

GENETIC IMPROVEMENT OF SOME BACTERIAL STRAINS PRODUCING VITAMIN B₁₂

By

Bigad El-Sayed Mahmoud Ahmed Khalil

B.Sc. Agric. Sci. (Biochemistry), Ain Shams University, 2002

M.Sc.Agric.Sci.(Genetics), Ain Shamus University, 2011

**A Thesis Submitted in Partial Fulfillment
Of
The Requirement for the Degree of**

**DOCTOR OF PHILOSOPHY
in
Agricultural Sciences
(Genetics)**

**Department of Genetics
Faculty of Agriculture
Ain Shams University**

2017

Approval Sheet

**GENETIC IMPROVEMENT OF SOME BACTERIAL
STRAINS PRODUCING VITAMIN B₁₂**

By

Bigad El-Sayed Mahmoud Ahmed Khalil

B.Sc. Agric. Sc. (Biochemistry), Ain Shams University, 2002

M.Sc.Agric.Sc.(Genetics), Ain Shamus University, 2011

This thesis for Ph.D. degree has been approved by:

Dr. Mohamed Abdel- Baith El- Seehy

Prof. Emeritus of Genetics, Faculty of Agriculture, Alexandria
University

Dr. Khaled Abdel-Aziz Abdel-Aty Soliman

Prof. of Genetics, Faculty of Agriculture, Ain Shams University

Dr. Ashraf Bakry Abdel-Razik

Prof. of Genetics, Faculty of Agriculture, Ain Shams University

Dr. Samir Abdel-Aziz Ibrahim

Prof. Emeritus of Genetics, Faculty of Agriculture, Ain Shams
University

Date of Examination:

GENETIC IMPROVEMENT OF SOME BACTERIAL STRAINS PRODUCING VITAMIN B₁₂

By

Bigad El-Sayed Mahmoud Ahmed Khalil

B.Sc. Agric. Sci. (Biochemistry), Ain Shams University, 2002

M.Sc.Agric.Sci.(Genetics), Ain Shamus University, 2011

Under the supervision of:

Dr. Samir Abdel-Aziz Ibrahim

Prof. Emeritus of Genetics, Department of Genetics, Faculty of Agriculture, Ain Shams University (Principal supervisor)

Dr. Ashraf Bakry Abdel-Razik

Prof. of Genetics, Department of Genetics, Faculty of Agriculture, Ain Shams University

Dr. Abd El-Hamid Abd El-Alim Haggran

Researcher Prof. of Genetics, Department of Microbial Genetics, National Research Center

ABSTRACT

Bigad El-Sayed Mahmoud Ahmed Khalil: Genetic Improvement of Some Bacterial Strains Producing Vitamin B₁₂. Unpublished PhD. Thesis, Department of Genetics, Faculty of Agriculture, Ain Shams University, 2017

The present investigation aims to improving some bacterial strains for producing vitamin B₁₂ through genetic improvement methods *i.e* induction of mutation and protoplast fusion. In this study 18 bacterial cultures were isolated from three different areas (Menofia - Domiatt-Cairo) and tested for production of vitamin B₁₂. Three isolates which highly producing vitamin B₁₂ are identified as a *Pseudomonas* strains by some biochemical tests and 16s rDNA technique. The B₁₂ production was determined by spectrophotometric and HPLC methods. Multiple mutagenesis technique by Ethyl Methan Sulphonate (EMS) as a chemical mutagen and Ultraviolet (UV) as a physical mutagen used to increase vitamin B₁₂ production of strains. The strains treated with 200 mM EMS for different exposure time (15, 30, 45, 60 and 90 min). Mutant M 6 from (*P. aeruginosa*) produced 6 mg/L B₁₂ higher than their parent that produced 4 mg/L that increase the production by 50% in the first step mutation, in the second step mutation the mutant M 6.9 from (*P. aeruginosa*) produced 12.3 mg/L that increase the production by 207%, and in the third step mutation the mutant M 6.9.9 from (*P. aeruginosa*) produced 15.5 mg/L that increase the production by 287%.

