# INDUCTION OF AZOREDUCTASE FROM Aeromonas hydrophila FOR BIOLOGICAL TREATMENT OF DYES IN WASTEWATER

By

## **HEBA HAMED BAYOMY MAGHRAWY**

B.Sc. Agric. Sci., (Biotechnology), Fac. Agric., Cairo Univ., Egypt, 2004

## **THESIS**

**Submitted in Partial Fulfillment of the Requirements for the Degree of** 

## MASTER OF SCIENCE

In

Agricultural Sciences (Agric. Microbiology)

Department of Agricultural Microbiology
Faculty of Agriculture
Cairo University
EGYPT

2011

#### SUPERVISION SHEET

# INDUCTION OF AZOREDUCTASE FROM Aeromonas hydrophila FOR BIOLOGICAL TREATMENT OF DYES IN WASTEWATER

M.Sc. Thesis
In
Agric. Sci. (Agricultural Microbiology)

By

### HEBA HAMED BAYOMY MAGHRAWY

B.Sc. Agric. Sci. (Biotechnology), Fac. Agric., Cairo Univ., 2004

### SUPERVISION COMMITTEE

## Dr. SAMIR ABD EL WAHED EL GIZAWY

Emeritus Professor of Agricultural Microbiology, Fac. Agric., Cairo University

#### Dr. REFAE IBRAHIM REFAE

Professor of Agricultural Microbiology, Fac. Agric., Cairo University

#### Dr. HUSSEIN ABD EL KAREEM AHMED

Researcher Professor of Microbiology, NC for Radiation Research and Technology

#### APPROVAL SHEET

# INDUCTION OF AZOREDUCTASE FROM Aeromonas hydrophila FOR BIOLOGICAL TREATMENT OF DYES IN WASTEWATER

M.Sc. Thesis
In
Agric. Sci. (Agricultural Microbiology)

By

## HEBA HAMED BAYOMY MAGHRAWY

B.Sc. Agric. Sci. (Biotechnology), Fac. Agric., Cairo Univ., 2004

# **Approval Committee**

Dr. ALI AHMED IBRAHIM HAMMAD  Emeritus Researcher Professor of Microbiology, NC for Radiation Research and Technology
Dr. MOHAMED FAYEZ FOUAD  Emeritus Professor of Agric. Microbiology, Fac. Agric., Cairo University
Dr. SAMIR ABD EL WAHED EL GIZAWY  Emeritus Professor of Agric. Microbiology, Fac. Agric., Cairo University
Dr. REFAE IBRAHIM REFAE  Professor of Agric. Microbiology, Fac. Agric., Cairo University

Date: / / 2011

# تحفيز إنزيم الآزوردكتيز من بكتريا إيروموناس هيدروفيلا للمعالجة البيولوجية للصبغات في مياه الصرف

رسالة مقدمة من

هبه حامد بيومى مغراوى بكالوريوس فى العلوم الزراعية (بيوتكنولوجى)- كلية الزراعة- جامعة القاهرة، ٢٠٠٤ للحصول على درجة

الماجستير

فی

العلوم الزراعية (ميكروبيولوجيا زراعية)

قسم الميكروبيولوجيا الزراعية كلية الزراعة جامعة القا هرة مصر

# تحفيز إنزيم الآزوردكتيز من بكتريا إيروموناس هيدروفيلا للمعالجة البيولوجية للصبغات في مياه الصرف

# رسالة ماجستير فى العلوم الزراعية (ميكروبيولوجيا زراعية)

مقدمة من

# هبه حامد بيومى مغراوى بكالوريوس فى العلوم الزراعية (بيوتكنولوجى)- كلية الزراعة- جامعة القاهرة، ٢٠٠٤

# لجنة الحكم دكتور/ علي أحمد إبراهيم حماد أستاذ باحث الميكروبيولوجيا المتفرغ – المركز القومي لبحوث و تكنولوجيا الا شعاع دكتور/ محمد فايز فؤاد أستاذ الميكروبيولوجيا الزراعية المتفرغ – كلية الزراعة – جامعة القاهرة دكتور / سمير عبد الواحد الجيزاوي أستاذ الميكروبيولوجيا الزراعية المتفرغ – كلية الزراعة – جامعة القاهرة دكتور / رفاعي إبراهيم رفاعي أستاذ الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة أستاذ الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة

