PRODUCTION AND CHARACTERIZATION OF URICASE FROM *Pseudomonas otitidis* STRAIN SN1 ISOLATED FROM A HOT SPRING

NOR SAHSLIN BINTI IRWAN SHAH LEE

A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Bioscience)

Faculty of Science
Universiti Teknologi Malaysia

OCTOBER 2019

DEDICATION

This thesis is dedicated to my parents; Irwan Shah Lee bin Abdullah, Rahimah bt Ahmad and Ahmad bin Yaacub

My parents in law;Allahyarham Hj Nasir and Hjh Mek

For my lovely husband; Mohd Khairul Mizan bin Nasir

For my wonderful children; Muhammad Ammar Anaqi, Nur Aufa Azzahra and Muhammad Azfar Anaqi

ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious, The Most Merciful. Praise is to Allah S.W.T. by whose grace and blessing, I received guidance in completing this study. Thanks for His greatest love and blessings.

First and foremost, I wish to express my profound gratitude to my supervisors, Assoc. Prof. Dr Shafinaz Shahir and Dr Norahim, for guidance, encouragement and critics throughout the study. I am also indebted to Dr Haryati Jamaluddin for assisting me on the purification study. Without their continued support and interest, this thesis would not have been the same as presented here. I thanked foremost to Ministry of Higher Education for the scholarship award, Universiti Teknologi Malaysia for the research facilities and funding. Million thanks also go to my fellow postgraduate friends for their assistance, support and motivation. My sincere appreciation to all laboratory staffs in Bioscience Department especially Mr. Yusnizam and Mr. Afiezy for their assistance during my laboratory works.

Deepest thanks and unending gratitude go to my parents and parent in-laws for their continuous love, prayers and encouragement throughout my study. Finally, my greatest and warmest appreciation to my best friend and dearest husband, Mohd Khairul Mizan whose unending love, understanding, patience and constant support throughout my darkest day. Without them, this long journey will be very exhausting. A heartfelt thank you to my adorable children, Ammar, Aufa, and Azfar, for their sacrifices as three of you were born during my PhD studies and witnessed to all my sweat and tears to complete this study. All of you are my driving force to end this long journey.

ABSTRACT

Uricase is an enzyme that catalyzes the oxidation of uric acid to allantoin. This study aimed to exploit bacteria isolated from hot springs to produce uricase using uric acid as substrate. Sampling was conducted at Hulu Langat hot springs and the samples were enriched in LB medium followed by inoculation into uric acid minimal medium. The isolated bacteria were screened for uricase production using plate assay method. One-factor-at-a-time (OFAT) methodological optimization was used to enhance uricase production from the selected bacteria. Uricase was then purified using anion exchange and gel filtration chromatography prior to its characterization. Four bacterial strains positive for uricase enzyme activity were successfully isolated and were identified as *Pseudomonas otitidis* strain SN1, *Pseudomonas* sp. strain SN2, *Pseudomonas stutzeri* strain SN3 and *Pseudomonas* sp. strain SN4. *P. otitidis* strain SN1 showed the highest uricase activity (0.12 U/mg) and was selected for further study. Optimized uricase production of strain SN1 was achieved with uric acid minimal medium supplemented with 2% (w/v) glucose, 0.2% (w/v) uric acid, 2% (w/v) yeast extract, and adjusted to pH 7.2. Statistical analyses showed that all variables were significant parameters that affect the production of uricase. The uricase was successfully purified to homogeneity using 70% (w/v) ammonium sulfate precipitation, HiTrap™ Q Sepharose HP anion exchange chromatography and Superdex G-75 gel chromatography. The specific activity of uricase was increased by 3.52 fold, from 0.058 U/mg for crude to 0.204 U/mg for purified uricase. The purified uricase showed a size of approximately 35 kDa band on SDS PAGE gel. The kinetics of purified uricase was 16.54 µM and 0.2 U/mg (V_{max}). The purified uricase exhibited an optimum activity at 35°C and pH 8.5. The enzyme was stable from pH 8.0-9.0 and was able to retain 50% of its total activity after incubation for 30 minutes at 50°C. Activity of uricase was retained in the presence of metal ions (Cu^{2+}, Fe^{2+}, Mn^{2+}) and detergent (urea). The band corresponding to the expected size of uricase, was sent for mass spectrometry analysis for protein identification. However, the results showed that the peptide sequences matched that of ornithine carbamoyltransferase (score=780). The discrepancy was probably due to the presence of low abundance of uricase which resulted in low peptides spectra. Further literature search revealed that both uricase and ornithine carbamoyltransferase are involved in the metabolic pathway producing urea. It was thus postulated that *P. otitidis* strain SN1 may possess two metabolic pathways (uricolysis and argininolysis) and thus, able to exhibit dual enzyme activity (uricase and ornithine carbamoyltransferase). This study is the first to report the characteristic of uricase producing bacterium, *P. otitidis* strain SN1 isolated from hot spring, and also suggesting the hot spring as potential source of new uricase.

ABSTRAK

Urikase ialah enzim pemangkin yang mengoksidakan asid urik kepada alantoin. Matlamat kajian ini adalah untuk mengeksploitasi bakteria yang dipencilkan dari kolam air panas untuk menghasilkan urikase dengan asid urik sebagai substrat. Pensampelan dilakukan di kolam air panas di Hulu Langat dan sampel diperkaya dalam medium LB diikuti oleh inokulasi ke dalam medium minimal asid urik. Bakteria yang dipencilkan disaring untuk penghasilan urikase menggunakan kaedah cerakinan plat. Pengoptimuman metodologi satu faktor-pada-masa (OFAT) digunakan untuk meningkatkan pengeluaran urikase daripada bakteria terpilih. Urikase kemudiannya ditulenkan menggunakan kaedah kolum kromatografi penukaran cas anion dan kolum kromatografi penapisan gel sebelum penciriannya. Empat bakteria positif aktiviti urikase telah berjaya dipencilkan dan telah dikenalpasti sebagai *Pseudomonas otitidis* strain SN1, *Pseudomonas* sp. strain SN2, *Pseudomonas stutzeri* strain SN3 dan *Pseudomonas* sp. strain SN4. *P. otitidis* strain SN1 menunjukkan aktiviti urikase paling tinggi (0.12 U/mg) dan telah dipilih untuk ujian selanjutnya. Penghasilan optimum urikase oleh strain SN1 telah dicapai dengan medium minimal asid urik yang ditambahi dengan 2% (b/i) glukosa, 0.2% (b/i) asid urik, 2% (b/i) ekstrak yis pada pH 7.2. Analisis statistikal menunjukkan semua faktor adalah signifikan dalam mempengaruhi penghasilan urikase. Urikase telah berjaya ditulenkan kepada homogen menggunakan pemendapan ammonium sulfat, kolum kromatografi penukaran cas anion HiTrap™ Q Sepharose HP dan kolum kromatografi penapisan Superdex G-75. Aktiviti spesifik urikase meningkat sebanyak 3.52 kali ganda, daripada 0.058 U/mg pada enzim kasar kepada 0.204 U/mg pada enzim tulen. Urikase tulen mempunyai saiz kira-kira 35 kDa telah ditujukkan dalam gel SDS PAGE. Kinetik enzim tulen adalah 16.54 µM (K_m) dan 0.2 U/mg (V_{max}). Urikase tulen menunjukkan aktiviti optimum pada suhu 35°C dan pH 8.5. Enzim ini didapati stabil antara pH 8.0-9.0 dan mampu mengekalkan 50% aktiviti asalnya selepas dieramkan selama 30 minit dalam suhu 50°C. Aktiviti urikase juga tidak terjejas oleh kehadiran ion logam (Cu^{2+}, Fe^{2+}, Mn^{2+}) dan juga detergen (urea). Jalur protein sepadan dengan saiz yang dianggapkan sebagai urikase telah dianalisa spektrofotometer jisim untuk pengenalpastian protein. Walau bagaimanapun keputusan analisis menunjukkan peptida tersebut berpadanan dengan karbamoiltransferase ornitin (skor=780). Perbezaan ini mungkin berpunca daripada kehadiran peptida urikase yang terlalu rendah lalu menghasilkan spektrum rendah. Carian literatur menunjukkan kedua-dua enzim ini terlibat dalam laluan metabolik menghasilkan urea. Oleh itu, *P. otitidis* strain SN1 dicadangkan mempunyai dua laluan metabolik (urikolisis dan argininolisis) dan oleh sebab itu, mampu mempamerkan aktiviti enzim ganda (urikase dan karbamoiltransferase ornitin). Kajian ini adalah yang pertama melaporkan pencirian bakteria yang menghasilkan urikase, *P. otitidis* strain SN1 yang dipencilkan daripada kolam air panas, dan seterusnya mencadangkan kolam air panas sebagai sumber yang berpotensi untuk pencarian urikase baru.

TABLE OF CONTENTS

	TITLE	PAGE
	DECLARATION	**ii**
	DEDICATION	**iii**
	ACKNOWLEDGEMENT	**iv**
	ABSTRACT	**v**
	ABSTRAK	**vi**
	TABLE OF CONTENTS	**vii**
	LIST OF TABLES	**xiii**
	LIST OF FIGURES	**xv**
	LIST OF ABBREVIATIONS	**xix**
	LIST OF SYMBOLS	**xx**
	LIST OF APPENDICES	**xxi**
CHAPTER 1	**INTRODUCTION**	**1**
1.1	Background of Study	1
1.2	Problem Statement	4
1.3	Objectives of Study	5
1.4	Scope of Study	5
1.5	Significance of Study	6
CHAPTER 2	**LITERATURE REVIEW**	**9**
2.1	Introduction	9
2.2	Biochemistry and Metabolism of Uric Acid	10
2.3	Uric Acid Related Disorders	11
	2.3.1 Asymptomatic Hyperuricemia	12
	2.3.2 Gout	13
	2.3.3 Tumor Lysis Syndrome	14
	2.3.4 Coronary Heart Disease (CHD)	15
2.4	Treatment of Uric Acid Related Disorders	15

	2.4.1	Allopurinol	16
	2.4.2	Febuxostat	17
	2.4.3	Colchicine	17
2.5	Uricase (Urate oxidase)		18
2.6	Uricase Reactions and Intermediates		20
2.7	Uricase Enzyme Microbial Producers		22
2.8	*Pseudomonas otitidis*		24
2.9	Hot Springs as Isolation Source		25
2.10	Application of Uricase		28
2.11	Optimization of Uricase Production in Microorganism		30
	2.11.1	Effect of Carbon and Nitrogen Sources in Microorganisms	31
	2.11.2	Effect of Environmental Factors	32
2.12	Purification of Uricase Enzyme		36
	2.12.1	Ammonium Sulfate Precipitation	37
	2.12.2	Ion Exchange Chromatography	38
	2.12.3	Gel Filtration Chromatography	39
2.13	Biochemical Characterization of Uricase		43
2.14	Protein Identification via Mass Spectrometry		48
CHAPTER 3	**RESEARCH METHODOLOGY**		**51**
3.1	Materials		51
	3.1.1	Chemicals and Biologicals	51
	3.1.2	Preparation of Media	51
		3.1.2.1 Luria-Bertani (LB) Medium	52
		3.1.2.2 Uric Acid Minimal Medium 1	52
		3.1.2.3 Uric Acid Minimal Medium 2	53
	3.1.3	PCR Primers	53
3.2	Sampling		53
3.3	Isolation of Uricase Producing Bacteria		54
3.4	Screening of Potential Uricase Producing Bacteria		54
3.5	Culture preservation of Isolated Bacteria and Storage		55

