI 58: 132-135, 1975.

Eaton, D., Chang, H.-m., Kirk, T.K., "Fungal Decolorization of Kraft Bleach Plant Effluents", TAPPI 63: 103-106, 1980.

EPA DEVELOPMENT DOCUMENT FOR INTERIM FINAL AND PROPOSED EFFLUENT LIMITATIONS, AND PROPOSED NEW SOURCE PERFORMANCE STANDARDS, VOL. 1, EPA 440/10-76/047-a, 1976.

Evans, C., Herbert, D., Tempest, D., "The Continuous Cultivation of Micro-Organisms. 2. Construction of a Chemostat", Chapter 13 in Methods in Microbiology, Vol. 2, Academic Press, New York, 1970.

Fahraeus, G., Reinhammar, B., "Large Scale Production and Purification of Laccase from Cultures of the Fungus Polyporus versicolor and Some Properties of Laccase A ", Acta Chemica Scandinavica 21: 2367-2378, 1967.

Freudenberg, K., Neish, A.C., Constitution and Biosynthesis of Lignin, Springer - Verlag, Berlin - Heidelberg - New York, 1968.

Ganczarczyk, J., Obiaga, T., "Mechanism of Lignin Removal in Activated Sludge Treatment of Pulp Mill Effluents", University of Toronto, Civil Engineering Publication 74-08, April, 1974.

Gierer, J., Opara, A.E., "Studies on the Enzymatic Degradation of Lignin. The Action of Peroxidase and Laccase on Monomeric and Dimeric Model Compounds," Acta Chemica Scandinavica 27: 2909-2922, 1973.

Gould, M., "Color Removal from Kraft Mill Effluent by an Improved Lime Process", TAPPI 56: 79-83, 1973.

Haars, A., Huttermann, A., "Function of Laccase in the White-Rot Fungus Fomes annosus", Archives of Microbiology 125: 233-237, 1980.

Hardell, H.-L., de Sousa, F., "Characterization of Spent Bleaching Liquors. Part 1. Spent Liquors form the Chlorine and Alkali Extraction Stages in the Prebleaching of Pine Kraft Pulp", Svensk Papperstidning 4: 110-120, 1977.

Harkin, J.M., Obst, J.R., "Syringaldazine, An Effective Reagent for Detecting Laccase and Peroxidase in Fungi", Experimentia 15: 381-387, 1973.

Ishihara, T., "The Role of Laccase in Lignin Biodegradation", in Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications, Vol. II, edited by T.K. Kirk, T. Higuchi, and H.-m. Chang, CRC Press, Inc., Boca Raton, Florida, 1980.

Kirk, T.K., Conners, W.J., Zeikus, J.G., "Advances in Understanding the Microbiological Degradation of Lignin", in Recent Advances in Phytochemistry, Vol. II, The Structure, Biosynthesis, and Degradation of Wood, edited by F.A. Loewus and V.C. Runeckles, Plenum Press, New York, pp. 369-394, 1977.

Klibanov, A.M., Alberti, B.N., Morris, E.D., Felshin, L.M., "Enzymatic Removal of Toxic Phenols and Anilines from Waste Waters", Journal of Applied Biochemistry 2: 414-421, 1980.

Klibanov, A.M., Morris, E.D., "Horseradish Peroxidase for the Removal of Carcinogenic Aromatic Amines from Water", Enzyme and Microbial Technology 3: 119-122, 1981.

The Kline Guide to the Paper Industry, 4th edition, Fairfield, N.J., Charles H. Kline and Company Inc., 1980.

Lang, E.W., Miller, R.L., "Color Increase of Treated Kraft Mill Effluents", Proceedings of the 1977 TAPPI Environmental Conference, Tappi Press, Atlanta, Georgia, pp. 117-124, 1977.

Leatham, G.F., Stahmann, M.A., "Studies on the Laccase of Lentinus edodes: Specificity, Localization and Association with the Development of Fruiting Bodies", Journal of General Microbiology 125: 147-157, 1981.

Leonowicz, A., Grzywnowicz, K., "Quantitative Estimation of Laccase Forms in Some White-Rot Fungi Using Syringaldazine as a Substrate", Enzyme and Microbial Technology 3: 55-58, 1981.