اسم الطالب: هيه حامد بيومي مغراوى الدرجة: ماجستير

عنوان الرسالة: تحفيز إنزيم الأزوردكتيز من بكتيريا إيروموناس هيدروفيلا للمعالجة البيولوجية

للصبغات في مياه الصرف

المشرفون: دكتور: سمير عبد الواحد الجيزاوي

دكتور: رفاعي إبراهيم رفاعي دكتور: حسين عبد الكريم أحمد

قسم: المكيروبيولوجيا الزراعية فرع: تاريخ منحة الدرجة:

#### المستخلص العربى

الهدف من هذا البحث هو تكسير الصبغات الناتجة من مصانع النسيج بواسطة سلالات بكتيرية معزولة من مصادر محلية. تم عزل اثنين وعشرين عزلة من نوع الإير وموناس من مصادر محلية مختلفة. أظهرت سبع سلالات القدرة علي إزالة لون الصبغة في بيئة بويون مغذى بعد ٤٨ ساعة تحضين، ولكن اظهرت سلالة واحدة فقط معزولة من سمك البلطي قدرة عالية على ازالة اللون (٩٩%). تم تعريف هذه السلالة مور فولوجياً ،فسيولوجياً و بواسطة RNA على ازالة اللون إير وموناس هيدروفيلا. وقد وجد أن الظروف المثلى لازالة اللزن هي شحيحة الأكسجين ودرجة حرارة ٣٠٥م وأس هيدروجيني لاو تستطيع هذه السلالة ان تزيل لون صبغة الأزو ذات المجموعة الكبريتيه (كونجو حمراء) من ٥٠ جزء في المليون فما فوق. أيضاً هذه السلالة تستطيع إزالة انواع مختلفة من صبغة الأزو بعد ٤٨ ساعة تحضين.

وجد أن زيادة تركيز مستخلص الخميرة في البيئة كمصدر للنيتروجين اكبر من ٢% لا يعمل بقوة على نحسين من كفاءة عملية ازالة اللون. اثبتت النتائج أن وجود اى نوع من الكربون بتركيزات مختلفة يعمل على تثبيط عملية ازالة اللون نتيجة لتحول الكربون المستهلك الي أحماض عضوية تخفض من قيمة الأس الهيدروجيني للبيئة، وبالتالي تؤثر علي نمو الخلية وكفاءتها في إزالة اللون.

تستطيع السلالة إز الة اللون عند عدة تركيزات مختلفة من كلوريد الصوديوم (V = A / U عده تركيزات مختلفة من كلوريد التكسير.

وجد أن إنزيم الآزوريدكتيز مستحث، ويفرز خارج الخلية، وهو من نوع فلافوبروتين، ويستخدم NADH كمعطي للإلكترونات اثناء عملية التكسير. ووجد ان المعالجة المثالية للازالة الكاملة لصبغات الازو هي المعاملة تحت الظروف شحيحة الأكسيجين تليها مرحلة معالجة تحت ظروف هوائية وفيزيائية (إشعاع).

أثبتت دراسة السمية على بذور الفول بأنه تم تكسير صبغة الكونغو الحمراء الى مركبات غير سامة بواسطة الأيروموناس هيدروفيلاً.

الكلمات الدالة: إيروموناس هيدروفيلا- الكونجو الحمراء – تكسير الصبغة – الازوردكتيز – اختبار السمية على النبات

**Name of Candidate:** Heba Hamed Bayomy Maghrawy **Degree:** M.Sc. **Title of Thesis:** Induction of Azoreductase from *Aeromonas hydrophila* for

