Development of a Physical-Chemical and Enzymatic Methods for the Removal of Phenolic Pollutant: Application of Chitosan and Laccase.

Gbekeloluwa Oguntimein[1], Victoria Aladejana[1], Gregory Payne[2]

[1]Morgan State University, Baltimore / [2]Centre for Agricultural Biotechnology, University of Maryland, College Park.

Abstract

This paper reports the result of an undergraduate student involvement in research in an Historically Black College and University (HBCU) in a Science Engineering and Mathematics (SEM) Summer Research Training program sponsored by the national Science Foundation and the Office of Naval Research (ONR). The student had an opportunity of working with scientists in two Universities collaborating on an environmental engineering project.

Water pollution has been a growing problem for all nations as a result of industrialization. Most of the industrial pollutants are toxic and have been classified as hazardous and carcinogenic. Typical examples generated from dyes used in the textile industry and found in wastewater are phenolic compounds. The development of economically treatment processes to remove these substances has been of research interest worldwide. A physical-chemical method using chitosan and a biochemical method using laccase for the potential removal of phenolic compounds from an aqueous medium was investigated. The dual state behavior of chitosan in acidic and basic medium was taken advantage of in the physical-chemical method. At pH 4, azo dye reacted with chitosan and on increasing the pH to 8, chitosan was precipitated removing the dye from solution. In the biochemical method, at pH 4, laccase degraded the dye. The Michealis Menten constant (K_m) and the maximum velocity (V_m) for this reaction at room temperature were 8.5 X 10^{-2}g/L and 150 g/L·min respectively.

I. Introduction

National concern have been expressed about the status of the U. S. science and engineering base-specifically the human talent, knowledge and infrastructure that generate innovations and undergird technological advances to achieve national objectives. Analyses have shown that there may be a significant shortage in the entry level science and engineering labor pool, and that scientific and technical fields could be significantly affected. Demographic data show a future with proportionately fewer young people and a work force comprised of growing numbers of minorities and the economically disadvantaged. These groups, which the economy must increasingly rely, have been historically underrepresented in science, engineering and related fields. The added dimension of a projected shortage of qualified science and mathematics instructors at the pre-college and undergraduate levels could have serious consequences for the nation's scientific and technological literacy and, therefore for our capabilities to complete economically with other industrialized counties [1]. In 1990 less than 2% of the Science Engineering and Mathematics (SEM) workforce hails from the African-American community. While African-Americans Hispanic/Latinos and American Indians comprise 23% of the U.S. population, they make up only 4.5% of those holding scientific doctorates [2]. In a report to the Maryland Higher Education Commission in March 1992, the Task force on Engineering Education wrote " The representation of Africa-American almost disappears at the graduate level, with only 3% of all Maryland master's degrees granted in 1990 going to African-Americans. There are no doctorates awarded to African-Americans" [3]. Based on these facts a proposal with the specific objective directed at increasing the number of minority graduates prepared to effectively contribute to the U.S. SEM workforce was submitted by Morgan State University (MSU) to the Department of Defense to establish the Infrastructure Support Education Program (ISEP), NSF and ONR. The primary objectives of the program are:

1. To double the number of SEM degrees awarded to African –Americans from 140 t0 280 in three years and to triple them in five years.
2. To achieve this by increasing the first and second year retention rates of SEM majors from 75 to 85% (1st year) and 60 –80% (2nd year) respectively
3. To maintain a 30% graduate school going rate for SEM graduates. The number which is about 10% percentage points above the National average, should have a significant impact on future industries leaders and SEM faculty
4. To increase the number of graduates in each of the SEM majors offered so as to become the largest per capita producer of African-American receiving the degrees in each of the fields in the MSU curriculum base.
5. To raise two year retention rate in Engineering from 1993 rate of 60% to 80% in 1996
6. To raise the number of engineering degrees awarded from 50 in 1993 to 80 in 1996
7. To graduate students from a program that incorporates a more applied, product-oriented curriculum.

