

# Some genetic attempts to construct a highly effective hemicellulase producer

*Thesis*

SUBMITTED IN PARTIAL FULFILMENT  
FOR THE DEGREE OF MASTER OF SCIENCE

*By*

**Manal Moosa Mohammad Hassan**

*Research Assistant*

*National Research Centre*

*Under The supervision of*

**Dr. Mehreshan T. El-Mokadem**

*Prof. of Microbiology*

*Women's College - Ain Shams University*

**Dr. Osama H. El-Sayed**

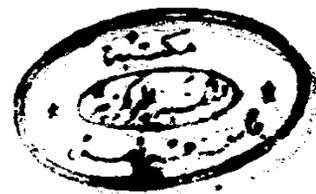
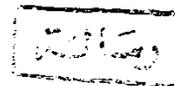
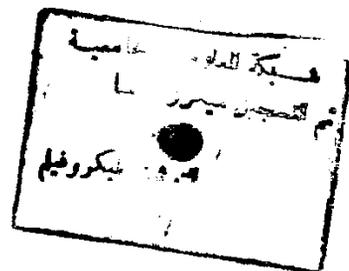
*Prof. of Microbial Chemistry*

*National Research Centre*

**Dr. Assem M.M.Ali**

*Prof. of Microbial Genetics*

*Mubark Institute*



51206

1994

## Approval Sheet

**Name:** Manal Moosa Mohammad Hassan

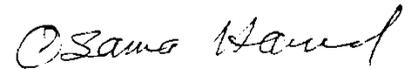
**Title:** Some genetic attempts to construct a highly effective hemicellulase producer

*This thesis has been approved by:*

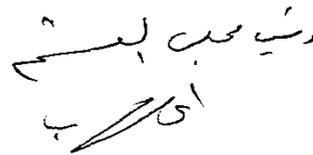
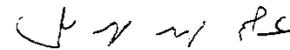
**1- Prof. Dr. Mehreshan T. El-Mokadem**



**2- Prof. Dr. Osama H. El-Sayed**



**3- Prof. Dr. Assem M. M. Ali**





## Acknowledgment

I would like to express my deepest gratitude & thanks to *Dr. Mehreshan T. El-Mokadem*, Prof. of Microbiology, Botany Department, Women's College, Ain Shams University for her valuable discussions & constructive suggestions.

Sincere thanks & gratitude are also to *Dr. Osama Hamed El-Sayed*, Prof. of Microbial Chemistry, and The head of Genetic Engineering & Biotechnology Division, National Research Center, for suggestion the point of this study.

I would like to express my sincerest thanks to *Dr. Assem M. Ali*, Prof. of microbial genetics & the head of Genetic Engineering Researches, Mubark Institute, for his kind supporting & pervasion.

I would like also to express my deepest appreciation & thanks to *Dr. Yosri M. Ahmed* for his kind encouragement through the course of work.

# CONTENT

	<i>page</i>
<b>Chapter (1)</b>	
<b>Review of literatures</b>	
1.1.The hemicellulosic polysaccharides	1
1.2.The hemicellulase enzymes	4
1.3.The process of N <sub>2</sub> fixation	16
1.4.Gene transfer systems in azotobacters	19
1.5.Molecular cloning of xylanase genes	24
<b>Chapter (2)</b>	
<b>Material &amp; Methods</b>	
2.1.Straains & media	
2.1.1.Donor strain	27
2.1.2.Recipient strains	27
2.2.Preparative methods	
2.2.1. Preparation of xylan from corncobs	28
2.2.2. Preparation of DNA	29
2.3.Transformation procedure	30
2.4.Staining reaction for detection of xylanase producing transformants	30
2.5.Assay of xylanase activity	31
2.6.Analytical methods	
2.6.1. Determination of protein concentration	32
2.6.2. Determination of DNA concentration	33
2.6.3. Determination of total nitrogen	34
2.7.Factors affecting xylanase production by <u>Azotobacter vinelandii</u> transformed strain.	