3.6 Morphological Characterization of Isolated Bacteria Using Gram Staining 55

3.7 Identification of Potential Uricase Producing Bacteria Based on 16S rRNA Analysis 56

3.7.1 Genomic DNA Extraction 56

3.7.2 Qualitative Analysis of DNA 57

3.7.3 Quantitative Analysis of DNA 58

3.7.4 Polymerase Chain Reaction (PCR) 58

3.7.5 Purification of PCR Product 60

3.7.6 16S rRNA Gene Analysis 60

3.7.7 Construction of Phylogenetic Tree 61

3.7.7.1 Multiple Sequence Alignment Using ClustalW 61

3.7.7.2 Construction of Phylogenetic Tree Using MEGA Software 61

3.8 Growth Profile of Potential Uricase Producing Bacteria 62

3.9 Study on the Effects of Uric Acid Concentration towards the Bacterial Growth 63

3.10 Determination of Uricase Activity 63

3.11 Localization of Possible Uricase Enzyme 64

3.11.1 Extraction of Possible Uricase from Extracellular Fraction 64

3.11.2 Extraction of Possible Uricase from Intracellular Fraction 64

3.11.3 Extraction of Possible Uricase from Periplasmic Fraction 65

3.12 Optimization of Medium Components for Uricase Production Efficiency by *Pseudomonas otitidis* strain SN1 65

3.12.1 Cell Harvesting 65

3.12.2 Effect of Carbon Sources and Its Concentration on Uricase Production 66

3.12.3 Effect of Varying Uric Acid Concentration on Uricase Production 67

3.12.4 Effect of Nitrogen Source and Its Concentration on Uricase Production 67

3.12.5 Influence of pH on Uricase Production 68
3.12.6 Effect of Temperature on Uricase Production 68
3.12.7 Statistical Analysis 69
3.12.8 Microbial Biomass Production and Growth Kinetics 69
3.13 General Protein Techniques 70
3.13.1 Mechanical Lysis 70
3.13.2 Determination of Protein Concentration (Lowry Protein Assay) 70
3.13.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) 71
3.14 Purification of Uricase Enzyme 72
3.14.1 Screening of Ammonium Sulfate Precipitation 72
3.14.2 Dialysis of Protein Sample 73
3.14.3 Anion Exchange Chromatography (Hi Trap Q High Performance) 73
3.14.4 Ultrafiltration for Pooled Fractions (Vivaspin® 20 Sartorius Stedim) 74
3.14.5 Gel Filtration Chromatography on Superdex 75 75
3.15 Characterization of Uricase 75
3.15.1 Uricase Kinetic Study 75
3.15.2 Effect of pH on Purified Uricase 76
3.15.3 Effect of pH Stability on Purified Uricase 76
3.15.4 Effect of Temperature on Purified Uricase 76
3.15.5 Effect of Temperature Stability on Purified Uricase 77
3.15.6 Effect of Metal Ions and Chemical Reagents on Purified Uricase 77
3.15.7 Statistical Analysis 77
3.15.8 Quantitative Screening on Purified Uricase 77
3.16 Mass Spectrometry Analysis and Instrumentation 78
3.16.1 Protein Sample Preparation 78
3.16.2 Data Analysis of Mass Spectrometry 79

CHAPTER 4 RESULTS AND DISCUSSION 81

4.1 Introduction 81

4.2 Results and Discussion 82

4.2.1 Isolation, Screening and Characterization of Uricase Producing Bacteria 82

4.2.1.1 Physical Parameters of the Hot Springs 82

4.2.1.2 Isolation of Uricase Producing Bacteria 86

4.2.1.3 Qualitative Screening of Uricase Producing Bacteria 90

4.2.1.4 Identification of Isolated Strain Based on 16S rRNA Gene Analysis 92

4.2.1.5 Growth Profile of Potential Uricase Producing Bacteria 99

4.2.1.6 Effects of Uric Acid Concentration 102

4.2.1.7 Localization of Uricase Enzyme 104

4.2.2 Optimization of Uricase Production by *Pseudomonas otitidis* strain SN1 107

4.2.2.1 Effect of Types of Carbon Sources on Bacterial Growth and Uricase Production 107

4.2.2.2 Effect of Concentration of Carbon Source on Bacterial Growth and Uricase Production 110

4.2.2.3 Effect of Uric Acid Concentration on Uricase Production 113

4.2.2.4 Effect of Types of Nitrogen Sources on Bacterial Growth and Uricase Production 116

4.2.2.5 Effect of Concentration of Nitrogen Source on Bacterial Growth and Uricase Production 119

4.2.2.6 Influence of Initial pH on Bacterial Growth and Uricase Production 121

4.2.2.7 Effect of Temperature on Uricase Production 125

4.2.3 Purification and Characterization of Uricase 130

4.2.3.1 Ammonium Sulfate Precipitation Screening 131
4.2.3.2 Anion Exchange Chromatography 134
4.2.3.3 Gel Filtration Chromatography 137
4.2.3.4 Purification of Uricase 139
4.2.3.5 Characterization of Uricase 141
4.2.3.6 Quantitative Screening on Purified Uricase 151
4.2.3.7 Mass Spectometry Analysis and Protein Identification 153
4.2.3.8 Prediction of Metabolic Pathway 155

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS 159
5.1 Conclusions 159
5.2 Future Works 161

REFERENCES 162

LIST OF PUBLICATIONS 215

LIST OF TABLES

TABLE NO.	TITLE	PAGE
Table 2.1	Uricase producing microorganisms isolated from various sources	22
Table 2.2	Applications of uricase	30
Table 2.3	Optimum condition of uricase producing microorganisms	34
Table 2.4	Purification scheme to purify uricase enzyme	41
Table 3.1	The universal primers used for the amplification of 16S rRNA gene	59
Table 3.2	Components of reaction mixtures in PCR tube	59
Table 3.3	PCR profile	59
Table 3.4	Parameters and conditions for sonication process	70
Table 4.1	Location and descriptions of the sampling sites (Hamzah *et al.*, 2013; Chan *et al.*, 2017)	83
Table 4.2	Summary of the physical parameters of the hot springs	84
Table 4.3	Chemical analysis of the hot springs (Hamzah *et al.*, 2013)	85
Table 4.4	The potential of uricase producing bacteria and their isolation source	87
Table 4.5	Morphological characterization of the potential uricase producing bacteria	88
Table 4.6	Clearing zones produced from potential uricase producing bacteria	91
Table 4.7	Species of bacteria determined by amplification of 16S rRNA	93
Table 4.8	Total protein concentration and uricase activity extracted from the isolates	105
Table 4.9	Effects of types of carbon source on cell growth and uricase production from *Pseudomonas otitidis* strain SN1	109
Table 4.10	Effects of different initial concentration of carbon source on cell growth and uricase production from *Pseudomonas otitidis* strain SN1	112
Table 4.11	Effects of different initial concentration of uric acid on cell growth and uricase production from *Pseudomonas otitidis* strain SN1	115
Table 4.12	Effects of types of nitrogen source on cell growth and uricase production from *Pseudomonas otitidis* strain SN1	118

Table 4.13 Effect of different initial concentration of nitrogen source on cell growth and uricase production from *Pseudomonas otitidis* strain SN1 120

Table 4.14 Effect of different initial pH on cell growth and uricase production from *Pseudomonas otitidis* strain SN1 123

Table 4.15 Effect of incubation temperature on uricase production and growth of *P. otitidis* strain SN1 126

Table 4.16 Production of biomass and kinetics growth of *P. otitidis* strain SN1 incubated at different temperature 129

Table 4.17 Screening ammonium sulfate saturation for precipitation of uricase enzyme from *P.otitidis* strain SN1. NS denotes as No Saturation. 132

Table 4.18 Summary of purification of uricase from wild type *Pseudomonas otitidis* strain SN1 141

Table 4.19 Comparison of kinetic parameter apparent K_m and apparent V_{max} from other microorganisms 143

Table 4.20 Source and optimum temperature of uricase 148

Table 4.21 Clearing zones of purified uricase 152

Table 4.22 Identified match proteins from MS analysis 154

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
Figure 2.1	Production of uric acid from purines (Hafez *et al.*, 2017)	11
Figure 2.2	Major causes of hyperuricemia. A) High consumption of high-fructose foods and drinks. B) High consumption of high purine food. C) Body metabolism. D) Medications. E) Increase blood lead level. F) Kidney disease. G) Genetic factors (Chen *et al.*, 2016)	12
Figure 2.3	Example of chronic gout. Numerous large tophi with joints deformity (Pascual *et al.*, 2017)	13
Figure 2.4	Pathogenesis of Tumor Lysis Syndrome (Pession *et al.*, 2008)	14
Figure 2.5	Purine catabolic pathway and site of action of allopurinol. Barred box (-----) indicate inhibition. Arrows indicates activation or consequences (Pession *et al.*, 2008).	16
Figure 2.6	Schematic diagram of uric acid conversion into allantoin and hydrogen peroxide (Zhou *et al.*, 2016)	19
Figure 2.7	Pathway for formation and degradation of uric acid. Degradation of purine nucleotide converges to the nitrogen base xanthine; 1) oxidation of xanthine occurred and yield uric acid. 2) oxidized to 5-hydroxyisourate (HIU)-very unstable product. 3) (a) spontaneous conversion of HIU to racemic allantoin through the 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU). 3) (b) Proposed pathway for the enzymatic conversion of HIU to S-(+)-allantoin.4) Allantoin formed (Ramazzina *et al.*, 2006).	21
Figure 2.8	Location of hot springs located in Peninsular Malaysia (Chow *et al.*, 2010)	27
Figure 2.9	General view of experimental steps of the data in mass spectrometry analysis (Resing and Ahn, 2005; Nesvizhskii, 2007)	49
Figure 2.10	Main components in mass spectrometer (Cottrell, 2011)	50
Figure 3.1	Overview of the research methodology in achieving three objectives	80
Figure 4.1	Electrophoresis of amplified genomic DNA. 1: DNA ladder; 2: Amplified product of SN1; 3: Amplified product of SN2; 4: Amplified product of SN3; 5: Amplified product of SN4	92
Figure 4.2	Phylogenetic tree based on 16S rRNA gene sequences showing evolutionary relationships of strain SN1, SN2, SN3 and SN4 among related taxa. *Cellvibrio japonicus* was used as an outgroup. Percentages at nodes are level of boostrap support based on 1000	

resamplings. Scale bar indicate 0.01 substitutions per nucleotide positions. Numbers in bracket represents the Genbank accession number. 95

Figure 4.3 Growth profile of SN1, SN2, SN3 and SN4 strain in LB medium incubated at 37°C, agitated at 150 rpm 100

Figure 4.4 Growth profile of SN1, SN2, SN3 and SN4 strain in uric acid minimal medium incubated at 37°C, agitated at 150 rpm 100

Figure 4.5 The growth of isolate SN1 (A), SN2 (B), SN3 (C) and SN4 (D) in medium broth containing different concentrations of uric acid. Data are represented as means ± standard deviation, n=3 103

Figure 4.6 Effect of carbon sources towards uricase production and growth of P.otitidis strain SN1 at 18 hours incubation. Data are represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 109

Figure 4.7 Effect of varying concentration of glucose towards uricase production and growth of *P.otitidis* strain SN1 at 18 hours incubation. Data are represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 111

Figure 4.8 Effect of varying uric acid concentration as sole nitrogen source towards uricase production and growth of *P.otitidis* strain SN1 at 18 hours incubation. Data are represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 114

Figure 4.9 Effect of additional nitrogen source towards uricase production and growth of *P.otitidis* strain SN1 at 18 hours incubation. Data are represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 118

Figure 4.10 Effect of varying concentration of nitrogen source (yeast extract) source towards uricase production and growth of *P. otitidis* strain SN1 at 18 hours incubation. Data are represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 120

Figure 4.11 Effect on initial pH of the medium on uricase production and growth of *P.otitidis* strain SN1 at 18 hours of incubation. Data are represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 123

Figure 4.12 Effect on incubation temperature on uricase production and growth of *P.otitidis* strain SN1 at 18 hours of incubation. Data are

represented as means ± standard deviation, n=3. The values with different letters over bars denotes significantly different by Duncan's Multiple range tests ($p < 0.05$) 126

Figure 4.13 The growth curve of *P. otitidis* strain SN1 at different temperatures. The strain SN1 was grown in optimized uric acid minimal medium. 128