In pursuit of the third objective the SEM Summer Research / Trainee program was started two years ago to provide financial support to Morgan State University underrepresented minority students in science, engineering and mathematics the opportunity to gain research experience by allowing them under the supervision of a faculty member or professional scientist mentor at an industrial, governmental or university laboratory site. The objectives of this program are:

- To increase the number of students who participate in undergraduate research.
- To enhance students' learning and commitment to their studies.
- To increase the number of students attending graduate schools.
- To provide students with professional development training.

MSU, one of the Nation's 114 Historically Black Colleges and Universities (HBCU) was founded in 1867 as a private institution of higher education. It became a public institution in 1939 when the state of Maryland acquired it for the purpose of affording its African- American citizens access to public higher education at a time when access to this population sector was limited. Over the years, under committed and dedicated leadership, the University has seized the opportunity to develop MSU into a highly competitive educational institution which now possess a national reputation for excellence in SEM related fields. Among its many achievements in support of this claim are that MSU

- Continues to graduate more African-American undergraduates in chemistry than all other State institutions combine.
- Awarded 10% of all the nations B.S. Degrees in Physics earned by African-American students, in the last 15 yeas.
- Enrolls over 60% of all the State's African-America engineering students.
- Graduated 100% of the United States' African-American majors in Physics.
- Largest producer of African-American engineers in Maryland and among the top ten Institutions graduating the highest number of Africa-American Engineers in the nation.
- Led all public institutions nationally (between 1986 –1990) in awarding bachelor's degree to African-American students who subsequently went to receive doctorates in their chosen fields of study.

Research in the undergraduate engineering curriculum remains a significant contributor to the educational preparation of new practitioners for an increasingly complex technical society. Continued specialization is needed to provide the basic foundations of new and emerging technologies. It is therefore, important to modify curricula continuously to incorporate more applied, product-oriented programs while maintaining the concepts of basic science, mathematics and engineering sciences. It is also important to periodically refocus the research paradigm. Today, that means a shift towards the nation's domestic welfare needs as well as global economic competitiveness. The engineering research programs at MSU have a discipline-based focus in various sub-specialties in civil, electrical and industrial engineering. However, increasing attention is being paid to cross-discipline studies. Specifically, we are looking at urban development; transportation and manufacturing enterprises as interconnected systems that affect the quality of life. Our undergraduate research agenda includes increased emphasis on applied topics, including total quality management, design for manufacturing, and continuous product/process improvement with attention to client satisfaction, environmental considerations, and global economics.

I.I. Description of the Program

The SEM Undergraduate Summer Research/Training program is a ten-week long. The participants are selected based on the following eligibility criteria:

- Underrepresented minorities who are US citizens
- Full-time student during the academic year
- At least a sophomore (25 credits or more by the end of Spring 2001 semester)
- Minimum CUM GPA of 3.0 (exceptions may be made based on the review of the applicant's last coursework and faculty recommendation)
- Show significant interest in research and participating in ongoing research.

The financial support depends on the students academic classification. Seniors and juniors are awar $5000 and $4000 for rising sophomores over the 10-week period. This includes a research stipend of $350 juniors and seniors and $2500 for sophomores, and a $1500 allotment toward room and board for resident participants.

Student required to abide by the following regulations:

- Students selected for the program may NOT enroll in summer courses.
- Students may not work at any other employment during the course of the program.
- Each student will be required to participate in the research training, and attend and give technical presentations throughout the program.
- Participants will be required to submit weekly journals.
- Each student will be required to prepare a written and oral final report describing the results of her/his research.
- Each student is required to participate in the research symposium once a week prior to the end of the program.
- Students not living in close proximity to the University must live on campus. On campus housing will not be provided for students living close to the University, unless there are extenuating circumstances.