Biological Treatment of Dyes in Wastewater

Supervisors: Dr. Samir Abd-El Wahed El Gizawy

Dr. Refae Ibrahim Refae

Dr. Hussein Abdel Kareem Ahmed

**Department:** Agricultural Microbiology

**Approval:** / / 2011

#### **ABSTRACT**

The aim of this work is to evaluate textile dyes degradation by novel bacterial strains isolated from different local sources. Twenty two isolates of Aeromonas species were isolated from different local sources. Among those, seven showed the ability to decolorize Congo red in nutrient broth medium after 48 h incubation. The only one isolate from fish (AF) showed the highest efficiency in decolorization (98%). This isolate was identified as Aeromonas hydrophila (AF) based on Gram staining, morphology characters, biochemical tests and the 16S rRNA sequencing. Color removal was highest in microaerophilic culture of pH 7 at 30 °C. The isolate was able to decolorize sulphonated azo dye (Congo red) in a wide range (up to 50 ppm). This isolate could also decolorize the medium containing different types of azo dyes after 48 h incubation. High concentration of yeast extract (as a Nsource) did not enhance strongly the decolorization efficiency. Similarly, carbon source inhibited decolorization activity because the consumed carbon was converted to organic acids that might decrease the pH of the culture medium, thus inhibiting the cell growth and decolorization activity. The isolate was capable to decolorize in the presence of NaCl concentrations up to 10 g/l. The biodegradation was monitored by UV-vis, IR spectroscopy and TLC. Azoreductase has shown to be inducible extracellular, flavoprotein and use NADH as electron donor. Sequential microaerophilic, aerobic and physical (radiation) treatment seemed the most logical strategy for the complete removal of azo dyes in biological systems. The phytotoxicity study revealed the degradation of Congo red into non-toxic product by Aeromonas hydrophila (AF).

**Key words:** Aeromonas hydrophila, Congo red, degradation of azo dyes, azoreductase, phytotoxicity.

# ACKNOWLEDGMENT

First, I thank "GOD", the Beneficent, the Merciful, for my success in the completion of this work.

My sincere gratitude is due to Dr. Samir El Gizawy Professor of Agricultural Microbiology, Faculty of Agriculture, Cairo University, for his kind assistance, support, patience, sincere advices, encouragement and helpful supervision. Deeply thanks to Dr. Refae I. Refae Professor of Microbiology, Faculty of Agriculture, Cairo University, for his assistance, kind support, helpful supervision, valuable discussion during all stages of fulfillment of the thesis, encouragement and continuous guidance. I wish to express my sincere thanks and gratitude to Dr. Hussein Abd El Kareem Professor of Microbiology, National Center for Radiation Research and Technology (NCRRT), for supervising the present work, great encouragement, continuous quidance, invaluable advices and valuable critical reading and revising the manuscript. I also like to thank him for giving me the chance to learn. I am deeply grateful also to Dr. Ola Mohammed Gomma Assistant Professor of Microbiology, National Center for Radiation Research and Technology (NCRRT), who suggested the point of research, for her effective supervision, fruitful discussion and valuable advice. She has guided me into this field and continuously helped and encouraged during the whole work.

I'm deeply grateful to my colleagues in (NCRRT).

Also I would like to thank my colleague Fatma Hemaia, Researcher Assistant, NRC, Giza for her kind help.

Sincere thanks to all the members of Microbiology Department, Faculty of Agriculturae, Cairo University. Great thanks to every one who offered me help and assistance.

# تحفيز إنزيم الآزوردكتيز من بكتريا إيروموناس هيدروفيلا للمعالجة البيولوجية للصبغات في مياه الصرف

رسالة ماجستير فى العلوم الزراعية (ميكروبيولوجيا زراعية)

مقدمة من

هبه حامد بيومى مغراوى بكالوريوس فى العلوم الزراعية (بيوتكنولوجى) - كلية الزراعة - جامعة القاهرة، ٢٠٠٤

لجنة الإشراف

الدكتور / سمير عبد الواحد الجيزاوى أستاذ الميكروبيولوجيا الزراعية المتفرغ - كلية الزراعة- جامعة القاهرة

الدكتور / رفاعى إبراهيم رفاعى أستاذ الميكروبيولوجيا الزراعية- كلية الزراعة- جامعة القاهرة

الدكتور / حسين عبد الكريم أحمد المتاذ باحث الميكروبيولوجيا – المركز القومي لبحوث وتكنولوجيا الاشعاع

# **CONTENTS**

			Page
IN	TR	ODUCTION	1
		EW OF LITERATURE	5
1.		ktile industry	5
2.		es as pollutants	
3.	•	Dyes and Intermediates	
4.		xicity of dyestuff	
5.		lor removal techniques	11
	a.	Non-biological color removal	11
	b.	Biological techniques	12
6.		chanism of color removal	13
7.		colorisation of azo dyes in an anaerobic system	16
8.		ctors affecting color removal	19
	a.	Oxygen	20
	b.	Temperature	
	c.	PH	
	d.	Dye concentration	
	e.	Dye structure	23
	f.	Carbon source	
	g.	Nitrogen source	24
	h.	Salt concentration	25
9.	Co	mbined anaerobic- aerobic biodegradation of azo dyes	<b>26</b>
		robic oxidation of aromatic amines	
$\mathbf{M}$	ATE	ERIAL AND METHODS	31
		als	
1.	Dy	es used	31
2.		croorganisms used	33
3.	Pla	nt seed (Vicia faba) and Roket leaves	33
4.		raffin oil	
5.	An	aerobic indicator sachets (oxoid)	33
6.	Soi	l sample	33
7.	NA	DH for determination of azoreductase	<b>34</b>
8.	Cu	lture media and buffers	<b>34</b>
Me	etho	ds	<b>37</b>
1.	Sci	reening of suspected Aeromonas sp. isolates capable of	
		colorization	<b>37</b>
2	Ido	antification of isolates	40