Figure 4.14 SDS PAGE analysis of crude ricase enzyme after 70% (w/v) ammonium sulfate precipitation. Lane M: Prestained protein marker; Lane 1: Crude enzyme extract from *P.otitidis* strain SN1; Lane 2: Crude enzyme extract at 70% (w/v) 132

Figure 4.15 Chromatogram of the purified uricse applied on Hi Trap Q Hi Performance (1mL) column as plotted by Primeview™ software. The purification was operated with 1 mL/min flow rate and operated at room temperature. The chromatogram represents the UV absorbance (blue) and NaCl gradient elution (green). Fraction numbers are labeled in red. 136

Figure 4.16 Coomassie stained 12% SDS PAGE of dialysate after ammonium sulfate precipitation (AS), unbound sample (FT), wash (W) and fractions from elutions (P1-P4), Lane M: protein marker. 136

Figure 4.17 SDS PAGE analysis of eluates from anion exchange purification by optimizing flow rate to 0.5 mL/min and binding and elution buffer pH 8.5. 137

Figure 4.18 Chromatogram of gel filtration of uricase. Eluate from Hi Trap Q High Performance (1 mL) was concentrated and loaded onto HiLoad 16/60 Superdex column. The chromatogram represents the UV absorbance (blue) and fraction numbers are labelled in red. 138

Figure 4.19 SDS PAGE analysis of eluates from gel filtration 139

Figure 4.20 Kinetic plot against uric acid concentration. Data are expressed as mean value of triplicates with their corresponding standard deviation 143

Figure 4.21 Effect of pH on uricase activity produced *Pseudomonas otitidis* strain SN1. The highest uricase was taken as 100%. Each point indicate the mean, $p<0.05$. Error bars represent mean standard deviation of triplicate in each individual experiment. 145

Figure 4.22 The pH stability on the activity of purified uricase produced by *Pseudomonas otitidis* strain SN1. Each point indicate the mean, $p <0.05$. Error bars represent mean standard deviation of triplicate in each individual experiment. 146

Figure 4.23 Optimum temperature of uricase activity produced by *Pseudomonas otitidis* strain SN1. The highest uricase was taken as 100%. Each point indicate the mean, $p<0.05$. Error bars represent mean standard deviation of triplicate in each individual experiment. 147

Figure 4.24 Temperature stability of the purified uricase. Each point indicate the mean, $p<0.05$. Error bars represent mean standard deviation of triplicate in each individual experiment. 149

Figure 4.25 Effect of different metal ions and chemical reagents on purified uricase. Error bars represent mean standard deviation of triplicate in each individual experiment. 150

Figure 4.26 Lane 1: Protein marker; Lane 2: Purified uricase. Protein bands divided into 3 sections according to the range of protein marker 153

Figure 4.27 The alternative metabolic pathways producing urea. (1) The purine degradation pathway is composed of xanthine oxidase, urate oxidase, allantoinase and allantoicase, generating glyoxylate and urea. (2) The ornithine-urea cycle uses either glutamine or ammonia as substrates to first synthesize arginine via carbamoyl phosphate synthase, ornithine carbamoyltransferase, argininosuccinate synthase and argininosuccinate lyase (Lemoine and Walsh, 2015). 156

Figure 4.28 The multiple alternative metabolic pathways generated by BioCyc. The database proposed an integration of uricolytic pathway and ornithine-urea cycle 158

LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
BSA	-	Bovine Serum Albumin
CV	-	Column volume
DCW	-	Dry Cell Weight
DMSO	-	Dimethyl sulfoxide
E.coli	-	*Escherichia coli*
LB	-	Luria Bertani
PCR	-	Polymerase Chain Reaction
DNA	-	Deoxyribonucleic acid
DEAE	-	Diethylaminoethyl
IKBN	-	Institut Kemahiran Belia Negara
MEGA	-	Molecular Evolutionary Genetics Analysis
mw	-	Molecular weight
MWCO	-	Molecular Weight Cut Off
NaCl	-	Sodium chloride
nm	-	Nanometer
OD	-	Optical density
OFAT	-	One-Factor-at-a-Time
OHCU	-	2-oxo-4-hydroxy-5-ureidaimidazoline decarboxylase
rpm	-	Rotation per minute
SDS PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TAE buffer	-	Tris-Acetate-EDTA buffer
Tris-HCl	-	Tris-(hydroxyl)-aminomethane hydrochloride
UV-Vis	-	Ultraviolet visible
V	-	Volt
16s rRNA	-	16 small subunit of ribosomal Ribonucleic Acid

LIST OF SYMBOLS

%	-	Percentage
μ_{max}	-	Maximum specific growth rate
μ	-	Specific growth rate
μg	-	Microgram
μL	-	Microlitre
μm	-	Micron/micrometer
μM	-	Micromolar
μmol	-	Micromole
°C	-	Degree Celcius
g	-	Gram
g	-	Gravitational force
g/L	-	Gram per litre
M	-	Molar
mM	-	miliMolar
mg	-	Milligram
mm	-	Milimeter
ng	-	Nanogram
L	-	Litre
V	-	Volt
v/v	-	Volume per volume
w/v	-	Weight per volume

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
Appendix A	Colony Morphology	183
Appendix B	The 16S nucleotide sequence of SN1, SN2, SN3 and SN4	184
Appendix C	TAE DNA electrophoresis buffer	188
Appendix D	Determination of protein content by Lowry assay	189
Appendix E	Assay and calculation of uricase assay	190
Appendix F	Chemical solution for SDS PAGE	193
Appendix G	Statistical Analysis	195
Appendix H	Calculation of carbon and nitrogen mass	204
Appendix I	Microbial growth kinetics calculation	207
Appendix J	Ammonium sulphate nomogram	208
Appendix K	Calculation in purification table	210
Appendix L	Statistical analysis	211

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Uric acid ($C_5H_4N_4O_3$) is an organic heterocyclic compound which commonly present in all organisms. In the human body, uric acid is produced when the body breaks purine or naturally obtained from certain foods contain uric acid such as meat and seafood. A proper balance of diet is needed to maintain uric acid level in the body. Excessive intake of food containing high level of uric acid may cause precipitation of uric acid in blood, joints and formation of kidney stones. Overproduction of uric acid in the body may lead to few illnesses such as gout, hyperuricemia and renal disease. Patients that suffer with these illnesses should avoid food with high purine contents.

The normal level of uric acid in blood is between 3-7mg/100 mL (Yeap *et al.*, 2009; Hafez *et al.*, 2017). However, an average meal intake for adult in United States contains 600-100 mg of purines daily (Yeap *et al.*, 2009). The prevalence of gout has increased worldwide approximately two fold increase for over 30 years. It is estimated about 8 million people in United States suffer with gout and other uric acid related diseases (Mikuls *et al.*, 2005). In New Zealand, gout problems have increased about 5.5% per year while in England recorded 7.2% yearly increased starting from 1999-2009. Unfortunately, published reports regarding gout patients in Malaysia are yet available (Chow *et al.*, 2008; Teh *et al.*, 2014). Therefore, practicing healthy diet and monitoring uric acid level is very important. When levels of uric acid exceed its solubility limit, uric acid starts to accumulate and crystals of uric acid may be formed over time. The uric acid may deposits in joints and cause painful inflammation and swelling joints. This condition is known as gout attack. The gout patients will feel intense pain, functional impairment and thus hampered quality of life. This sickness can only be reduced with aid from drug treatment. Allopurinol and colchicine are the

examples of drugs used in the treatment of gout attack. However, these medicines are expensive and patients will have undesirable side effects such as allergic reactions and internal hypersensitivity (Teh *et al*., 2014). Due to that, health practitioners are progressively searching for another type of treatment that is less risk and able to relief pain efficiently.

Uricase (urate oxidase) is an enzyme involved in purine metabolism. This enzyme is responsible to start a chain of reactions that transforms uric acid to more soluble compound known as allantoin. Initially, uricase catalyzes the reaction of uric acid and converting it to unstable product, 5-hydroxy-isourate (HIU). This unstable compound then goes through non-enzymatic hydrolysis to 2-oxo-4-hydroxy-4-carboxy-5-uredoi-midazoline (OHCU) and spontaneously decarboxylates to form allantoin (Kahn and Tipton, 1998; Ramazzina *et al.*, 2006). Uricase is mostly present in most vertebrates but absent in humans (Anderson and Vijayakumar, 2012; Garay *et al.*, 2012). The absence of uricase in humans was due to the disruption of uricase gene occurred during molecular evolution and thus, caused inactivation of the uricase gene in human (Oda *et al.*, 2002). As a result, humans lost functional uricase and excrete uric acid as the end product of purine degradation.

The microbial production of uricase has been extensively studied. Several studies reported uricase produced from bacteria such as *Microbacterium sp*. ZZJ4-1 (Zhou *et al.*, 2005), *Bacillus thermocatenulatus* (Lotfy, 2008), *Pseudomonas aeruginosa* (Anderson and Vijayakumar, 2012) and *Streptomyces exfoliates* UR10 (Magda *et al.*, 2013). Several fungi also demonstrated to produce uricase such as *Aspergillus carbonarius*, *Aspergillus sydowii* and *Botrytis fabae* (Mahmoud and El-Fallal, 1996). Tanaka *et al.*, (1977) also suggested that uric acid was effective for inducing uricase in *Candida tropicalis*. The uricase from these microorganisms can be isolated from environment such as soil, poultry waste and marine sediment. Besides that, uricase is also purified from leaves of chickpea (*Cicer arietimum L.*), broad beans (*Vicia faba major L.*) and wheat (*Triticum aestivum L.*) (Pineda *et al.*, 1997). To date, there are two clinically approved uricases, Elitek® (Rasburicase) (Coiffier *et al.*, 2003) and Krystexxa® (Pegloticase) (Sundy *et al.*, 2011). Rasburicase is a recombinant uricase enzyme produced by a genetically modified

Saccharomyces cerevisiae strain. The cDNA coding for rasburicase was cloned from a strain of *Aspergillus flavus*. On the other hand, pegloticase is a fusion protein constituted uricase from porcine and baboon. This pegloticase is also hyper-PEGylated to minimize immunogenicity and extend shelf life. Both of these uricase are used in the treatment of severe and chronic gout by metabolizes the conversion of uric acid to allantoin. The advantage of uricase is they allow more rapid resorption of urate kidney stone compared to allopurinol treatment (Nyborg *et al.*, 2016).

The biology of uricase producing bacteria and characterization of purified uricase was investigated as it can build knowledge on novel strain on producing uricase. Hence, it is very important to screen the diverse microbial populations in the environment for more uricase producing microorganisms. Isolation and characterization of uricase producing bacteria from the environment would provide the fundamental studies for improving therapeutic treatment of gout and hyperuricemia. At present, there are limited studies that describe uricase producing microorganisms.

Hot springs are water that brings the heat from the Earth's mantle to the surface through a permeable path of the aquifer (Belkova *et al.*, 2007). Chemical content of the water in hot springs changes according to the chemical composition of rocks situated on the path of the hot water flow. When the water temperature increases, it causes the dissolving rate of minerals to increase. Therefore, it has been proposed that this increases the treatment value of water in hot springs (Rajapaksha *et al.*, 2014). Thus, the water of the hot springs is well known for relief of gout, rheumatism, paralysis and skin diseases. Hot spring water is rich in magnesium, calcium, sodium and all sorts of naturally occurring trace minerals (Etani *et al.*, 2016). However, bacterial enumerations from the hot springs water are scarce and restricted. Microorganisms that exist in the hot springs were left abundance without further studied of their potential (Mohammad *et al.*, 2017). Interestingly, to the best of our knowledge, uricase producing microorganisms have to be isolated from the hot springs. Therefore, it is essential to investigate the physiology and the new properties of uricase from *Pseudomonas otitidis* strain SN1 for providing insights into the potential of therapeutic enzyme. In addition, comparison of different uricase

from various organisms can be a great importance for comparison of protein structure and its properties.