The program was divided into two phases. The first phase, which lasts 1-2 weeks, includes a series of lectures on research methodology, technical writing (e.g. proposals, reports and papers), keeping research records in journals and effective communications. Each student presented a proposal of his/her research project. The second phase, which lasted eight weeks, involved students working on the projects under faculty supervision. The program ended with a one-day symposium during which students gave either an oral presentation or a poster presentation. The symposium was attended by professionals from the funding agencies and other governmental agencies. These papers were published as proceedings.

The profile of students that have participated in the program in the last two years are shown in the tables 1 & 2.

Table 1. Profile of Students

Year	No. of Males	No. of Females	Sophomores	Juniors	Seniors	Total
1999	14	14	9	14	5	28
2000	12	12	5	12	7	24

Table 2. Students Profile by Discipline

Year	CE	ECE	IE	Chemistry	Eng. Physics	Biology	Total
1999	1	18	4	4	1	--	28
2000	--	16	1	4	--	3	24

The result of the work carried out by one of the students is presented below; The work was carried out at both MSU and at the University of Maryland College Park.

II. Student Research

II.1 Introduction

Water is our most precious natural resource and it is impossible to live without it. Water pollution has been a growing problem for all nations as a result of industrialization. The potential beneficial uses of water are lost due to changes in its composition as a result of human activity. Water pollution causes waterborne diseases including hepatitis, yellow fever, malaria, typhoid fever and cholera. Diseases spread by polluted water affect a lot of people around the world. In the industrialized world, the most serious threat to people is water contaminated with hazardous chemicals. When the water supply becomes contaminated, there are increases in cancer rates[1].

Many industrial pollutants such as textile industries produce toxic waste that are usually synthetic organic compounds that can be easily absorbed by the surrounding environment. Industrial dyes can be released into the environment from two major sources: as effluents from plants and from dye-using industries such as textile factory [2]. Between 10 and 15% of the total dye used in the dyeing process may be found in wastewater[3]. Azo dyes are the world's most commonly used dyes in the dye using industries. Azo dyes are characterized by the one or more azo groups (-N=N-) present in the dye molecules. They play an important part in almost every type of application and account for almost 60% of the total number of commercially manufactured dyes. Their high industrial usage, together with about 10% loss of their total production makes them an environmental pollution problem[4]. Some azo dyes are manufactured from certain chemicals aromatic amines that are known or suspected carcinogenic[5]. Once azo dyes have undergone azo bond cleavage they form aromatic amines, which are mostly toxic and carcinogenic[4]. Their discharge to the environment may cause serious and long lasting damages. Azo dyes, after passing through the washing process in the textile industries are passed into the environment; it is estimated that between 10 and 15% of the total dye used in the dyeing process may be found in wastewater[6].

Chitin is the second most abundant polysaccharide in nature (after cellulose). Chitin mainly consists of the amino sugar N-acetylglucosamine, which is partially deacetylated. The mostly deacetylated form of chitin is called chitosan[7]. Chitosan is a modified carbohydrate polymer derived from chitin component of the shell of crustacean such as crab, shrimp, and shellfish. Shellfish waste from food processing are decalcificated in diluted aqueous HCl solution, deproteinated in diluted aqueous NaOH solution, decolorized in 0.5% $KMnO_4$ aq. or sunshine to form chitin. Deacetylation of chitin in hot concentrated NaOH solution forms chitosan[8]. Chitosan is very similar to chitin (figure 2 and 3). The difference is that chitosan has an amine group instead of an amide group which means that chitosan doesn't have any carbons double bonded to oxygen and chitin does[9]. Chitosan has primary amino groups that confer interesting pH-solubility behavior; at low pH, it is polycationic, water soluble, and unreactive while at neutral pH, it is insoluble in water, nucleophilic and reactive (figure 4)

Laccase is a special polyphenol oxidase. It has wide substrate specificity for phenolic compound[10,11]. The capabilities of laccase can be greatly enhanced through the inclusion of suitable mediator compound. In the presence of some of its primary substrate, laccase can catalyze the oxidation of non-phenolic compounds[12,13]. Laccase is among the first non-food consumer products that can be produced in genetically enhanced plants. This high-value industrial enzyme is a prime example of a natural, renewable and bio-based product that, in some cases, can replace synthetic, chemical-based product[14]. Laccase differs from peroxidases in that it does not require hydrogen peroxide to oxidize substrates. Instead, electrons are transferred to molecular oxygen (O_2), yielding water.