	a. Morphological and biochemical testes.	40
<b>3.</b>	Identification of isolated strain by 16S rRNA gene	
	a. DNA extraction.	
	<b>b</b> . PCR amplification of bacterial 16S rDNA.	
	<b>c.</b> Cloning and sequencing	
	<b>d</b> . Phylogenetic analysis.	
4.	Cell Dry weight.	43
<b>5.</b>	Decolorization and biodegradation of dyes under	
	different conditions	<b>4</b> 4
	<b>a.</b> Effect of aeration	<b>4</b> 4
	<b>b.</b> Effect of dye concentration	45
	c. Effect of temperature	45
	<b>d.</b> Effect of pH	
	e. Effect of azo bonds number	46
	<b>f.</b> Effect of salt concentration	46
	<b>g.</b> Effect of carbon and nitrogen source on color removal	46
	<b>h.</b> Effect of inoculum size	46
	i. Effect of growth phase.	47
6.	Growth curve determination	47
7.	Determination of azoreductase activity	47
	<b>a</b> . Separation of the enzyme	47
	<b>b</b> . Azoreductase assay	48
8.	Quantitative estimation of protein content	48
9.	Decolorization of Congo red under consecutive	
	microaerophilic /aerobic conditions	51
<b>10.</b>	Biodegradation analysis	51
11.	Determination of dye and its products	51
	<b>a.</b> UV/Visible spectrometer	51
	<b>b.</b> Thin Layer Chromatography (TLC)	
	<b>c.</b> Fourier Transform Infrared Spectroscopy (FTIR)	52
<b>12.</b>	Effect of radiation on the degraded product after	
	aerobic stage	53
13.	Toxicity assessment of dye and their biodegraded	
	products on plants (Phytotoxicity study)	53
<b>14.</b>	The proposed pathway for degradation of Congo red	54
	Statistical analysis	
RE	ESULTS AND DISCUSSION	
1.	Isolation of dye - degrading microorganisms	55
	<b>a.</b> Isolation and screening of <i>Aeromonas</i> species capable of	

	dye decolorziation	55
	<b>b.</b> The relationship between the growth of <i>Aeromonas spp</i> .	
	and their capabilities of azo dye color removal	<b>56</b>
	<b>c.</b> Identification of <i>Aeromonas</i> isolates (AF) to species level	<b>60</b>
2.	Factors affecting growth and color removal efficiency of	
	Aeromonas hydrophila	<b>62</b>
	<b>a.</b> Effect of aeration	<b>62</b>
	<b>b.</b> Effect of dye concentrations	64
	<b>c.</b> Effect of temperature	<b>67</b>
	<b>d.</b> Effect of initial pH	<b>69</b>
	e. Effect of azo bonds number	<b>71</b>
	<b>f.</b> Effect of salt concentration	<b>73</b>
	<b>g.</b> Effect of carbon sources	77
	<b>h.</b> Effect of yeast extract	84
	i. Effect of culture age	<b>87</b>
	<b>j.</b> Effect of inoculum size	<b>89</b>
<b>3.</b>	<b>Determination of Protein content and azoreductase activity</b>	91
4.	Decolorization of congo red under consecutive	
	microaerophilic/aerobic conditions	93
<b>5.</b>	Biodecolorization and biodegradation analysis	96
	<b>a.</b> UV- VIS analysis	96
	<b>b.</b> TLC chromatography	<b>97</b>
	c. FTIR analysis	98
6.	Effect of radiation on the degraded product after aerobic	
	stage	103
7.	The proposed pathway for degradation of Congo red	109
8.	Phytotoxicity studies	111
CC	ONCLUSION	115
SU	MMARY	117
RE	FERENCES	123
	RABIC SUMMARY	