1.2 Problem Statement

Uricase is an endogenous enzyme found in most mammals but absent in human. This occurs because of the nonsense mutation occurred during molecular evolution. Thus, due to high level of uric acid produced in human body, it contributes to many negative effects. This is caused from deposition of urate in the joints and tissues. The prevalence of diseases related to uric acid are rising yearly and become as major health issue in developed countries (Winnard *et al.*, 2012). Varieties of medicines are available in the market. For example, allopurinol is the most commonly used as urate lowering medication. Allopurinol works by inhibit xanthine oxidase enzyme during synthesis of uric acid and thus reducing production of uric acid in the body (Dubreuil *et al.*, 2015). However, most of the drugs are prone to show adverse effects such as allergic reaction, severe abdominal pain, nausea or painful during urination. Without effective urate lowering drugs, many patients developed to chronic gout by persistent pain, enlarging tophi and functional disabilities (Sundy *et al.*, 2011).

Currently, health practitioners become interested with the use of uricase enzyme in the treatment of diseases related to uric acid. Uricase is used as biodrug for therapeutic treatment associated with gout and hyperuricemia (Crittenden and Pillinger, 2013; Xiaolan *et al.*, 2013) as well as important tools for uric acid assay in clinical laboratories (Zhao *et al.*, 2009; Huang *et al.*, 2015). In addition, uricase is able to convert uric acid into allantoin with higher solubility and no obvious adverse actions were reported. Thus, uricase becomes a promising therapeutic enzyme for handling uric acid related diseases (Sundy *et al.*, 2011). However, the introduction of uricase enzyme for treating this problem, the limited capacity concurrent with the low uricase efficacy in commercially available uricase remains a problem (Gurung *et al.*, 2013). Researchers undertook to manage these problems by genetically

engineering those available uricase as it could enhance the rate of its activity. However, the high cost of engineered enzyme production, specificity and reliability problem circumscribe their widespread use (Liu *et al.*, 1994). In light of this, it is important to identify and investigate new uricase enzyme that are more specific and safe for therapeutic treatment of gout and hyperuricemia. Furthermore, the uses of microbial enzymes acquiring many attentions because of the rapid development of enzyme technologies. The use of microbial enzymes are preferred because of their economic feasibility, rapid growth of microbes using inexpensive media, ease of product modification and optimization and greater catalytic activity (Gurung *et al.*, 2013).

1.3 Objectives of Study

This study was carried out to investigate the ability of new uricase producing bacteria from local hot spring that is responsible for uricase production. The specific objectives of this research were as follows:

i) To study the biology of the organisms with respect to uric acid

ii) To optimize the uricase production by selected bacteria in batch culture

iii) To purify and characterize the uricase produced by selected bacteria

1.4 Scope of Study

In this study, a few bacteria that can produce uricase were successfully isolated and identified from local hot springs. The isolates were investigated for their ability to produce uricase. As a result, a novel bacterium, *Pseudomonas otitidis* strain SN1 was selected for study of effects of different nutritional components on maximizing uricase production. This research was then proceeds with purification

works and enzymatic studies where the isolated microbial uricase was purified by using ammonium sulfate precipitation, anion exchange and gel filtration chromatography. Purity of isolated uricase was verified using SDS PAGE. Characterization of uricase was carried out in terms of biochemical studies. At the end of the study, highly possible uricase was identified using tandem mass spectrometry.

1.5 Significance of Study

Microbial uricase enzymes isolated from environment has significant potential and hopes for patients that suffer with uric acid related diseases. The uricase enzyme was known to provide better effect compared to commercial medicine available in markets. This is because injection of uricase permits rapid dissolution of urate kidney stone and thus relief intense pain promptly (Nyborg *et al.*, 2016). In addition, uricase from environments are considered low-risk compared to the use of medicine drug (Garay *et al.*, 2012).

Investigation to find new isolates from new sources (hot springs) with the capability to produce uricase enzyme are relatively good strategies to obtain uricase producing microorganisms. This is because the hot springs were known as places that left unexplored. Due to their increased importance, potential applications and roles in different fields, scientists have focused their studies to discover new genus and species from hot springs (Mohammad *et al.*, 2017). To date, no study has reported on the isolation of uricase producing bacteria from hot springs. The study of uricase enzyme from other bacterial strains and their properties are important, because the diversity may produce beneficial knowledge towards expanding uricase enzymes with high value potential. To the best of our knowledge, the biology of novel *Pseudomonas otitidis* in producing uricase isolated from hot springs has yet to be discovered and reported.

Besides that, this study provides important environment parameters in increasing the production of uricase isolated from hot springs. The uricase

production was affected by physicochemical parameters such as carbon and nitrogen source, pH and temperatures. Thus, by studying the parameters, this will provide information of the growth rate and kinetics in enhancing the production of uricase of the isolated bacteria. This study also presents the purification work and characterization of uricase from *Pseudomonas otitidis* strain SN1. This anticipates additional information for uricase purified from wild type strain. This is because only limited studies on purification of uricase and its properties from wild type were reported.

At the end of the study, the mass spectrometry analysis revealed the potential uricase also shared the similarity function with ornithine carbamoyltransferase in producing urea via alternative metabolic pathway. The analysis also shows that working with protein originated from wild type is more diverse and challenging because wild type bacteria exhibited different physiologies and unpredicted metabolic pathway. The new findings about relationships of uricase and ornithine carbamoyltransferase enzyme promote additional knowledge and value to the novel strain of *Pseudomonas otitidis*.

REFERENCES

Abdel-Fattah, Y.R., Saeed, H.M., Gohar, Y.M. and El-Baz, M.A. (2005). Improved Production of *Pseudomonas aeruginosa* Uricase by Optimization of Process Parameters Through Statistical Experimental Designs. *Process Biochemistry*. 40(5), 1707–1714.

Abdullah, S.K. and Flayyih, M.T. (2015). Production , Purification and Characterization of Uricase Produced by *Pseudomonas aeruginosa*. *Iraqi Journal of Science*. 56(3), 2253–2263.

Abeles, A.M. (2012). Febuxostat Hypersensitivity. *Journal of Rheumatology*. 39(3), 659.

Aguilar, M., Montalbini, P. and Pineda, M. (2002). Urate Oxidase from The Rust *Puccinia recondita* Is A Heterotetramer with Two Different-Sized Monomers. *Current Microbiology*. 44, 257–261.

Alfred, A. and John, N. (1974). Factors Affecting Urate Solubility *In vitro*. *Annual Rheumatology Disease*. 33, 721–735.

Allen, M.B. and Grove, P. (1952). A Note on *Pseudomonas stutzeri*. 64, 413–422.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST : A New Generation of Protein Database Search Programs. 25(17), 3389–3402.

Ames, G., Prody, C. and Kustu, S. (1984). Simple, Rapid, and Quantitative Release of Periplasmic Proteins by Chloroform. *Journal of Bacteriology*. 160(3), 1181–1183.

Amirthanathan, A. and Subramaniyan, V. (2012). Studies on Uricase Production by Marine *Bacillus cereus* and its Optimum Conditions. *International Journal of Medicine and Biosciences*. 1(3), 5–12.

Amirthanathan, A. and Vijayakumar, S. (2011). Purification and Optimization of Uricase Enzyme Produced by *Pseudomonas aeruginosa*. *Journal of Experimental Sciences*. 2(11), 5–8.

Anastasia, S., Binita, S., Michael, H.P. and Svetlana, K. (2015). Colchicine: Old and New. *The American Journal of Medicine*. 128(5), 461–470.

Anderson, A. and Vijayakumar, S. (2012). Isolation and Optimization of

Pseudomonas aeruginosa for Uricase Production. *International Journal of Pharma and Bio Sciences*. 3(1), B143–B150.

Ang, S.K., Yahya, A., Abd Aziz, S. and Md Salleh, M. (2015). Isolation, Screening and Identification of Potential Cellulolytic and Xylanolytic Producers for Biodegradation of Untreated Oil Palm Trunk and Its Application in Saccharification of Lemongrass Leaves. *Preparative Biochemistry and Biotechnology*. 45(3), 279–305.

Aoki, M., Tsujino, Y., Kano, K. and Ikeda, T. (1996). Significance of Uricase in Oxidase-Induced Oxidative Coloring Reaction of p-Phenylenediamine. *Journal of Organic Chemistry*. 61(16), 5610–5616.

Arima, K and Nose, K. (1968). Studies on Bacterial Urate: Oxygen Oxidoreductase I. Purification and Properties of The Enzyme. *Biochimica et Biophysica Acta*. 151(1968), 649–653.

Atalla, M. and Farag, M. (2009). Optimum Conditions for Uricase Enzyme Production by *Gliomastix gueg*. *Malaysian Journal of Mocrobiology*. 5(1), 45–50.

Atiqah, A., Syafawanie, A., Syafiqah, A., Izhar, I., Zarif, M., Abdelazim, A., Syafiq, A. and Wei, O.Q. (2017). Hydrogeological and Environmental Study of Sungai Serai, Hulu Langat. *Pakistan Journal of Geology*. 1(1), 8–11.

Bachrach, B.Y.U. (1957). The Aerobic Breakdown of Uric Acid by Certain *Pseudomonads*. *Journal of General Microbiology*. 16(3), 1–11.

Bahar, T. and Celebi, S.S. (1998). Characterization of Glucoamylase Immobilized on Magnetic Poly(styrene) Particles. *Enzyme and Microbial Technology*. 64(12), 1003–1008.

Baioumy, H., Nawawi, M., Wagner, K. and Arifin, M.H. (2015). Geochemistry and Geothermometry of Non-volcanic Hot springs in West Malaysia. *Journal of Volcanology and Geothermal Research*. 290(December), 12–22.

Balasubramani, G., Induja, K., Aiswarya, D., Deepak, P., Arul, D., Kavitha, M. and Perumal, P. (2018). Isolation and Characterization of Human Foot Crack – Associated Bacterium , *Pseudomonas Otitidis* , and Its Biological Propensity. *Smart Science*. 00(00), 1–12.

Baldwin, M.A. (2004). Protein Identification by Mass Spectrometry. *Molecular & Cellular Proteomics*. 3(1), 1–9.

Ballesta-Claver, J., Díaz Ortega, I.F., Valencia-Mirón, M.C. and Capitán-Vallvey,

L.F. (2011). Disposable Luminol Copolymer-based Biosensor for Uric Acid in Urine. *Analytica Chimica Acta*. 702(2), 254–261.

Bardin, T., Chalès, G., Pascart, T., Flipo, R.M., Korng Ea, H., Roujeau, J.C., Delayen, A. and Clerson, P. (2016). Risk of Cutaneous Adverse Events with Febuxostat Treatment in Patients with Skin Reaction to Allopurinol. A Retrospective, Hospital-based Study of 101 Patients with Consecutive Allopurinol and Febuxostat Treatment. *Joint Bone Spine*. 83(3), 314–317.

Bayol, A., Capdevielle, J., Malazzi, P., Buzy, A., Bonnet, M.C., Colloc'h, N., Mornon, J.P., Loyaux, D. and Ferrara, P. (2002). Modification of A Reactive Cysteine Explains Differences between Rasburicase and Uricozyme (R), A Natural *Aspergillus flavus* Uricase. *Biotechnology and Applied Biochemistry*. 36, 21–31.

Belkova, N.L., Tazaki, K., Zakharova, J.R. and Parfenova, V. V. (2007). Activity of Bacteria in Water of Hot Springs from Southern and Central Kamchatskaya Geothermal provinces, Kamchatka Peninsula, Russia. *Microbiological Research*. 162(2), 99–107.

Berg J.M., Tymoczko, J.L. and Stryer, L. (2002). The Michaelis-Menten Model Accounts for the Kinetic Properties of Many Enzymes. In *Biochemistry*. New York: W H Freeman, p.22430.

Bhargava, A.K., Lal, H. and Pundir, C.S. (1999). Discrete Analysis of Serum Uric Acid with Immobilized Uricase and Peroxidase. *Journal of Biochemical and Biophysical Methods*. 39(3), 125–136.