Azo dyes can be removed from the environment physically and/or through the process of biochemical degradation. It is the objective of this research is to investigate the two methods of azo dye removal before applying them to the environment and have detrimental affect.

II. 2. Materials and Methods:

Chitosan and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma Chemical Co., (St Louis, MO). Remazol Brilliant Blue R and Reactive Blue 19, industrial dyes, were obtained from BASF (Ludwigshafen, Germany). Dibasic and Monobasic Potassium Phosphate were anhydrous ACS Reagents grade. All solution were prepared in 60mM acetic Buffer pH 4.5.

Laccase was obtained from the Instituto de Biotecnologia, University of New Mexico, Mexico and enzyme activity was determined by measuring the increase in absorbance at 436 nm of ABTS and as decrease in absorbance at 622 nm for the dye in 60 mM acetate buffer, pH 4.5 at room temperature. Protein was determined by Bradford method using the BioRad protein assay kit.
The physical method involved adding Reactive Blue 158 (0.5mM) to chitosan. This solution was vortex and the pH was increased from pH 4 to 8 using 0.1mM Sodium Hydroxyl (NaOH) and centrifuged at 9400rpm.

The enzymatic degradation activity was determined by measuring the decrease in dye absorbance at 622 nm in with presence of laccase.

II. 3. Results and discussion

The reaction between chitosan and dye shows that chitosan is unable to react with the dye due to its water solubility at pH of 4. While in the presence of a base (NaOH) which increased the pH of the solution, chitosan was able to precipitate the dye because at high pH, Chitosan is very reactive and insoluble in water. (Figure 5). This method can only remove Reactive Blue 158 by physical adsorption, which means that the dye is still present in the reaction.

With the aim of breaking down the dye, laccase was added to the dye and it degraded the dye (figure 6). The kinetic parameters of Laccase, the K_m and the V_m for this reaction were determined, the low K_m indicate a high affinity for the dye (figure 7).

When the physical and biochemical degradation methods were combined together at pH 8 it was observed that the chitosan precipitated the dye indicating that this reaction was not only physical but also chemical (figure 8). The Dye was not broken down because chitosan reacted with the dye and made it unavailable for laccase to reactive with.

In conclusion, physical and enzymatic degradation of Azo dyes were investigated; chitosan was able to precipitate Reactive Blue 158 at a high pH while laccase broke it down. In the process, of combining these two methods, the physical method showed that a physical-chemical reaction was involved in the removal of the dye.

III. Conclusion

It is too early to measure the outcome of this program in terms of enrollment in graduate school by participants in the program but one of the students who participated in 1999 have gone on to graduate school and will be finishing in May 2001**.** Some of the sophomores and junior participants have indicated their intention in participating in the program this summer and going on to graduate school. The feedback from the participants is that they have gained research and technical experience under the supervision of a faculty member which has kindled their interest in pursuing a graduate study.

IV. Acknowledgement

V.A. would like to acknowledge the Science, Engineering and Mathematics Undergraduate Research Trainee Program funded by National Science Foundation and the Office of Naval Research for supporting her summer internship.