# LIST OF FIGURES

No.	Title	Page
1.	Schematic representation of the operations involved in textile cotton industry and the main pollutants from each step	6
2.	Proposed mechanism for reduction of azo dyes by whole bacterial cells	16
3.	General overview of the fate of azo dyes and aromatic amines during anaerobic–aerobic treatment	27
4.	Flow chart indicating the identification and screening profile of <i>Aeromonas</i> spp. isolates	39
5.	Standard curve of congo red	45
6.	Standard curve from different concentrations of bovin serum albumin	50
7.	Average percentages of dye removal by <i>Aeromonas</i> spp. isolated from different sources	59
8.	The relationship between biomass yield and decolorization efficiency of different isolates after 48 h	59
9.	Phylogenetic tree representing the strain under study compared to the whole bacterial database.	61
10.	Average of percentages of decolorization of Congo red under different aeration conditions after 48 h	63
11.	Linear regression among Congo red concentration and biomass.	66
12.	The relationship between biomass yield and decolorization efficiency of <i>Aeromonas hydrophila</i> at different temperatures after 48 h.	68
13.	The relationship between biomass yield and decolorization efficiency of <i>Aeromonas hydrophila</i> at different pH values after 48 h.	71

14.	The relationship between biomass yield and decolorization of mono, di and poly azo dyes by <i>Aeromonas hydrophila</i> after 48h	73
15.	Linear regression among NaCl concentrations and decolorization rate of Congo red	75
16.	Linear regression among NaCl concentrations and bacterial biomass yield	76
17.	The relationship between biomass yield and decolorization efficiency of <i>Aeromonas hydrophila</i> at different concentrations of glucose after 48 h.	80
18.	The relationship between biomass yield and decolorization efficiency of <i>Aeromonas hydrophila</i> at different concentration of sucrose after 48 h.	80
19.	The relationship between biomass yield and decolorization efficiency of <i>Aeromonas hydrophila</i> at different concentration of mannitol after 48 h	81
20.	The relationship between biomass yield and decolorization efficiency of <i>Aeromonas hydrophila</i> at different concentration of starch after 48 h.	81
21.	Linear regression among concentrations of glucose and pH of the culture after 48 h.	82
22.	Linear regression among concentrations of sucrose and pH of the culture after 48 h.	82
23.	Linear regression among concentrations of mannitol and pH of the culture after 48 h.	83
24.	Linear regression among concentrations of starch and pH of the culture after 48 h.	83
25.	Linear regression among concentrations of yeast extract and decolorization of Congo red after 48 h.	86
26.	Linear regression among concentrations of yeast extract and biomass after 48h.	86

27.	The relationship between biomass yield and decolorization efficiency of Congo red by different culture ages of <i>Aeromonas hydrophila</i> .	88
28.	Growth curve of Aeromonas hydrophila	89
29.	The relationship between biomass yield and decolorization efficiency of Congo red by different inocula size of <i>Aeromonas hydrophila</i>	91
30.	Decolorization of Congo red under consecutive microarophilic conditions at 24, 48 hrs and aerobic conditions.	95
31.	The UV-Visible spectrum for the Congo red in cultivation media before decolorization (zero time) and after decolorization (48 h) by <i>Aeromonas hydrophila</i>	97
32.	Breakdown of Congo red observed by UV lamp	98
33.	Detection of aromatic ring observed using iodine pellets	98
34.	The FT-IR spectrum for control culture (uninoculated)	99
35.	The FT-IR spectrum for anaerobic treatment by <i>Aeromonas hydrophila</i>	100
36.	The FT-IR spectrum for aerobic treatment by <i>Aeromonas hydrophila</i>	10
37.	The FT-IR spectrum for aerobic treatment by <i>P. putida</i>	102
38a.	The FT-IR spectrum for radiation treatment at 2KGy	104
38b	The FT-IR spectrum for radiation treatment at 4 KGy	104
38c	The FT-IR spectrum for radiation treatment at 6 KGy	10:
38d	The FT-IR spectrum for radiation treatment at 8 KGy	10:
38e	The FT-IR spectrum for radiation treatment at 10KGy	100
38f	The FT-IR spectrum for radiation treatment at 12 KGy	100
38g	The FT-IR spectrum for radiation treatment at 14 Kg	10′
38h	The FT-IR spectrum for radiation treatment at 16KGy	10′