Binz, P.A., Barkovich, R., Beavis, R.C., Creasy, D., Horn, D.M., Julian, R.K., Seymour, S.L., Taylor, C.F. and Vandenbrouck, Y. (2008). Guidelines for Reporting the Use of Mass Spectrometry Informatics in Proteomics. *Nature Biotechnology*. 26(8), 862.

Bollag, D.M., Rozycki, M.D. and Edelstein, S.J. (1997). *Protein Methods* 2nd ed., New York: WIley-Liss, Inc.

Bongaerts, G.P.A. and Vogels, G.D. (1979). Mechanism of Uricase Action. *Biochimica et Biophysica Acta*. 567(2), 295–308.

Bringans, S., Eriksen, S., Kendrick, T., Gopalakrishnakone, P., Livk, A., Lock, R. and Lipscombe, R. (2008). Proteomic Analysis of the Venom of *Heterometrus longimanus* (Asian black scorpion). *Proteomics*. 8(5), 1081–1096.

Bru, R. and Titgemeyer, F. (2002). Carbon Catabolite Repression in Bacteria :

Choice of The Carbon Source and Autoregulatory Limitation of Sugar Utilization. *Federation of European Microbiological Societies (FEMS) Microbiology Letters*. 209.

Burgess, R.R. (2009). Protein Precipitation Techniques. In *Methods in Enzymology*. USA: Elsevier Inc., pp.331–342.

Burns, C.M. and Wortmann, R.L. (2011). Gout therapeutics : New Drugs for an Old Disease. *The Lancet*. 377(9760), 165–177.

Chan, C.S., Chan, K., Ee, R., Hong, K., Urbieta, M.S., Donati, E.R., Shamsir, M.S. and Goh, K.M. (2017). Effects of Physiochemical Factors on Prokaryotic Biodiversity in Malaysian Circumneutral Hot Springs. *Frontiers in Microbiology*. 8(July).

Chan, C.S., Chan, K.G., Tay, Y.L., Chua, Y.H. and Goh, K.M. (2015). Diversity of Thermophiles in A Malaysian Hot Spring Determined Using 16S rRNA and Shotgun Metagenome Sequencing. *Frontiers in Microbiology*. 6(MAR), 1–15.

Chen, C., Lü, J.-M. and Yao, Q. (2016). Hyperuricemia-Related Diseases and Xanthine Oxidoreductase (XOR) Inhibitors: An Overview. *Medical Science Monitor*. 22, 2501–2512.

Chen, J.-C., Chung, H.-H., Hsu, C.-T., Tsai, D.-M., Kumar, A.S. and Zen, J.-M. (2005). A Disposable Single-use Electrochemical Sensor for the Detection of Uric Acid in Human Whole Blood. *Sensors and Actuators B: Chemical*. 110(2), 364–369.

Chen, Z., Wang, Z., He, X., Guo, X., Li, W. and Zhang, B. (2008). Uricase Production by A Recombinant *Hansenula polymorpha* strain Harboring *Candida utilis* Uricase Gene. *Applied Microbiology and Biotechnology*. 79(4), 545–554.

Chohan, S. (2011). Safety and Efficacy of Febuxostat Treatment in Subjects with Gout and Severe Allopurinol Adverse Reactions Safety and Efficacy of Febuxostat Treatment in Subjects with Gout and Severe Allopurinol Adverse Reactions. *The Journal of Rheumatology*. 38(9).

Chow, S.K., Mohd Shahdan, M.S., Yeap, S.S., Heselynn, H. (2008). The Clinical Practice Guidelines on the Management of Gout. , 1–36.

Chow, W.S., Irawan, S. and Fathaddin, M.T. (2010). Hot Springs in the Malay Peninsula. *Proceedings World Geothermal Congress 2010*. (April), 25–29.

Clark, L.L., Dajcs, J.J., McLean, C.H., Bartell, J.G. and Stroman, D.W. (2006).

Pseudomonas otitidis sp. nov., Isolated from Patients with Otic Infections. *International Journal of Systematic and Evolutionary Microbiology*. 56(4), 709–714.

Coiffier, B., Mounier, N., Bologna, S., Fermé, C., Tilly, H., Sonet, A., Christian, B., Casasnovas, O., Jourdan, E., Belhadj, K. and Herbrecht, R. (2003). Efficacy and Safety of Rasburicase (Recombinant Urate Oxidase) for the Prevention and Treatment of Hyperuricemia during Induction Chemotherapy of Aggressive Non-Hodgkin's Lymphoma: Results of the GRAAL1. *Journal of Clinical Oncology*. 21(23), 4402–4406.

Cottrell, J.S. (2011). Protein Identification Using MS/MS Data. *Journal of Proteomics*. 74(10), 1842–1851.

Crittenden, D.B. and Pillinger, M.H. (2013). New Therapies for Gout. *Annual Review of Medicine*. 64(1), 325–337.

Dako, E., Bernier, A.-M., Jankowski and K., A.T.D.C. (2012). The Problems Associated with Enzyme Purification. In P. D. Ekinci, ed. *Chemical Biology*. Croatia: In Tech, pp.19–37.

Deriase, S.F. and El-Gendy, N.S. (2014). Mathematical Correlation between Microbial Biomass and Total Viable Count for Different Bacterial Strains Used in Biotreatment of Oil Pollution. *Biosciences Biotechnology Research Asia*. 11(1), 61–65.

Dong, C.-H. and Yao, Y.-J. (2005). Nutritional requirements of mycelial growth of Cordyceps sinensis in Submerged Culture. *Journal of Applied Microbiology*. 99(3), 483–492.

Dubreuil, M., Zhu, Y., Zhang, Y., Seeger, J.D., Lu, N., Hee, Y., Choi, H.K. and Unit, E. (2015). Allopurinol Initiation and All-Cause Mortality in the General Population. *Annual Rheumatology Disease*. 74(7), 1368–1372.

Dwivedi, H., Agrawal, K. and Saraf, S.A. (2012). Screening of Uricase Producing Microorganisms and Uricase Estimation: A Simple and Novel Approach. *International Journal of Pharmacy and Pharmaceutical Sciences*. 4, 7–9.

El-Naggar, N.E.A. (2015). Isolation, Screening and Identification of Actinobacteria with Uricase Activity: Statistical Optimization of Fermentaion Conditions for Improved Production of Uricase by *Streptomyces rochei* NEAE-25. *International Journal of Pharmacology*. 11(7), 644–658.

Essam, A.A., Magda, M.A. and Mervat, F.F. (2005). Studies on Uricase Induction in

Certain Bacteria. *Egyptian British Biological Society*. 226(204), 219–226.

Etani, R., Kataoka, T., Kanzaki, N., Sakoda, A., Tanaka, H., Ishimori, Y., Mitsunobu, F. and Yamaoka, K. (2016). Difference in The Action Mechanism of Radon Inhalation and Radon Hot Spring Water Drinking in Suppression of Hyperuricemia in Mice. *Journal of Radiation Research*. 57(3), 250–257.

Fazel, R., Zarei, N., Ghaemi, N., Namvaran, M.M., Enayati, S., Mirabzadeh Ardakani, E., Azizi, M. and Khalaj, V. (2014). Cloning and Expression of *Aspergillus flavus* Urate Oxidase in *Pichia pastoris*. *SpringerPlus*. 3(1), 1–7.

French, J.B. and Steven, E. (2011). Structural and Kinetic Insights into The Mechanism of 5-Hydroxyisourate Hydrolase from *Klebsiella pneumoniae*. *Acta Crystallographica*. 67, 671–677.

Garay, R.P., El-Gewely, M.R., Labaune, J.P. and Richette, P. (2012). Therapeutic Perspectives on Uricases for Gout. *Joint Bone Spine*. 79(3), 237–242.

GE Healthcare (2011). *GE Healthcare Lıfe Sciences Instruction 28-9920-17 AB HiLoad ™ 16 / 600 and 26 / 600 Superdex ™ 30 prep grade HiLoad 16 / 600 and 26 / 600 Superdex 75 prep grade HiLoad 16 / 600 and 26 / 600 Superdex 200 prep grade Introduction Connecting the column*, Uppsala, Sweden.

GE Healthcare (2010). *Strategies for Protein Purification Handbook*, Uppsala, Sweden.

Geweely, N.S. and Nawar, L.S. (2011). Production, Optimization, Purification and Properties of Uricase Isolated from Some Fungal Flora in Saudi Arabian Soil. *Australian Journal of Basic and Applied Sciences*. 5(10), 220–230.

Ghosh, T. and Sarkar, P. (2014). Isolation of A Novel Uric-Acid-Degrading microbe *Comamonas* sp. BT UA and Rapid Biosensing of Uric Acid from Extracted Uricase Enzyme. *Journal of Biosciences*. 39(5), 805–819.

Ghosh, T., Sarkar, P. and Turner, A.P.F. (2015). A Novel Third Generation Uric Acid Biosensor Using Uricase Electro-Activated with Ferrocene on A Nafion Coated Glassy Carbon Electrode. *Bioelectrochemistry*. 102(October 2015), 1–9.

Giffard, M., Ferté, N., Ragot, F., El Hajji, M., Castro, B. and Bonneté, F. (2011). Urate Oxidase Purification by Salting-in Crystallization: Towards an Alternative to Chromatography. *PLoS ONE*. 6(5), e19013.

Gomila, M., Peña, A., Mulet, M., Lalucat, J. and García-Valdés, E. (2015). Phylogenomics and Systematics in *Pseudomonas*. *Frontiers in Microbiology*.

6(MAR), 1–13.

Green, A. A. and Hughes, W.L. (1955). Protein Fractionation on the Basis of Solubility in Aqueous Solutions of Salts and Organic Solvents. *Methods in Enzymology*. 1, 67–90.

Gurung, N., Ray, S., Bose, S., Rai, V. and K, W.F. (2013). A Broader View : Microbial Enzymes and Their Relevance in Industries , Medicine and Beyond. *Biomedical Research International*. 2013(2), 1–14.

Gustafsson, D. and Unwin, R. (2013). The Pathophysiology of Hyperuricaemia and Its Possible Relationship to Cardiovascular Disease, Morbidity and Mortality. *BMC Nephrology*. 14(1), 1–9.

Guttmann, A., Krasnokutsky, S., Pillinger, M.H. and Berhanu, A. (2017). Pegloticase in Gout Treatment - Safety Issues , Latest Evidence and Clinical Considerations. *Therapeutics Advances in Drug Safety*. 8(12), 379–388.

Hafez, R.M., Abdel-Rahman, T.M. and Naguib, R.M. (2017). Uric Acid in Plants and Microorganisms: Biological Applications and Genetics - A Review. *Journal of Advanced Research*. 8(5), 475–486.

Hamzah, Z., Abd Rani, H. L., Saat, A. and Wood, K. (2013). Determination of Hot Springs Physico-Chemical Water Quality Potentially Use for Balneotherapy. *Malaysian Journal of Analytical Sciences*. 17(January 2018).

Hamzah, H.H., Zain, Z.M., Musa, N.L.W., Lin, Y.-C. and Trimbee, E. (2013). Spectrophotometric Determination of Uric Acid in Urine Based-Enzymatic Method Uricase with 4-Aminodiphenylamine Diazonium Sulfate (Variamine Blue RT Salt). *Analytical and Bioanalytical Techniques*. 11, 1–6.

Handayani, I., Utami, T., Hidayat, C. and Rahayu, E.S. (2017). Enhancement of An Intracellular Uricase Produce by *Lactobacillus plantarum* Dad-13 Which has Stability in Gastrointestinal System. *Research Journal of Microbiology*. 12(3), 202–209.

Howard, S., Jones, D. and Pui, C. (2011). The Tumor Lysis Syndrome. *The New England Journal of Medicine*. 364(19), 1844–1854.

Huang, Q., Jiang, H., Briggs, B.R., Wang, S., Hou, W., Li, G., Wu, G., Solis, R., Arcilla, C.A., Abrajano, T. and Dong, H. (2013). Archaeal and Bacterial Diversity in Acidic to Circumneutral Hot Springs in the Philippines. *Federation of European Biochemical Societies (FEBS) Letters*. 85, 452–464.