Lindberg, S., Lund, L.-B., "A Nonpolluting Bleach Plant", TAPPI 63: 65-67, 1980.

Lundahl, H., Mansson, I. "Ultrafiltration for Removing Color from Bleach Plant Effluent", TAPPI 63: 97-101, 1980.

Mayer, A.M., Harel, E., "Polyphenol Oxidases in Plants", Phytochemistry 18: 193-215, 1979.

McDonald, R.G., Pulp and Paper Manufacture, Vol. I. - The Pulping of Wood, 2nd Edition, McGraw-Hill Book Co., New York, 1969.

Moggio, W.A., "Color Removal from Kraft Mill Effluent",TAPPI 38: 564-567, 1955.

Moll, R., "Centrifuge Plus Limits Lignin in Waste Water", Southern Pulp and Paper Manufacturer 39: 14-15, 1976.

Naish, V.A., Sandilands, L.M., "Lime Treatment of Bleached Softwood Kraft Mill Effluents", Cooperative Pollution Abatement Research (CPAR) Project Report 475-1, March, 1977.

National Council of the Paper Industry for Air and Stream Improvement, "An Investigation of Improved Procedures for Measurement of Mill Effluent and Receiving Water Color", Technical Bulletin No. 253, Dec., 1971.

Obiaga, T., Ganczarczyk, J., "Biological Removal of Lignin from Kraft Mill Effluents - Changes in Molecular Size Distribution", TAPPI 57: 137-138, 1974.

Petroski, R.J., Peczynska - Czoch, W., Rosazza, J.P., "Analysis, Production, and Isolation of an Extracellular Laccase from Polyporus anceps", Applied and Environmental Microbiology 40: 1003-1006, 1980.

Puiu, M., "Investigations of the Decolorization of Wastewaters Generated in Conifer Sulfate Pulp Mills," Celuloza Hirtie 27(2):60, 1978.

Rankin, P.R., Benedek, A., "Lignin Adsorption on Activated Carbon", Paper presented at the Fourth Joint AIChE/CSChE Engineering Conference, Vancouver, Canada, Sept., 1973.

Rock, S.L., Bruner A., Kennedy, D.C., "Decolorization of Kraft Mill Effluents with Polymeric Adsorbents", TAPPI 57: 87-92, 1974.

Rush, R.J., Shannon, E.E., Review of Colour Removal Technology in the Pulp and Paper Industry, Environmental Protection Service, Environment Canada Report No. EPS 3-WP-76-5, 1976.

Sarkanen, K.V., "Dehydrogenative Polymerization and Structure of Lignins", in Lignins, edited by K.V. Sarkanen and C.H. Ludwig, John Wiley and Sons, Inc., New York, 1971.

Schmidt, R.L., "The Effect of Horseradish Peroxidase on the Lime Precipitability of Biologically Treated Pulp Mill Effluents", Master's project report, the Institute of Paper Chemistry, Appleton, Wisconsin, October, 1979.

Schmidt, R.L., Joyce, T.W., "An Enzymatic Pretreatment to Enhance the Lime Precipitability of Pulp Mill Effluents", TAPPI 63: 63-67, 1980.

Sjöström, Eero, Wood Chemistry - Fundamentals and Applications, Academic Press, New York, 1981.

Spruill, E.L., "Color Removal and Sludge Recovery from Total Mill Effluent", TAPPI 56: 98-100, 1973.

Vedralova, E., Pechan, Z., Duchon, J., "Removing of Phenol from Waste Waters by Oxidation to Melanin with Mushroom Polyphenoloxidase", Collection of Czechoslovakian Chemical Communications 45: 623-627, 1980.

Wong, A., "Physical-Chemical Treatment", Proceedings of Seminars on Water Pollution Abatement Technology in the Pulp and Paper Industry. Canadian Environmental Protection Service Report No. EPS 3-WP-76-4, pp. 125-169, 1976.

Wong, A., Tenn, T., Dorica, J., Prahacs, S., "Detoxification and Decolorization of Kraft Pulp Mill Effluents using Activated Carbon", Pulp and Paper Canada 78 (4): T74 - T79, 1977.

Wright, R.S., Oswalt, J.L., Land, J.G., "Color Removal from Kraft Pulp Mill Effluents by Massive Lime Treatment", TAPPI 57: 126 , 1974.