References

1. Report of the Institute for Science, Space and Technology, Strengthening American Science and Technology, February 1990.
2. Mervis, J. 1998. "Wanted: A better Way to boost number of minority Ph.D.s. Science 281, 1268 -1270.
3. Report of the Task force on Engineering Education.to The Maryland Higher Education Commission March 1992
4. Barss, K. Clean Water. New York: Chelsea House Publishers, (1999).[1]
5. Rodriguez, E., Pickard, M.A., and Vazquez-Duhalt, R., "Industrial Dye Decolorization by Laccase from Ligninolytic Fungi" (1998) Current Microbiology 38:27-32

6. DH Brown, HR Hitz, L Schafer (1981) The assessment of the possible inhibitory effect of dyestuffs on aerobic wastewater bacteria. Experiment with a screening test. Chemosphere 10:245-26
7. http://www.brad.ac.uk/acad/envsci/ET/conf_98/yuan.htm
8. Danish Environmental Protection Agency. file://A/azo 4a_files/kap01_eng.htm
9. http://www.biotechsupportindia.com/biotextile.htm
10. http://www.uni-ulm.de/biologie1/Forschung/Chitin/chitine.htm
11. http://user.chollian.net/~chitin/prechitosan.gif
12. http://www.psrc.usm.edu/macrog/sea/chitin.htm
13. http://www.ensia.inra.fr/~courtois/fidel/Lisbon/enzymatic/1/an_apps/laccase.html
14. Kaiwai, S., Umezawa, T., Shimada, M., and Higuchi, T. (1998) "Aromatic Ring Cleavage of 4,6-Di(tert-butyl)guaiacol, a Phenolic Lingnin Model Compound, by Laccase of Coriolus versicolor," FEBS Letters 236,309-311.
15. Bourbonnais, R., and Paice, M.G. (1990) "Oxidation of Non-Phenolic Substrate: An Expanded Role for Laccase in Lingin Biodegradation," FEBS Letters 267,99-102.
16. Kawai, S., Umezawa, T., and Higuchi, T. (1989) "Oxidation of Methoxylated Benzyl Alcohols by Laccase of Coriolus Versicolor in the Presence of Syringaldehyde," Wood Research 76,10-16
17. http://www.prodigene.com/news_releases/00-04-05_ProdiGene_patents.html

GBEKELOLUWA OGUNTIMEIN

Gbekeloluwa Oguntimein is an Associate Professor in the Department of Civil Engineering at Morgan State University in Baltimore. He is the Director of the Science Engineering and Mathematics Research Trainee program funded by The Office of Naval Research (ONR) and National Science Foundation. Dr Oguntimein received his B.S. and Ph.D. degrees in Chemical Engineering from Iowa State University in 1974 and 1979 respectively.

VICTORIA ALADEJANA

Victoria Aladejana is an undergraduate student in the Chemistry Department at Morgan State University. She hopes to pursue a degree in Pharmacy.

GREGORY PAYNE

Gregory Payne is a Professor in the Department of Chemical and Biochemical Engineering at the University of Maryland, Baltimore County and Research Professor at the Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park. Dr. Payne received a B.S and M.S in Chemical Engineering from Cornell University in 1979 and 1981 respectively and a Ph.D. from the University of Michigan in 1984