Huang, Y., Chen, Y., Yang, X., Zhao, H., Hu, X., Pu, J., Liao, J., Long, G. and Liao,

F. (2015). Optimization of pH Values to Formulate The Bireagent Kit for Serum Uric Acid Assay. *Biotechnology and Applied Biochemistry*. 62(1), 137–144.

Inouye, E., Park, K.S. and Asaka, A. (1984). Blood Uric Acid Level and IQ: A Study in Twin Families. *Acta Geneticae Medicae et Gemellologiae*. 33(2), 237–242.

Inskeep, W.P., Jay, Z.J., Tringe, S.G., Herrgard, M.J. and Rusch, D.B. (2013). The YNP Metagenome Project : Environmental Parameters Responsible for Microbial Distribution in The Yellowstone Geothermal Ecosystem. *Frontiers in Microbiology*. 4(May), 1–15.

Iribarren, C., Sharp, D.S., Curb, J.D. and Yano, K. (1996). High Uric Acid: A Metabolic Marker of Coronary Heart Disease Among Alcohol Abstainers? *Journal of Clinical Epidemiology*. 49(6), 673–678.

Isoe, J. and Scaraffia, P.Y. (2013). Urea Synthesis and Excretion in *Aedes aegypti* Mosquitoes Are Regulated by A Unique Cross-Talk Mechanism. *PLoS ONE*. 8(6).

Iswantini, D., Rachmatia, R., Diana, N.R., Nurhidayat, N., Akhiruddin and Saprudin, D. (2016). Activity and Stability of Biofilm Uricase of *Lactobacillus plantarum* for Uric Acid Biosensor. *IOP Conference Series: Earth and Environmental Science*. 31, 012028.

Ivanova, E.P., Gorshkova, N.M., Sawabe, T., Hayashi, K., Kalinovskaya, N.I., Lysenko, A.M., Zhukova, N. V, Nicolau, D. V, Kuznetsova, T.A., Mikhailov, V. V and Christen, R. (2002). *Pseudomonas extremorientalis* sp. nov., Isolated From a Drinking Water Reservoir. *International Journal of Systematic and Evolutionary Microbiology*. 52(5), 2113–2120.

Jagadeesan, Y. and Meenakshisundaram, S. (2019). Combinatorial Approach for Screening and Assessment of Multiple Therapeutic Enzymes from Marine Isolate *Pseudomonas aeruginosa* AR01. *Royal Society of Chemistry*. 9, 16989–17001.

Jianguo, L.I.U. and Gaoxiang, L. (1994). Purification and Properties of Uricase from *Candida* sp. and Its Application in Uric Acid Analysis in Serum. *Applied Biochemistry and Biotechnology*. 47.

Kahn, K. and Tipton, P.A. (1998). Spectroscopic Characterization of Intermediates in the Urate Oxidase Reaction. *Biochemistry*. 37(33), 11651–11659.

Kai, L., Ma, X.H., Zhou, X.L., Jia, X.M., Li, X. and Guo, K.P. (2008). Purification and Characterization of A Thermostable Uricase from *Microbacterium* sp. strain

ZZJ4-1. *World Journal of Microbiology and Biotechnology*. 24(3), 401–406.

Kasper, I.R., Juriga, M.D., Giurini, J.M. and Shmerling, R.H. (2016). Treatment of Tophaceous Gout: When Medication is Not Enough. *Seminars in Arthritis and Rheumatism*. 45(6), 669–674.

Ken, O., Bryan, T.E., Tomoko, N., Emil, F., and Takeshi, N. (2008). Mechanism of Inhibition of Xanthine Oxidoreductase by Allopurinol : Crystal Structure of Reduced Bovine Milk Xanthine Oxidoreductase Bound with Oxypurinol. *Nucleosides, Nucleotides & Nucleic Acids*. 27, 888–893.

Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M. and Schleifer, K.-H. (1996). Recent Changes in the Classification of the Pseudomonads: An Overview. *Systematic and Applied Microbiology*. 19(4), 465–477.

Khade, S. and Srivastava, S.K. (2015). Uricase and Its Clinical Applications. *International Journal of Biological and Medical Research*. 6(2), 5211–5215.

Khade, S., Srivastava, S.K. and Dutt, A. (2016). Biocatalysis and Agricultural Biotechnology Production of Clinically Efficient Uricase Enzyme Induced from Different Strains of *Pseudomonas aeruginosa* under Submerged Fermentations and Their Kinetic Properties. *Biocatalysis and Agricultural Biotechnology*. 8, 139–145.

Khucharoenphaisan, K. and Sinma, K. (2011). Production and Partial Characterization of Uric Acid Degrading Enzyme from New Source *Saccharopolyspora* sp. PNR11. *Pakistan Journal of Biological Sciences*. 14(3), 226–231.

Kirchman, D. (2001). Measuring Bacterial Biomass Production and Growth Rates from Leucine Incorporation in Natural Aquatic Environments. In *Methods in Microbiolgy*. United States of America: Academic Press Ltd, pp.227–237.

Kornberg, A. (2009). Why Purify Enzymes? In *Methods in Enzymology*. pp.3–6.

Kostakioti, M., Newman, C.L., Thanassi, D.G. and Stathopoulos, C. (2005). Mechanisms of Protein Export across the Bacterial Outer Membrane. *Journal of Bacteriology*. 187(13), 4306–4314.

Kotb, E. (2016). Improvement of Uricase Production from *Bacillus subtilis* RNZ-79 by Solid State Fermentation of Shrimp Shell Wastes. *Biologia (Poland)*. 71(3), 229–238.

Kratzer, J.T., Lanaspa, M.A., Murphy, M.N., Cicerchi, C., Graves, C.L., Tipton, P.A., Ortlund, E.A., Johnson, R.J. and Gaucher, E.A. (2014). Evolutionary

History and Metabolic Insights of Ancient Mammalian Uricases. *Proceedings of the National Academy of Sciences*. 111(10), 3763–3768.

Kumar Sen, S., Mohapatra, S.K., Satpathy, S. and Rao, G. (2010). Characterization of Hot Water Spring Source Isolated Clones of Bacteria and Their Industrial Applicability. *International Journal of Chemical Research*. 2(1), 975–3699.

Lalucat, J., Bennasar, A., Bosch, R., Garcia-Valdes, E. and Palleroni, N.J. (2006). Biology of *Pseudomonas stutzeri*. *Microbiology and Molecular Biology Reviews*. 70(2), 510–547.

Lee, I.R., Yang, L., Sebetso, G., Allen, R., Doan, T.H.N., Blundell, R., Lui, E.Y.L., Morrow, C.A. and Fraser, J.A. (2013). Characterization of the Complete Uric Acid Degradation Pathway in the Fungal Pathogen *Cryptococcus neoformans*. *PLoS ONE*. 8(5), 1–13.

Leisinger, T., Itoh, Y., Soldati, L., Stalon, V., Falmagne, P., Terawaki, Y., Leisinger, T. and Haas, D. (2014). Anabolic Ornithine Carbamoyltransferase of Pseudomonas aeruginosa : Nucleotide Sequence and Transcriptional Control of the argF Structural Gene. *Journal of Bacteriology*. 170(6), 2725–2734.

Lemoine, C.M.R. and Walsh, P.J. (2015). Evolution of Urea Transporters in Vertebrates : Adaptation to Urea's Multiple Roles and Metabolic Sources. *The Journal of Experimental Biology*. 218, 1936–1945.

Leplatois, P., Le Douarin, B. and Loison, G. (1992). High-level Production of A Peroxisomal Enzyme: *Aspergillus flavus* Uricase Accumulates Intracellularly and Is Active in *Saccharomyces cerevisiae*. *Gene*. 122(1), 139–145.

Li, E. and Mira De Orduña, R. (2010). A Rapid Method for The Determination of Microbial Biomass by Dry Weight using A Moisture Analyser with An Infrared Heating Source and An Analytical Balance. *Letters in Applied Microbiology*. 50(3), 283–288.

Li, M., Liao, X., Zhang, D., Du, G. and Chen, J. (2011). Yeast Extract Promotes Cell Growth and Induces Production of Polyvinyl Alcohol-Degrading Enzymes. *Enzyme Research*. 2011, 1–8.

Lipkowitz, M.S. (2012). Regulation of Uric Acid Excretion by The Kidney. *Current Rheumatology Reports*. 14(2), 179–188.

Liu, J., Li, G., Liu, H. and Zhou, X. (1994). Purification and Properties of Uricase from *Candida* sp. and Its Application in Uric Acid Analysis in Serum. *Applied Biochemistry and Biotechnology*. 47(1), 57–63.

Lord, M. (2003). Gel Electrophoresis of Proteins. *Essential Cell Biology*., 197–268.

Lotfy, W.A. (2008). Production of A Thermostable Uricase by A Novel *Bacillus thermocatenulatus* strain. *Bioresource Technology*. 99(4), 699–702.

Lysenko, O. (1961). Pseudomonas--An Attempt at A General Classification. *Journal of General Microbiology*. 25(3), 379–408.

Madigan, M.T., Martinko, J.M. and Parker, J. (2014). *Brock Biology of Microrganisms* 14th ed., Benjamin Cummings.

Magda, A., Sanaa, T., Saleh, A.G. and Reda, A. (2013). Production and Characterization of Uricase from *Streptomyces exfoliatus* UR10 Isolated from Farm Wastes. *Turkish Journal of Biology*. 37, 520–529.

Mahdhi, A., Chaieb, K., Kammoun, F. and Bakhrouf, A. (2010). Use of *Pseudomonas stutzeri* and *Candida utilis* in The Improvement of The Conditions of *Artemia* Culture and Protection Against Pathogens. *Brazilian Journal of Microbiology*. 41, 107–115.

Mahler, B.Y.H.R., The, W., Assistamze, T. and Germille, O.F. (1955). Studies on Uricase: I; Preparation, Purification and Properties of A Cuproprotein. *Journal Biology Chemistry*. (216), 625–642.

Mahler, J.L. (1970). A New Bacterial Uricase for Uric Acid Determination. *Analytical Biochemistry*. 84(38), 65–84.

Mahmoud, M.N. and El-Fallal, A. (1996). Screening of Some Fungi for Uricolytic Activity. *Qatar Univesity Science Journal*. 16(1), 71–76.

Maier, R.M. (2009). Bacterial Growth. *Environmental Microbiology*., 37–54.

Maiuolo, J., Oppedisano, F., Gratteri, S., Muscoli, C. and Mollace, V. (2015). Regulation of Uric Acid Metabolism and Excretion. *International Journal of Cardiology*. 213, 8–14.

Maples, K.R. and Mason, R.P. (1988). Free Radical Metabolites of Uric Acid. *Journal Biology Chemistry*. 263(4), 1709–1712.

Markus, R.W. and Aaron, Z.F. (2007). Protein Purification. In *A Manual for Biochemistry Protocols*. Singapore: World Scientific Publishing Co. Pte. Ltd., p.142.

Matt, B. , Raj, V., Naga, C. (2016). Febuxostat-Induced Acute Liver Injury. *Hepatology*. 63(3), 87–92.

McClory, J. and Said, N. (2009). Gout in Women. *Medicine and Health*. 92(11), 363–4, 368.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., Desantis, T.Z., Probst, A., Andersen, G.L., Knight, R. and Hugenholtz, P. (2011). An Improved Greengenes Taxonomy with Explicit Ranks for Ecological and Evolutionary Analyses of Bacteria and Archaea. *The ISME Journal*. 6(3), 610–618.

McKee and Mckee, J.R. (2015). *Biochemistry: The Molecular Basis of Lıfe* 6th ed., Oxford University Press, USA.

Mehrotra, P. (2016). Biosensors and Their Applications – A Review. *Journal of Oral Biology and Craniofacial Research*. 6(2), 153–159.