The strains were treated with UV with different exposure time (30, 60, 90, 120, 150 and 180 sec) at fixed distance of 15 cm from light source, mutant coded P1 (M6.9.9.uv7), giving 20.3 mg/L that increase the production by 408 % in first step mutation. In in the second step mutation, the mutant coded P1 (M6.9.9.uv7.5) was the most B₁₂ producer mutant,

giving 21 mg/L showed 425% improvement over the wild-type strain (P1). Intra and inter specific protoplast fusion between auxotrophic mutants isolated after treatment with mutagen was carried out, twenty fusants were successfully obtained, fusant F10 produced 7 mg/L, from intra specific protoplast fusion, fusant F4 produced 7.9 mg/L, from inter specific protoplast. Finally ISSR-PCR marker technique were used to differentiation among parents and its mutants.

Keywords: *Pseudomonas*, vitamin B₁₂, mutation induction, EMS, UV, ISSR.

ACKNOWLEDGEMENT

First and above all, I praise Allah, the almighty for providing me this opportunity and granting me the capability to proceed successfully this thesis to appears in its current form.

I would like to express my sincere gratitude to my supervisor **Prof. Dr. Samir Abdel-Aziz Ibrahim** prof. Emeritus of Genetics, Faculty of Agriculture, Ain Shams University for the continuous support of my M.Sc and Ph.D study and research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. Words are not enough to express how grateful I am to him.

I am grateful to my supervisor warmly **Prof. Dr. Ashraf Bakry Abdel-Razik** prof. of Genetics, Faculty of Agriculture, Ain Shams University, whose encouragement and supervision and support from the initial to the final level enabled me to develop and understanding of the introduced and gave me moral support needed to complete the thesis.

The author is greatly indebted to **Dr. Abd El-Hamid Haggran** Professor of Microbial Genetics, Department of Microbial Genetics, National Research Center for suggesting the problem, sincere support, effective, valuable help, encouragement and his help in preparation the manuscript.

Sincere thanks and0 appreciation to **Dr. Tahany Mohamed Abo Elfath El-kawokgy** Researcher Professor Emeritus of Microbial Genetics, Microbial Genetics Department, National Research Centre for her excellent advises, and her help in writing and reviewing the manuscript.

Thanks should be extended to **(ACGEB), Genetics Department** members, Faculty of Agriculture, Ain Shams University and **Microbial Genetics** Department members, National Research Center for their help and continuous encouragement to complete this work.

To my family and my friends, you should know that your support and encouragement was worth more than I can express.

CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	3
1. Vitamin B₁₂	3
1.1.Chemistry of B ₁₂	4
1.2. Forms of B ₁₂	5
1.3. Vitamin B ₁₂ deficiency	6
1.4. Physiology of vitamin B ₁₂	7
1.5. Sources of vitamin B ₁₂ and bioavailability	8
1.5.1.Vitamin B ₁₂ in animal food	9
1.5.2.Vitamin B ₁₂ in plant food	10
1.6.Microbial production of vitamin B ₁₂	11
1.7. Biosynthetic pathways of vitamin B ₁₂	13
1.8. Determination of B ₁₂	13
1.8.1. Microbiological assay methods	13
1.8.2. Colorimetric methods.....	16
1.8.3. High-performance liquid chromatography (HPLC)....	16
2. Pseudomonas	17
2.1. Isolation and characterization of pseudomonas isolates..	18
2.2. Molecular analysis (16S rDNA).....	19
3. Genetic approaches for overproduction of B₁₂	19
3.1. Mutation	20
3.2. Recombinant DNA technology	22
MATERIALS AND METHODS	24
1. Materials	24
1.1. Bacterial strains.....	24
1.2. Primers	24
1.2.1. 16S rDNA universal primers	24
1.2.2.Inter Simple Sequence Repeat (ISSR) primers.....	24
1.3. Media.....	25
1.3.1.LB medium.....	25
1.3.2. Nutrient Agar	25
1.3.3. Minimal glucose.....	25