2.7.1. Carbon source	34
2.7.2. Xylan concentration	35
2.7.3. Nitrogen source	35
2.7.4. Phosphate concentration	35
2.7.5. Salinity	35
2.7.6. pH	35
2.8. Purification of the transformant xylanase	
2.8.1. Precipitation by ammonium sulphate	36
2.8.2. DEAE-cellulose column chromatography	37
2.8.3. Sephadex column chromatography	37
2.9. Some properties of the purified enzyme	
2.9.1. Determination of optimum temperature	38
2.9.2. Determination of optimum pH	38
2.9.3. Time course of enzymatic reaction	39
2.9.4. Effect of various reagents on enzyme activity	39

### ***Chapter (3)***

#### **Results & Discussion**

3.1. Transformation frequency	40
3.2. Staining reaction for detection of xylanase producing transformants	42
3.3. Xylanase activity in wild & transformant strains	45
3.4. Nitrogen contents in culture of wild & transformed <u><i>A. vinelandii</i></u>	48
3.5. Factors affecting xylanase production by <u><i>A. vinelandii</i></u> transformed strain	
3.5.1. Carbon source	48
3.5.2. Xylan concentration	53
3.5.3. Nitrogen source	56

3.5.4. Phosphate concentration	60
3.5.5. Salinity	63
3.5.6. pH	63
3.6. Purification of xylanase from transformed <u>A. vinelandii</u>	
3.6.1. Precipitation by ammonium sulphate	68
3.6.2. DEAE-cellulose column chromatography	70
3.6.3. Sephadex column chromatography	70
3.7. Some properties of the purified enzyme	
3.7.1. Determination of optimum temperature	73
3.7.2. Determination of optimum pH	76
3.7.3. Time course of enzymatic reaction	79
3.7.4. Effect of various reagent on enzymes activity	79

## **References**

## **Summary**

## **Arabic summary**

## List of Tables

	<i>Page</i>
1. Transformation frequency of accepting xylanase activity from <u>Bacillus subtilis</u> to <u>Azotobacter</u> sp.	41
2. Xylanase activity of wild, transformant & donor strains.	46
3. Nitrogen content of cultures of wild & transformed <u>A. vinelandii</u>	49
4. Effect of carbon source on xylanase production by transformed <u>A. vinelandii</u>	51
5. Effect of xylan concentration on xylanase production by transformed <u>A. vinelandii</u>	54
6. Effect of Nitrogen source on xylanase production by transformed <u>A. vinelandii</u>	57
7. Effect of phosphate concentration on xylanase production by transformed <u>A. vinelandii</u>	61
8. Effect of salinity on xylanase production by transformed <u>A. vinelandii</u>	64
9. Effect of pH on xylanase production by transformed <u>A. vinelandii</u>	66
10. Purification of xylanase from transformed <u>A. vinelandii</u>	69
11. Optimum temperature for the purified xylanase	74
12. Optimum pH for the purified xylanase	77
13. Time course of enzymatic reaction.	80
14. Effect of various reagent on the purified xylanase activity	83

## List of Figures & Photos

<i>Figure</i>	<i>Page</i>
1. Xylan structure with side chains attached	3
2. Photographs for donor & recipient strains	43
3. Staining reaction for detection of xylanase activity	44
4. Xylanase activity of wild , transformant & donor strains.	47
5. Effect of carbon on xylanase production by transformed <u>A. vinelandii</u>	52
6. Effect of xylan concentration on xylanase production by transformed <u>A. vinelandii</u>	55
7. Effect of nitrogen source on xylanase production by transformed <u>A. vinelandii</u>	58
8. Effect of phosphate concentration on xylanase production by transformed <u>A. vinelandii</u>	62
9. Effect of salinity on xylanase production by transformed <u>A. vinelandii</u>	65
10. Effect of pH on xylanase production by transformed <u>A. vinelandii</u>	67
11. Elution profile from a DEAE-cellulose column	71
12. Elution profile from a Sephadex G-200 column	72
13. Optimum temperature for the purified xylanase	75
14. Optimum pH for the purified xylanase	78
15. Time course of enzymatic reaction.	81
16. Effect of various reagent on enzyme activity	84

## Abstract

Azotobacter vinelandii & A. chroococcum accepted the ability to produce xylanase enzyme through genetic transformation using crude lysate DNA of Bacillus subtilis CAIM 1007. Of the two recipients, A. vinelandii was transformed with a higher frequency & showed more xylanase expression.