Meraj, M., Kalil-ur-Rahman, Amer, J., Muhammad, A., Ibrahim, R., Sadia, J. and Nazish, J. (2012). *Bacillus subtilis* Improvement Through UV and Chemical Mutagenesis for Indigenously Hyperproduced Urate oxidase. *Pakistan Journal of Lıfe and Social Sciences*. 10(2), 123–129.

Meraj, M., Javed, S. and Irfan, R. (2014). Hyperproduction of Urate Oxidase By Wild and Mutated *Bacillus substilis* (Bem-2). *Advance in Agriculture and Biology*. 1(3), 124–130.

Mikuls, T.R., Farrar, J.T., Bilker, W.B., Fernandes, S., Schumacher, H.R. and Saag, K.G. (2005). Gout Epidemiology: Results from the UK General Practice Research Database, 1990-1999. *Annals of the Rheumatic Diseases*. 64(2), 267–272.

Ming, J., Fan, Y., Irene, Y., Ying, Y., Jin, J.L., Hong, W. and Xiao-Feng, Y. (2012). Uric Acid, Hyperuricemia and Vascular Disease. *Frontiers Biosciences*. 17(2), 656–669.

Moat, A.G., Foster, J.W. and Spector, M.P. (2002). Metabolism of Substrates Other Than Glucose. In *Microbial Physiology*. Willey-Liss, Inc., pp.394–411.

Modric, N., Derome, A.E., Ashcroft, S.J.H. and Poje, M. (1992). Tracing and Identification of Uricase Reaction Intermediates. *Tetrahedron Letters*. 33(44), 6691–6694.

Mohammad, B.T., Daghistani, H.I. Al, Jaouani, A., Abdel-latif, S. and Kennes, C. (2017). Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs : *Bacillus lichenıformis* and *Thermomonas hydrothermalis* Isolates as Potential Producers of Thermostable Enzymes. *International Journal of Microbiology*. 2017.

Monod, J. (1949). The Growth of Bacterial Cultures. *Annual Review of Microbiology*. 3(1), 371–394.

Montalbini, P., Aguilar, M. and Pineda, M. (1999). Isolation and Characterization of Uricase from Bean Leaves and Its Comparison with Uredospore Enzymes. *Plant Science*. 147(2), 139–147.

Moriarity, J.T., Folsom, A.R., Iribarren, C., Nieto, F.J. and Rosamond, W.D. (2000). Serum Uric Acid and Risk of Coronary Heart Disease: Atherosclerosis Risk in Communities (ARIC) Study. *Annals of Epidemiology*. 10(3), 136–43.

Mucsi, Ashley D, Yan, S. and Gilbert, N. (2010). Monosodium Urate Crystals in Inflammation and Immunity. *Immunological Reviews*. 233, 203–217.

Munsch, P., Alatossava, T., Marttinen, N., Meyer, J.M., Christen, R. and Gardan, L. (2002). *Pseudomonas costantinii* sp. nov., Another Causal Agent of Brown Blotch Disease, Isolated from Cultivated Mushroom Sporophores in Finland. *International Journal of Systematic and Evolutionary Microbiology*. 52(6), 1973–1983.

Nakagawa, S., Oda, H. and Anazawa, H. (1995). High Cell Density Cultivation and High Recombinant Protein Production of *Escherichia coli* Strain Expressing Uricase. *Bioscience, Biotechnology and Biochemistry*. 59(12), 2263–2267.

Nanda, P. and Jagadeesh Babu, P.E. (2014). Isolation, Screening and Production Studies of Uricase Producing Bacteria from Poultry Sources. *Preparative Biochemistry and Biotechnology*. 44(8), 811–821.

Nanda, P., Jagadeeshbabu, P.E., Fernandes, J., Hazarika, P. and Dhabre, R. (2012). Studies on Production , Optimization and Purification of Uricase from *Gliocladium viride*. *Research in Biotechnology*. 3(4), 35–46.

Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*, New York: Oxford Univesity Press.

Nesvizhskii, A.I. (2007). Protein Identification by Tandem Mass Spectrometry and Sequence Database Searching. In R. Matthiesen, ed. *Methods in Molecular Biology*. Totowa,New Jersey: Humana Press Inc., pp.87–121.

Norashirene, M.J., Umi Sarah, H., Siti Khairiyah, M.H. and Nurdiana, S. (2013). Biochemical Characterization and 16S rDNA Sequencing of Lipolytic Thermophiles from Selayang Hot Spring , Malaysia. *International Conference on Agricultural and Natural Resources Engineering*. 5, 258–264.

Nyborg, A.C., Ward, C., Zacco, A., Chacko, B., Grinberg, L., Geoghegan, J.C., Bean, R., Wendeler, M., Bartnik, F., O'Connor, E., Gruia, F., Iyer, V., Feng, H., Roy, V., Berge, M., Miner, J.N., Wilson, D.M., Zhou, D., Nicholson, S., Wilker,

C., Wu, C.Y., Wilson, S., Jermutus, L., Wu, H., Owen, D.A., Osbourn, J., Coats, S. and Baca, M. (2016). A Therapeutic Uricase with Reduced Immunogenicity Risk and Improved Development Properties. *PLoS ONE*. 11(12), 1–23.

Oda, M., Satta, Y., Takenaka, O. and Takahata, N. (2002). Loss of Urate Oxidase Activity in Hominoids and Its Evolutionary Implications. *Molecular Biology and Evolution*. 19(5), 640–653.

Palleroni, N.J., Kunisawa, R., Contopoulou, R. and Doudoroff, M. (1973). Nucleic Acid Homologies in the Genus *Pseudomonas*. *International Journal of Systematic Bacteriology*. 23(4), 333–339.

Panda, M. K., Sahu, M. K., Tayung, K. (2013). Isolation and Characterization of A Thermophilic *Bacillus* sp . with Protease Activity Isolated from Hot Spring of Tarabalo , Odisha, India. *Iranian Journal of Microbiology*. 5(2), 159–165.

Pappin, D.J.C., Creasy, D.M. and Cottrell, J.S. (1999). Probability-based Protein Identification by Searching Sequence Databases Using Mass Spectrometry Data. *Electrophoresis*. 20, 3551–3567.

Park, S.W., Back, J.H., Lee, S.W., Song, J.H., Shin, C.H., Kim, G.E. and Kim, M. (2013). Successful Antibiotic Treatment of *Pseudomonas stutzeri* -Induced Peritonitis without Peritoneal Dialysis Catheter Removal in Continuous Ambulatory Peritoneal Dialysis. *Kidney Research and Clinical Practice*. 32(2), 81–83.

Pascual, E., Andrés, M., Vázquez-Mellado, J. and Dalbeth, N. (2017). Severe Gout: Strategies and Innovations for Effective Management. *Joint Bone Spine*. 84(5), 541–546.

Pession, A., Melchionda, F. and Castellini, C. (2008). Pitfalls, Prevention and Treatment of Hyperuricemia During Tumor Lysis Syndrome in the Era of Rasburicase (Recombinant Urate Oxidase). *Biologics: Targets and Therapy*. 2(1), 129–141.

Pineda, M., Montalbini, P., Redondo, J., Caballero, J.L., Ca, J., Perugia, Á., Giugno, B.X.X., Perugia, I.-, Vegetale, P. and Co, E.- (1997). Uricase from Leaves : Its Purification and Characterization from Three Different Higher Plants. *Planta*. (202), 277–283.

Pitts, O.M. and Priest, D.G. (1972). Uricase Reaction Intermediate. Mechanism of Borate and Hydroxide Ion Catalysist 0. *Biochemistry*. 12(7), 1358–1363.

Poovizh, T., Gajalakshmi, P., Nadu, T. and Nadu, T. (2014). Production of Uricase A Therapeutic Enzyme from *Pseudomonas putida* Isolated from Poultry Waste. *International Journal of Advanced Research*. 2(1), 34–40.

Pramanik, K., Sarkar, P. and Bhattachayay, D. (2015). Electrochemical Sensor for Uric Acid Estimation Using Uricase Biomimic. *International Journal of Advances in Engineering and Technology*. 8(1), 2084–2092.

Promega (2010). *Wizard ® Genomic DNA Purification Kit Wizard ® Genomic DNA Purification Kit*, Madison, USA.

Qiagen (2002). *QIAquick ® Spin Handbook QIAquick PCR Purification Kit QIAquick Nucleotide Removal Kit July 2002*,

Qu, X. and Wu, C.F.J. (2005). One-Factor-at-A-Time Designs of Resolution V. *Journal of Statistical Planning and Inference*. 131(2), 407–416.

Raina, M.M, Ian, L.P., Charles, P.G. (2009). Bacterial growth. In *Enviromental Microbiology*. London,United Kingdom: Elsevier Inc., pp.37–54.

Rajapaksha, B.M.M., Maithreepala, R.A. and Asanthi, H.B. (2014). Water Quality and Biology of Hot Springs Waters of Mahapelessa, Sri Lanka. *Scientific Research Journal*. 2(February), 1–6.

Rajoka, Muhammad I., Mehraj, M., Akhtar, M.W. and Zia, M.A. (2006). Purification and Properties of A Bovine Uricase. *Protein and Peptide Letters*. 13(4), 363–368.

Rajoka, M. I., Rehman, K.U., Tabish, T. and Zia, M.A. (2006). Purification and Characterization of Caprine Kidney Uricase, Possessing Novel Kinetic and Thermodynamic Properties. *World Journal of Microbiology and Biotechnology*. 22(3), 289–291.

Ram, S.K., Raval, K. and Jagadeeshbabu, P.E. (2015). Enhancement of A Novel Extracellular Uricase Production by Media Optimization and Partial Purification by Aqueous Three-Phase System. *Preparative Biochemistry and Biotechnology*. 45(8), 810–824.

Ramana, V.V. and Sastry, K.S. (1993). Chromium-uric acid Complexes as Growth Substrates and Inducers of Uricase in *Neurospora crassa*. *Journal of Inorganic Biochemistry*. 50(2), 107–117.

Ramazzina, I., Folli, C., Secchi, A., Berni, R. and Percudani, R. (2006). Completing the Uric Acid Degradation Pathway Through Phylogenetic Comparison of Whole Genomes. *Nature Chemical Biology*. 2(3), 144–148.

Ravichandran, R. Hemaasri, S., Swaranjit Singh Cameotra and Jayaprakash, N.S. (2015). Purification and Characterization of an Extracellular Uricase from A New Isolate of *Sphingobacterium thalpophilum* (VITPCB5). *Protein Expression and Purification*. 88(2), 214–220.

Reddy, M.V., Nikhil, G.N., Mohan, S.V., Swamy, Y. V and Sarma, P.N. (2012). *Pseudomonas otitidis* As A Potential Biocatalyst for Polyhydroxyalkanoates (PHA) Synthesis Using Synthetic Wastewater and Acidogenic Effluents. *Bioresource Technology*. 123, 471–479.

Rediers, H., Bonnecarre, V., Rainey, P.B., Hamonts, K. and Vanderleyden, J. (2017). Development and Application of a dapB -Based In Vivo Expression Technology System To Study Colonization of Rice by the Endophytic Nitrogen-Fixing Bacterium *Pseudomonas stutzeri* A15. *Applied and ENvironmental Microbiology*. 69(11), 6864–6874.

Reed, M.C., Lieb, A. and Nijhout, H.F. (2010). The Biological Significance of Substrate Inhibition: A Mechanism with Diverse Functions. *Bioesssays*. 32(5), 422–429.

Reischke, S., Kumar, M.G.K. and Baath, E. (2015). Threshold Concentration of Glucose for Bacterial Growth in Soil. *Soil Biology and Biochemistry*. 80, 218–223.

Resing, K.A. and Ahn, N.G. (2005). Proteomics Strategies for Protein Identification. *Federation of European Biochemical Societies (FEBS) Letters*. 579(4 SPEC. ISS.), 885–889.

Righetti, P.G. and Boschetti, E. (2013). Low-Abundance Protein Access by Combinatorial Peptide Libraries. In *Low-Abundance Proteome Discovery*. Milan, Italy: Elsevier Inc., pp.79–157.