II

1.3.4. Production medium.....	25
1.3.5. Pseudomonas agar base	26
1.4. Buffers, reagents and solution.....	26
1.4.1. For protoplast induction	26
1.4.1.1. Protoplast solution	26
1.4.1.2. Regeneration solution	26
1.4.1.3. P- solution and regeneration solution supplemented	26
1.4.1.4. Lysozyme.....	26
1.4.1.5. Polyethylene glycol buffer (PEG).....	27
1.4.2. DNA buffers.....	27
1.4.2.1. The Wizard® Genomic DNA Purification Kit.....	27
1.4.2.2. PCR-Pure Kit (BIORON).....	27
1.4.2.3. Agarose gel electrophoresis.....	27
1.4.2.4. TBE buffer (5X).....	27
1.4.2.5. Loading buffer.....	27
1.4.2.6. Ethidium bromide.....	28
1.5. Chemical mutagen.....	28
1.6. DNA marker	29
2. Methods	29
2.1. Isolation of bacteria.....	29
2.2. Molecular identification of bacterial isolates.....	30
2.2.1. DNA extraction.....	30
2.2.2. PCR amplification of 16s rDNA.....	30
2.2.3. Agarose gel electrophoreses.....	30
2.2.4. Phylogenetic analysis.....	31
2.3. Vitamin B12 assay	31
2.3.1. Spectrophotometric methods.....	31
2.3.2. HPLC methods.....	32
2.4. Induction of mutation.....	33
2.4.1. Induction of mutation using EMS.....	33
2.4.2. Induction of mutation using UV.....	33
2.5. Isolation of auxotrophic mutants.....	34
2.6. Preparation of protoplast.....	34
2.6.1. Protoplast formation.....	34

2.6.2. Protoplast fusion.....	35
2.6.3. Protoplast regeneration.....	35
2.7. Inter-simple Sequence Repeat (ISSR) PCR.....	36
2.7.1. DNA extractions.....	36
2.7.2. Thermal cycling.....	36
2.7.3. Agarose gel electrophoresis.....	36
RESULTS AND DISCUSSION	37
1.Isolation and identification of B ₁₂ producing bacteria	37
1.1. Isolation of B ₁₂ producing bacteria	37
1.2. Identification of bacterial isolates.....	37
1.3. Determination of vitamin B ₁₂ concentration for selected isolates.....	39
1.4. PCR amplification of the 16S rRNA gene.....	42
1.5. 16S rDNA gene sequence similarity and Phylogenetic analysis	43
2. Induction of mutation.....	52
2.1. Mutagenesis by EMS.....	52
2.1.1. First step mutagenesis by EMS.....	53
2.1.1.1. <i>P. aeruginosa</i> (P1) mutagenesis	53
2.1.1.2. <i>P. fluorescenc</i> (P3) mutagenesis	55
2.1.1.3. <i>P. putida</i> (P11) mutagenesis.....	58
2.1.2. Second step mutagenesis by EMS.....	59
2.1.2.1. <i>P. aeruginosa</i> (P1) M6 mutagenesis	60
2.1.2.2. <i>P. fluorescenc</i> (P3) M7 mutagenesis.....	61
2.1.2.3. <i>P. putida</i> (P11) M5 mutagenesis.....	63
2.1.3. Third step mutagenesis by EMS.....	64
2.1.3.1. <i>P. aeruginosa</i> (P1) M6.9 mutagenesis.....	64
2.2. Mutagenesis by UV irradiation.....	66
2.2.1. First step mutagenesis by UV.....	66
2.2.1.1. <i>P. aeruginosa</i> (P1) mutagenesis.....	67
2.2.1.2. <i>P. fluorescenc</i> (P3) mutagenesis.....	70
2.2.1.3. <i>P. putida</i> (P11) mutagenesis.....	72
2.2.1.4. <i>P. aeruginosa</i> (P1) M6.9.9 mutagenesis.....	74
2.2.2. Second step mutagenesis by UV.....	76

2.2.2.1. <i>P. aeruginosa</i> (P1) M.uv6 mutagenesis.....	76
2.2.2.2. <i>P. aeruginosa</i> (P1) M6.9.9.uv7 mutagenesis.....	77
3. Protoplast fusion.....	80
3.1. Characterization of auxotrophic <i>P. aeruginosa</i> and <i>P. fluorescence</i> mutants.....	80
3.2. Protoplast formation and fusion.....	81
3.2.1. Intra specific protoplast.....	82
3.2.2. Inter specific protoplast.....	85
4. Inter-simple sequence repeat (ISSR) PCR	89
4.1. Primer 1.....	89
4.2. Primer 2.....	90
4.3. Primer 3.....	92
4.4. Primer 4.....	93
4.5. Primer 5.....	95
4.6. Analysis of ISSR for the highest B12 producing mutants and their parents.....	96
SUMMARY	98
REFERENCES	101
ARABIC SUMMARY	