Furthermore, transformed A. vinelandii retained its capability for N<sub>2</sub> fixation as it was before transformation . so this strain was further studied for optimum conditions for production of xylanase enzyme & purification of the enzyme . The final purification fold of the enzyme was 22 regarding to the crude enzyme.

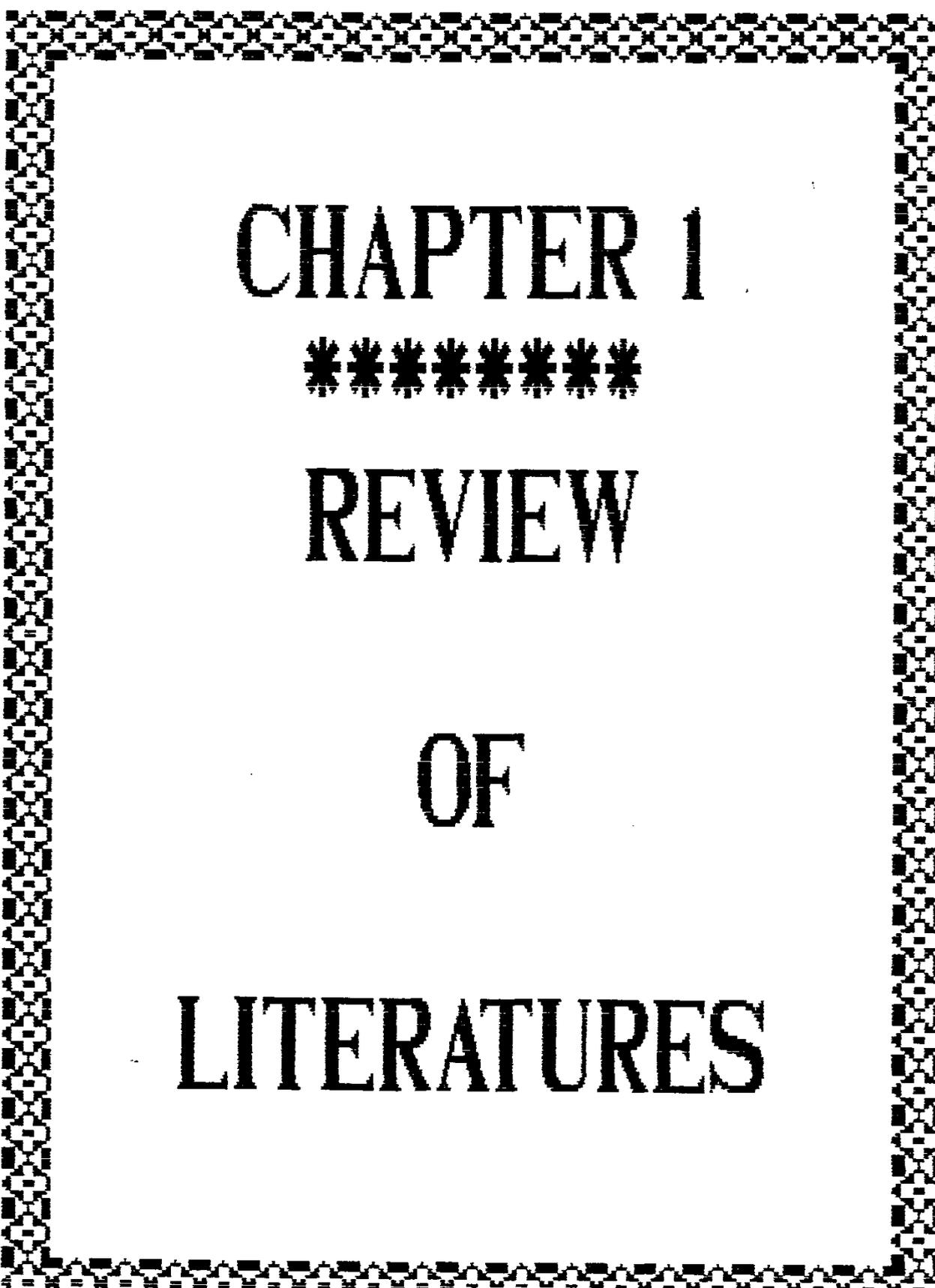
## I N T R O D U C T I O N

Nitrogen is lost from soils due to the processes of nitrification, denitrification and leaching. Unlike other plant nutrients, it can not be replaced by weathering of rocks or soil particles and has to be replaced by the small amounts of nitrogen present in rain water, by the processes of microorganisms, or artificially through fertilizers. With the current technology for fertilizer production and the inefficient methods employed for fertilizer application, both economic and ecological costs of fertilizer usage will eventually become prohibitive. Productive agriculture, therefore, should be more dependent on microbial nitrogen fixation. This can be accomplished by manipulating organisms to fix nitrogen more efficiently, or providing useful traits for such diazotrophs. The plant material in the form of agricultural wastes contribute naturally to the organic content of the soil or introduced as natural fertilizer depending on the action of soil microorganisms which serve in the conversion of complex organic compounds into simple inorganic forms or into their constituent elements.

In addition to the role of nitrogen fixing bacteria in increasing the nitrogen fertility of soil, it is more useful to render this group capable of degrading and utilizing the agricultural wastes, which are composed mainly of cellulose and

hemicellulose. The product of such degradation will provide nutrients for less versatile microbial species which have a role in biochemical processes in soil.

This work is an attempt to construct a genetically improved Azotobacter sp. (the well known nitrogen fixer) by acquiring an exogenous xylanase gene (the most famous group of hemicelluloses), in order to provide an additional source of renewable nitrogen for enhancing crop yield.



# CHAPTER 1

\*\*\*\*\*