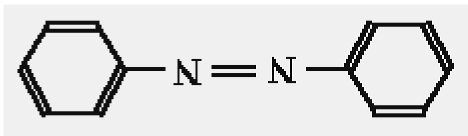

Figure 1: General Structure or Azo Dyes

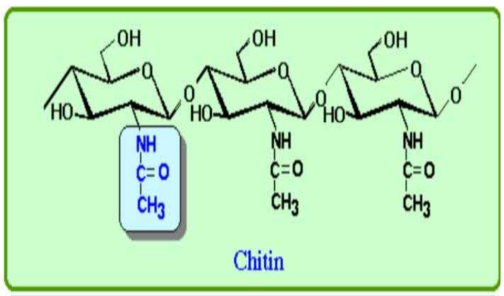

Figure 2: Structure of Chitin

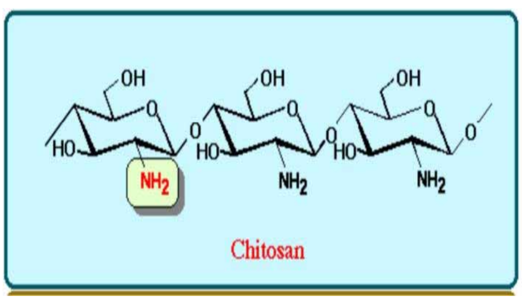

Figure 3: Structure of Chitosan

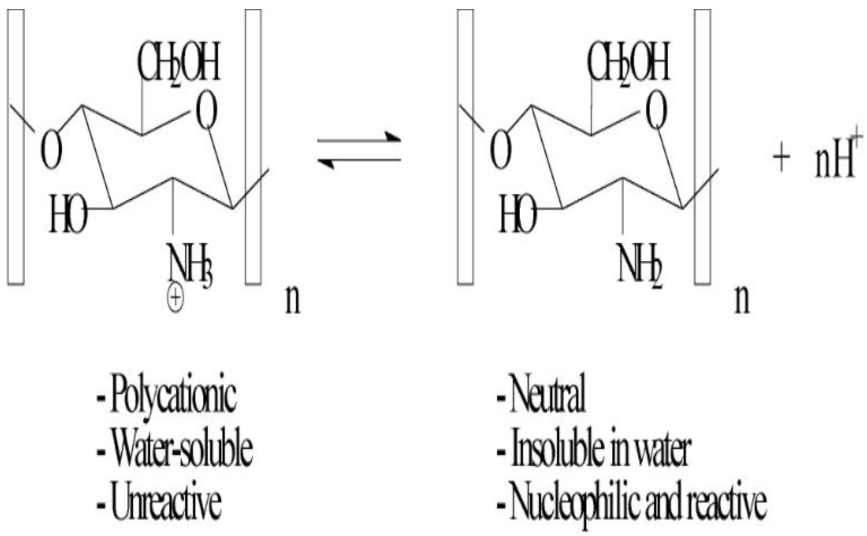

Figure 4: Behavior of Chitosan under different pH conditions.