LIST OF TABLES

No.		Page
1	Biochemical tests of bacterial isolates	38
2	The relationship between B ₁₂ concentration and absorption .	39
3	B ₁₂ concentration of isolates	41
4	Identification percentage between bacterial isolates and related species in Gen Bank database	51
5	The effect of EMS at different exposure time interval on the survival and auxotrophic mutation rate of P1.....	53
6	B ₁₂ production of P1 selected mutants	54
7	The effect of EMS at different exposure time interval on the survival and auxotrophic mutation rate of P3.....	56
8	B ₁₂ production of selected mutants from P3.....	57
9	The effect of EMS at different exposure time interval on the survival and auxotrophic mutation rate of P11.....	58
10	B ₁₂ production of selected mutants from P11.....	59
11	The effect of EMS at different exposure time interval on the survival rate of (P1) M6.....	60
12	B ₁₂ production of selected mutants from (P1) M6.....	60
13	The effect of EMS at different exposure time interval on the survival rate of (P3) M7.....	61
14	B ₁₂ production of selected mutants from (P3) M7.....	62
15	The effect of EMS at different exposure time interval on the survival rate of (P11) M5.....	63
16	B ₁₂ production of selected mutants from (P11) M5.....	63
17	The effect of EMS at different exposure time interval on the survival rate of (P1) M6.9.....	64
18	B ₁₂ concentration of selected mutants from (P1) M6.9.....	65
19	The effect of UV at different exposure time interval on the survival and auxotrophic mutation rate of P1.....	67
20	B ₁₂ production of selected mutants from P1.....	69

21	The effect of UV at different exposure time interval on the survival and auxotrophic mutation rate of P3.....	70
22	B ₁₂ production of selected mutants from P3.....	71
23	The effect of UV at different exposure time interval on the survival and auxotrophic mutation rate of P11	72
24	B ₁₂ production of selected mutants from P11.....	73
25	The effect of UV at different exposure time interval on the survival rate of (P1) M6.9.9.....	74
26	B ₁₂ production of selected mutants from (P1) M6.9.9.....	75
27	B ₁₂ production of selected mutants from (P1) M.uv6.....	77
28	The effect of UV at different exposure time interval on the survival rate of (P1) M6.9.9.uv7.....	78
29	B ₁₂ concentration of selected mutants from P1M6.9.9.uv7..	78
30	Protoplast fusion between <i>P. aeruginosa</i> . (asn-) & <i>P. fluorescence</i> .(gln-).....	83
31	B ₁₂ production of selected fusants from <i>P. aeruginosa</i> (asn-) & <i>P. fluorescence</i> (gln-).....	85
32	Protoplast fusion between <i>P. aeruginosa</i> . (asn-) & <i>P. aeruginosa</i> .(arg -).	87
33	B ₁₂ concentration of selected fusants from <i>P. aeruginosa</i> (asn-) & <i>P. aeruginosa</i> (arg-).	88
34	Polymorphism generated by primer 1 for parents and its high B ₁₂ producing mutants	90
35	Polymorphism generated by primer 2 for parents and its high B ₁₂ producing mutants	91
36	Polymorphism generated by primer 3 for parents and its high B ₁₂ producing mutants	93
37	Polymorphism generated by primer 4 for parents and its high B ₁₂ producing mutants	94
38	Polymorphism generated by primer 5 for parents and its high B ₁₂ producing mutants.	95
39	Similarity percentages between highest B ₁₂ producing mutants and their parents	97