# REVIEW

# OF

# LITERATURES

### 1.1. The hemicellulosic polysaccharides

Hemicellulose is the second most abundant fraction available in nature. It is a storage polymer in seeds ( Taiz and Honigman 1976 ) and it forms the structural component in cell walls of woody plants ( Eriksson 1990 ). The term " hemicellulose" was firstly introduced by Schulze ( 1891 ) for the fraction isolated or extracted from plant materials with dilute alkali. The classification of these hemicellulose fractions depends on the types of sugar moieties present. The principal monomers present in most hemicelluloses are D-xylose, D-mannose, D-galactose and L-arabinose. Hemicelluloses include xylan, mannan, galactan and arabinan as the main heteropolymers. Xylan contains D-xylose as its main monomeric unit, galactan contains D-galactose and mannan is made up of D-mannose units, while arabinan is composed of L-arabinose.

Hemicelluloses differ greatly in their molecular weights. The molecular weight of arabinoglucuronoxylan from wheat straw is 19400 Da, while that of glucuronoxylan from alder wood is 8600 ( Gorbacheva and Radionova 1977 ). Hemicellulose-like polysaccharides from marine brown algae have a range of molecular weights of 3800 - 75000 Da ( Hassan 1990 )

This indicates that the molecular weights of hemicelluloses are dependent on the source of isolation.

Xylans , the major portion of the hemicellulose of plant cell walls and grasses are heteropolymers. The homopolymer of xylan, which comprises only xylose units, is difficult to isolate. Normally xylan contains two to four different sugar monomers. The esparto grass xylan is composed of  $\beta$ -(2 $\rightarrow$ 4) linked branched as well as unbranched chains of D- xylopyranose units ( Chanda et al 1950 ). xylans from different plants and grasses have the same backbone structure of  $\beta$ -(1 $\rightarrow$ 4) glycosidic linkage between two adjacent xylose residues ( Whistler 1950, Aspinal 1959 ). The only differences in the branched residues is of D- glucuronic acid, L- arabinose and 4-O- methylesters of glucuronic acid ( Fig.1 ). Aspinal (1959), showed that xylan contains 85 to 93% of D-xylose, a small amount of L- arabinose and traces of glucuronic acid residues. Arabinoglucuronoxylan ( an acidic xylan ) prepared from wheat straw has the approximate composition of D-xylose 70%, L- arabinose 9%, D-glucose 5%, uronic acid 11% and D-galactose