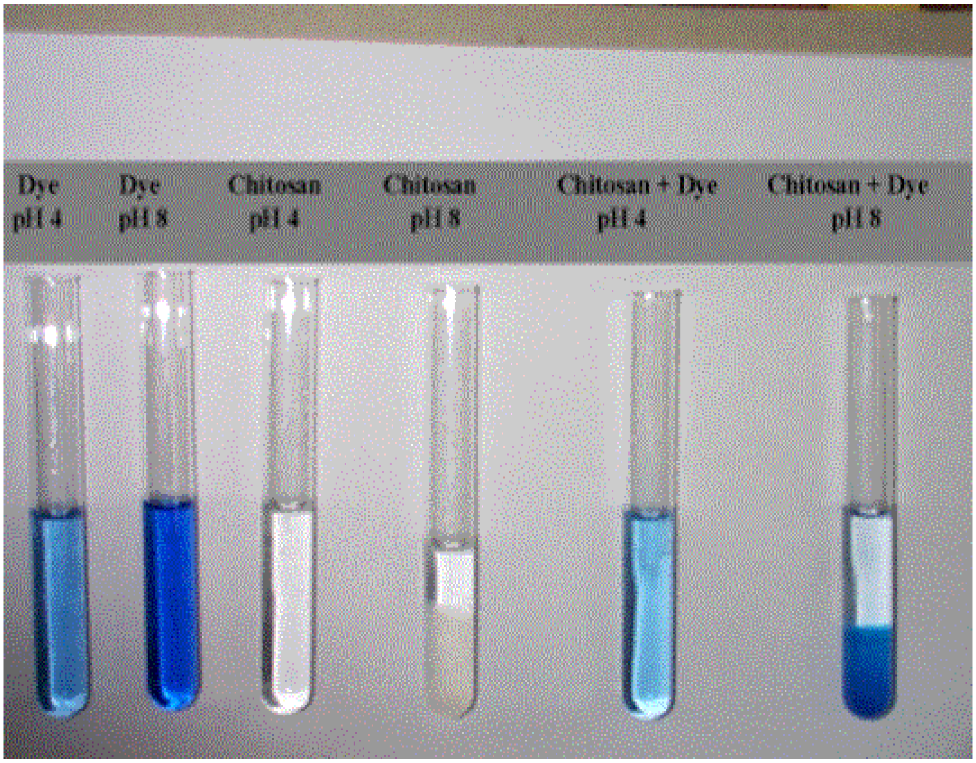

Figure 5: Results of Precipitation of Azo Dye with Chitosan

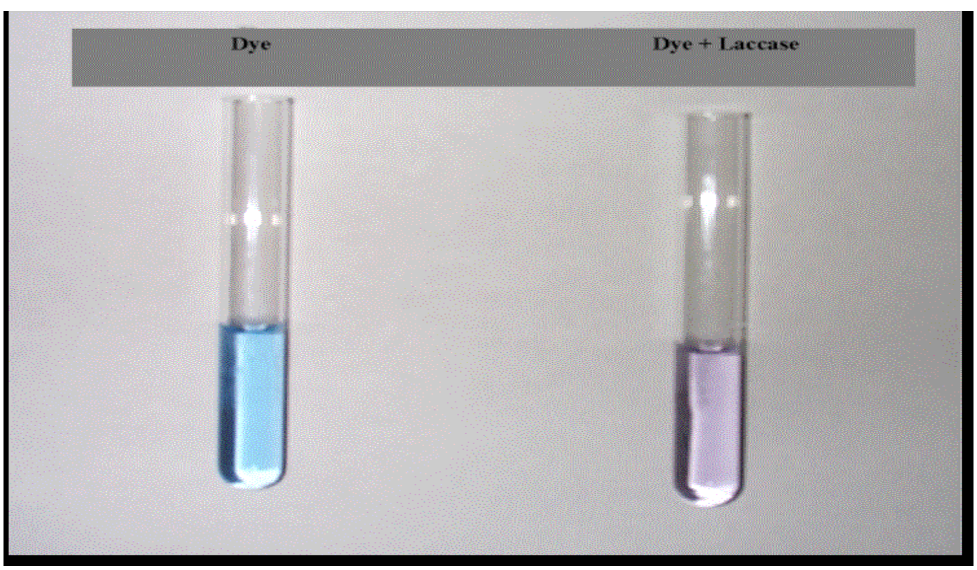

Figure 6: Enzymatic Degradation of Azo Dye