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D.S., Alston, M., Stringer, M.F., Betts, R.P., Baranyi, J., Peck, M.W. and Hinton, J.C.D. (2012). Lag Phase is A Distinct Growth Phase That Prepares Bacteria for Exponential Growth and Involves Transient Metal Accumulation. *Journal of Bacteriology*. 194(3), 686–701.

Rosendorff, C. and Jogendra, M.R.D. (2012). Uric Acid: Where Are We? *The Journal of Clinical Hypertension*. 15(1), 5–6.

Rouf, M.A. and Lomprey, R.F. (1968). Degradation of Uric Acid by Certain Aerobic Bacteria. *Journal of Bacteriology*. 96(3), 617–622.

Rudner, D.Z. and Losick, R. (2010). Protein Subcellular Localization in Bacteria. *Cold Spring Harbor Perspectives in Biology*., 1–14.

Saeed, H.M., Abdel-Fattah, Y.R., Berekaa, M.M., Gohar, Y.M. and Elbaz, M.A. (2004). Identification, Cloning and Expression of *Pseudomonas aeruginosa* Ps-x Putative Urate Oxidase Gene in Escherichia coli. *Polish Journal of Microbiology*. 53(4), 227–236.

Sahib, A.M.. and E.A.. (2012). Purification and Characterization of Uricase Enzyme Produced from *Pseudomonas aeruginosa* 7. *Kerbala Journal of Pharmaceutical Sciences*. 70(3).

Samsudin, A.R., Hamzah, U., Siwar, C., Fauzi, M., Jani, M. and Ab, R. (1997). Thermal Springs of Malaysia and Their Potential Development. *Journal of Asian Earth Sciences*. 15, 275–284.

Scaraffia, P.Y., Tan, G., Isoe, J., Wysocki, V.H., Wells, M.A. and Miesfeld, R.L. (2008). Discovery of An Alternate Metabolic Pathway for Urea Synthesis in Adult *Aedes aegypti* Mosquitoes. *Proceedings of the National Academy of Sciences*. 105(2), 518–523.

Scopes, R.K. (1994). *Protein Purification: Principles and Practice* 3rd ed., New York: Springer-Verlag.

Selvaraj, C. and Thirumalai, P. (2017). Screening, Production and Optimization of Uricase from *Pseudomonas aeruginosa*. *European Journal of Biotechnology and Bioscience*. 5(1), 57–61.

Seo, Y. K., James, P. G., Kyoung, M. K., Hyon, K. C., Daniel, F. H. and Daniel, A.A. (2012). Hypeuricemia and Coronary Heart Disease: A Systematic Review and Meta-Analysis. *Arthritis Care and Research*. 40(6), 1301–1315.

Shah, M.P. (2014). Exploited Application of *Pseudomonas stutzeri* ETL-4 in Microbial Degradation of Congo Red. *Open Access Biotechnology*. 3(1), 1–6.

Sikorski, J., Stackebrandt, E. and Wackernagel, W. (2001). *Pseudomonas kilonensis* sp. nov., A Bacterium Isolated from Agricultural Soil. *International Journal of Systematic and Evolutionary Microbiology*. 51(4), 1549–1555.

Sin, I.L. (1975). Purification and Properties of Xanthine Dehydrogenase from *Pseudomonas acidovorans*. *Biochimca et Biophysica Acta*. 0(410), 12–20.

Smith, J.S. and Scholtz, J.M. (1996). Guanidine Hydrochloride Unfolding of Peptide Helices: Separation of Denaturant and Salt Effects. *Biochemistry*. 35(22), 7292–7297.

Struthers, A. and Shearer, F. (2012). Allopurinol : Novel Indications in Cardiovascular Disease. *Pharmocology*. 98(21), 4–7.

Suhad, K.A. and May, T.F. (2015). Study the Effect of Purified Uricase From *Pseudomonas aeruginosa* on Hyperuricemia *In Vivo*. *International Journal of Biological & Pharmaceutical Research*. 6(6), 469–472.

Sundy, J.S., Baraf, H.S.B., Yood, R.A., Edwards, N.L., Gutierrez-Urena, S.R., Treadwell, E.L., Vazquez-Mellado, J., White, W.B., Lipsky, P.E., Horowitz, Z., Huang, W., Maroli, A.N., Waltrip, I.I.R.W., Hamburger, S.A. and Becker, M.A. (2011). Efficacy and Tolerability of Pegloticase for the Treatment of Chronic Gout in Patients Refractory to Conventional Treatment: Two Randomized Controlled Trials. *Journal of the American Medical Association*. 306(7), 711–720.

Suzuki, K., Sakasegawa, S.-I., Misaki, H. and Sugiyama, M. (2004). Molecular Cloning and Expression of Uricase Gene from *Arthrobacter globiformis* in *Escherichia coli* and Characterization of the Gene Product. *Journal of Bioscience and Bioengineering*. 98(3), 153–8.

Switzar, L., Giera, M. and Niessen, W.M.A. (2013). An Overview of the Available Techniques and Recent Developments. *Journal of Proteome Research*. 12(3), 1067–1077.

Szymanowska-powa, D. (2015). The Effect of High Concentrations of Glycerol on The Growth , Metabolism and Adaptation Capacity of *Clostridium butyricum* DSP1. *Electronic Journal of Biotechnology*. 18, 128–133.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6 : Molecular Evolutionary Genetics Analysis Version 6 . 0. 30(12), 2725–2729.

Tan, H., Zhang, Z., Hu, Y., Wu, L., Liao, F., He, J., Luo, B., He, Y., Zuo, Z., Ren, Z., Peng, G. and Deng, J. (2015). Isolation and Characterization of *Pseudomonas otitidis* TH-N1 Capable of Degrading Zearalenone. *Food Control*. 47, 285–290.

Tanaka, A., Yamamura, M., Kawamoto, S. and Fukui, S. (1977). Production of Uricase by *Candida tropicalis* Using n-alkane as a Substrate. *Applied and Environmental Microbiology*. 34(4), 342–346.

Taylor, R.C. and Coorssen, J.R. (2006). Proteome Resolution by Two-Dimensional Gel Electrophoresis Varies with the Commercial Source of IPG Strips. *Journal of Proteome Research*. 5(11), 2919–2927.

Teh, C.L., Chew, K.F. and Ling, G.R. (2014). Acute Gout in Hospitalized Patients in Sarawak General Hospital. *Medical Journal of Malaysia*. 69(3), 126–128.

Terkeltaub, R.A. (2009). Colchicine Update: 2008. In *Seminars in Arthritis and Rheumatism*. Elsevier Inc., pp.411–419.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W : Improving The Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting , Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Research*. 22(22), 4673–4680.

Tortora, G. J., Funke, B. R. and Case, C.L. (2007). *Microbiology: An Introduction* 9th ed., United States of America: Pearson Education.

Urbieta, M.S., Toril, E.G., Alejandra Giaveno, M., Bazán, Á.A. and Donati, E.R. (2014). Archaeal and Bacterial Diversity in Five Different Hydrothermal Ponds in the Copahue Region in Argentina. *Systematic and Applied Microbiology*. 37(6), 429–441.

Waterborg, J.H. and Matthews, H.R. (1984). The Lowry Method for Protein Quantitation. *Methods in Molecular Biology*. 1, 1–3.

Weijland, A., Harmark, K., Cool, R.H., Anborgh, P.H. and Parmeggiani, A. (1992). Elongation Factor Tu: A Molecular Switch in Protein Biosynthesis. *Molecular Microbiology*. 6(6), 683–8.

Weisburg, W.G., Barns S.M., Pelletier, D.A., Lane, D.J. (1991). 16S ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology*. 173(2), 697–703.

Wingfield, P.T. (2016). Protein Precipitation Using Ammonium Sulfate. *Current Protocol Protein Science*., 1–10.

Winnard, D., Wright, C., Taylor, W.J., Jackson, G., Te karu, L., Gow, P.J., Arroll, B., Thornley, S., Gribben, B. and Dalbeth, N. (2012). National Prevalence of Gout Derived from Administrative Health Aata in Aotearoa New Zealand. *Rheumatology*. 51(5), 901–909.

Wu, C.F.J. and Hamada, M. (2007). *Experiments : Planning , Analysis and Parameter Design Optimization*, New York: John Wiley & Sons, Inc.,.

Wu, J., Jung, B.G., Kim, K.S., Lee, Y.C. and Sung, N.C. (2009). Isolation and Characterization of *Pseudomonas otitidis* WL-13 and Its Capacity to Decolorize Triphenylmethane Dyes. *Journal of Environmental Sciences*. 21(7), 960–964.

Xiaolan, Y., Yonghua, Y., Chang-Guo, Z. and Fei, L. (2013). Uricases as

Therapeutic Agents to Treat Refractory Gout: Current States and Future Directions. *Drug Development Research*. 73(2), 66–72.

Yates, G.T. and Smotzer, T. (2007). On the Lag Phase and Initial Decline of Microbial Growth Curves. *Journal of Theoretical Biology*. 244(3), 511–517.

Yazdi, M.T., Zarrini, G., Mohit, E., Faramarzi, M.A., Setayesh, N., Sedighi, N. and Mohseni, F.A. (2006). *Mucor hiemalis*: A New Source for Uricase Production. *World Journal of Microbiology and Biotechnology*. 22(4), 325–330.

Ye, X. and Li, L. (2012). Microwave-Assisted Protein Solubilization for Mass Spectrometry-Based Shotgun Proteome Analysis. *Analytical Chemistry*. 84(14), 6181–6191.

Yeap, S.S., Goh, E.M.L. and Gun, S.C. (2009). A Survey on the Management of Gout in Malaysia. *International Journal of Rheumatic Diseases*. 12(4), 329–335.

Yokoyama, S., Ogawa, A. and Obayashi, A. (1988). Rapid Extraction of Uricase from *Candida utilis* Cells by Use of Reducing Agent Plus Surfactant. *Enzyme and Microbial Technology*. 10(1), 52–55.

Zhang, G., Ueberheide, B.M., Waldemarson, S., Myung, S., Molloy, K., Eriksson, J., Chait, B.T., Neubert, T.A. and Fenyö, D. (2010). Protein Quantitation Using Mass Spectrometry. *Methods in Molecular Biology*. 673(10), 1–11.

Zhao, C., Wan, L., Wang, Q., Liu, S. and Jiao, K. (2009). Highly Sensitive and Selective Uric Acid Biosensor Based on Direct Electron Transfer of Hemoglobin-Encapsulated Chitosan-Modified Glassy Carbon Electrode. *Analytical Sciences*. 25(8), 1013–1017.

Zhao, Y., Yang, X., Lu, W. and Liao, H. (2009). Uricase Based Methods for Determination of Uric Acid in Serum. , 1–6.

Zhou, X., Ma, X., Sun, G., Li, X. and Guo, K. (2005). Isolation of A Thermostable Uricase-producing Bacterium and Study on Its Enzyme Production Conditions. *Process Biochemistry*. 40(12), 3749–3753.

Zhou, X.L., Ma, X.H., Sun, G.Q., Li, X. and Guo, K.P. (2005). Isolation of a Thermostable Uricase-Producing Bacterium and Study on Its Enzyme Production Conditions. *Process Biochemistry*. 40(12), 3749–3753.

Zhou, Y., Zhang, M., He, D., Hu, X., Xiong, H., Wu, J., Zhu, B. and Zhang, J. (2016). Uricase Alkaline Enzymosomes with Enhanced Stabilities and Anti-Hyperuricemia Effects Induced by Favorable Microenvironmental Changes.

Scientific Reports. 7(December 2015), 1–15.

LIST OF PUBLICATIONS

Nor Sahslin, I.S.L., Hamed, M.K., Norahim, I. and Shafinaz, S. (2015). Isolation, Partial Purification and Characterization of Thermophilic Uricase from *Pseudomonas otitidis* strain SN4. Malaysian Journal of Microbiology. 11(4),352-357.