LIST OF FIGURES

No.	Page
1 The chemical structure of cobalamin (vitamin B ₁₂).....	5
2 Biosynthetic pathways of vitamin B ₁₂	15
3 Illustration of mutagenic effect of EMS on the nitrogen base Guanine.....	28
4 1Kb DNA ladder map.....	29
5 Standard curve of B ₁₂	40
6 Standard B ₁₂ diagram by HPLC	41
7 B ₁₂ concentration diagram of isolates P3 by HPLC.....	41
8 Agarose gel electrophoreses of 16S rRNA amplification of isolates. lane M, 1 kb DNA ladder.....	42
9 Forward DNA sequence of 16S rRNA of isolate P1	43
10 Reverse DNA sequence of 16S rRNA of isolate P1	44
11 Overlap DNA sequence of 16S rRNA of isolate P1.	44
12 Phylogenetic tree based on 16S rRNA sequence of isolate P1 and selected pseudomonas aeruginosa strain from the database using BLAST programe.	45
13 Forward DNA sequence of 16S rRNA of isolate P3	45
14 Reverse DNA sequence of 16S rRNA of isolate P3	46
15 Overlap DNA sequence of 16S rRNA of isolate P3.	46
16 Phylogenetic tree based on 16S rRNA sequence of isolate P3 and selected Pseudomonas fluorescens strain from the database using BLAST programe.	47
17 Forward DNA sequence of 16S rRNA of isolate P4.	47
18 Reverse DNA sequence of 16S rRNA of isolate P4	48
19 Overlap DNA sequence of 16S rRNA of isolate P4.	48
20 Phylogenetic tree based on 16S rRNA sequence of isolate P4 and selected Pseudomonas fluorescens strain from the database using BLAST programe.	49
21 Forward DNA sequence of 16S rRNA of isolate P11	49
22 Reverse DNA sequence of 16S rRNA of isolate P11.	50
23 Overlap DNA sequence of 16S rRNA of isolate P11	50
24 Phylogenetic tree based on 16S rRNA sequence of isolate P11	

VIII

and selected <i>Pseudomonas putida</i> strain from the database using BLAST programe.	51
25 The effect of EMS dose at different time interval on the survival and auxotrophic mutation rate of P1	54
26 Diagram B ₁₂ concentration of (P1) M6 by HPLC	55
27 The effect of EMS dose at different time interval on the survival and auxotrophic mutation rate of P3.	56
28 B ₁₂ concentration diagram of (P3) M7 by HPLC	57
29 The effect of EMS dose at different time interval on the survival and auxotrophic mutation rate of P11	58
30 B ₁₂ concentration diagram of (P1) M6.9 by HPLC.	61
31 B ₁₂ concentration diagram of (P3) M7.3 by HPLC	62
32 B ₁₂ concentration diagram of (P1) M6.9.9 by HPLC	65
33 The effect of UV dose at different time interval on the survival and auxotrophic mutation rate of P1	68
34 B ₁₂ concentration diagram of (P1)Muv6 by HPLC	69
35 The effect of UV dose at different time interval on the survival and auxotrophic mutation rate of P3.	71
36 The effect of UV dose at different time interval on the survival and auxotrophic mutation rate of P11.	73
37 The effect of UV dose at different time interval on the survival rate of (P1) M6.9.9	75
38 B ₁₂ concentration diagram of (P1)M6.9.9 uv7 by HPLC	76
39 B ₁₂ concentration diagram of (P1) M6.9.9 uv7.5 by HPLC	79
40 The form of prototroph (large) and auxotroph (small) colonies	81
41 B ₁₂ concentration diagram of Fusant F10 by HPLC	85
42 B ₁₂ concentration diagram of Fusant F4by HPLC	88
43 Agarose (1.8%) gel electrophoresis of ISSR-PCR products for primer 1 of mutants and their parents.	89
44 Agarose (1.8%) gel electrophoresis of ISSR-PCR products for primer 2 of mutants and their parents	91
45 Agarose (1.8%) gel electrophoresis of ISSR-PCR products for primer 3 of mutants and their parents.	92
46 Agarose (1.8%) gel electrophoresis of ISSR-PCR products for	

primer 4 of mutants and their parents	94
47 Agarose (1.8%) gel electrophoresis of ISSR-PCR products for primer 5 of mutants and their parents.	95
48 Dendrogram of ISSR based on unweighted pair group method with arithmetic averages algorithm (UPGMA) for highest B ₁₂ producing mutants and their parents.	96