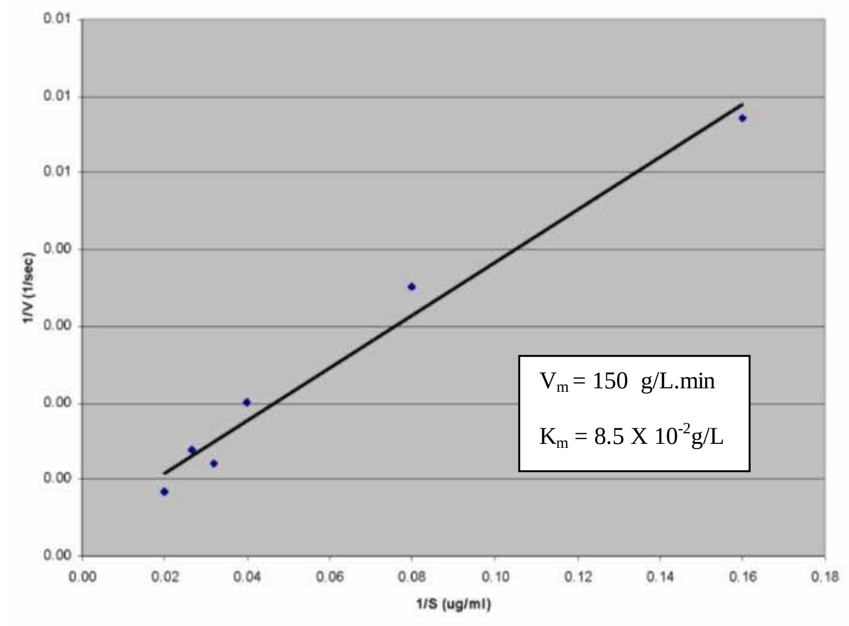

Figure 7: Detemination of Kinetic parameters of Laccase

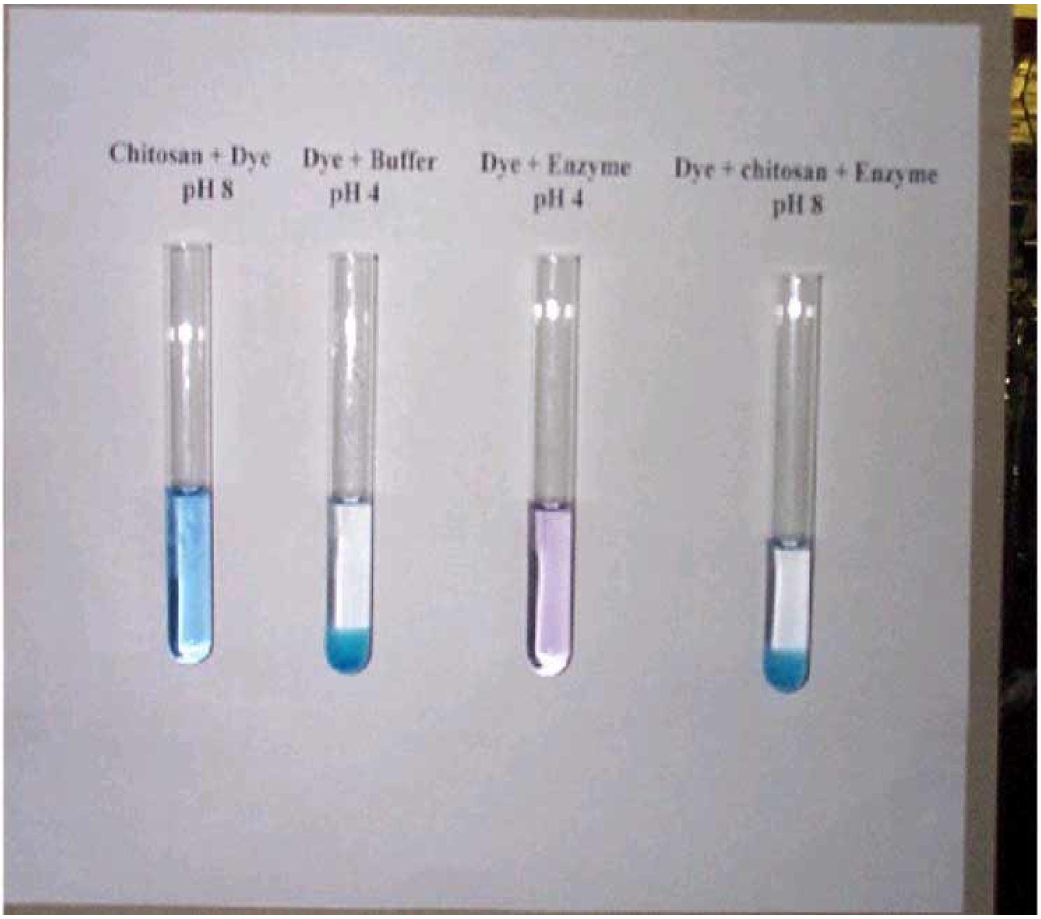

Figure 8: Physical and biochemical degradation of Reactive Blue 158