

TABLE OF CONTENTS

NO.	TITLE	PAGE
1	Introduction	1
2	Review of literature	9
2.1	Mechanism of action	10
2.2	History of urate oxidase	12
2.3	Microbial production of urate oxidase	12
2.3.1	Microorganism	12
2.3.1	Strain improvement techniques	13
2.4	substrate for urate oxidase production	16
2.5	Techniques for urate oxidase production	17
2.6	Factors effecting the production of urate oxidase	18
2.6.1	Chemical factors	18
2.6.2	Physical factors	20
2.7	Techniques for urate oxidase purification	20
2.8	Kinetics of urate oxidase	22
2.9	Determination of molecular mass	23
2.10	Determination of uric acid	24
2.11	Therapeutic effect of urate oxidase	25
3	Materials and Methods	27
3.1	chemicals, reagents and enzymes	27
3.2	Microorganism procurement and maintenance	27
3.2.1	Sporulation medium	27
3.2.2	Inoculum preparation	28
3.2.3	Spores Counting	28
3.3	Techniques to improve strain	28
3.3.1	Radiation mutagenesis	29
3.3.1.1	Mutagenesis by UV lamp	29
3.3.2	Chemical mutagenesis	29
3.3.2.1	Mutagenesis by EMS and EB	29
3.4	Mutant selection	29
3.4.1	Colony restrictor selection	30
3.4.2	Log kill mutant dose selection by kill/survival curve	30
3.4.2.1	Calculation of colony forming units (C.F.U. mL ⁻¹)	30
3.4.3	Screening Methods	30
3.4.3.1	Plate screening procedure	30
3.4.3.2	Mutants isolation of selective marker	30
3.4.4	Mutant identification	31
3.4.4.1	Enzyme diffusion zone analysis	31
3.4.4.2	Analytical examination	31
3.5	Urate oxidase production	31

3.5.1	Inoculum preparation	31
3.5.2	Urate oxidase production by liquid-state fermentation	31
3.5.3	Optimization conditions for urate oxidase production	32
3.5.3.1	Substrate level	32
3.5.3.2	Fermentation period	32
3.5.3.3	pH	33
3.5.3.4	Temperature	33
3.5.3.5	Inoculum size	34
3.5.3.6	Effect of nitrogen sources	34
3.5.3.7	Effect of carbon sources	35
3.5.4	Sample harvesting	35
3.6	Enzyme assay	35
3.6.1	Preparation of borate buffer (0.1 M)	36
3.6.2	HCl (1 M)	36
3.6.3	NaOH (1 M)	36
3.6.4	Uric acid (0.12 mM)	36
3.6.5	Procedure	36
3.6.6	Uric acid analysis	37
3.6.6.1	Preparation of uric acid standard curve	37
3.6.7	Determination of protein contents	37
3.6.7.1	Preparation of biuret reagent	37
3.6.7.2	Preparation of protein standard curve	38
3.7	Purification of urate oxidase	38
3.7.1	Partial purification by $(\text{NH}_4)_2\text{SO}_4$ precipitation	38
3.7.1.1	Dialysis	39
3.7.1.2	Partial purification by gel filtration	39
3.7.2.1	Column preparation	39
3.7.2.2	Swelling of resin	39
3.7.2.3	Filling the column	39
3.7.2.4	Equilibration of the column	40
3.7.2.5	Application of the sample	40
3.7.2.6	Elution	40
3.7.3	Purification by ion exchange chromatography	40
3.7.3.1	Preparation of NaOH (4 %)	40
3.7.3.2	Perpetration of HCl (4%)	40
3.7.3.3	Preparation of column	40
3.7.3.4	Washing of Column with Base	40
3.7.3.5	Washing of column with acid	41
3.7.3.6	Equilibration of the column	41
3.7.3.7	Application of sample	41
3.7.3.8	Elution	41
3.8	Molecular mass determination	41

3.8.1	Electrophoresis	41
3.8.1.1	Stock solutions	41
3.8.1.2	Resolving gel preparation	41
3.8.1.3	Stacking gel preparation	42
3.8.1.4	Sample buffer	42
3.8.1.5	Stock Electrode Buffer	43
3.8.1.6	For SDS-PAGE prepare urate oxidase	43
3.8.1.7	For SDS-PAGE prepare protein markers ladder	43
3.8.1.8	Running of PAGE	43
3.8.1.9	Protein staining of sodium dodecyl sulfate-polyacrylamide gel	43
3.9	Kinetic and Thermodynamic Studies	44
3.9.1	Optimum pH	44
3.9.2	Optimum Temperature	44
3.9.3	Activation energy (E_a)	44
3.9.4	Determination of Michealis-Menten constants	44
3.9.5	Irreversible thermal denaturation	44
3.9.6	Activation energy of thermal denaturation	44
3.9.7	Thermodynamics of irreversible thermal inactivation	45
3.10	Stability Determination	45
3.11	Uric acid estimation	45
3.11.1	Comparison with standard kit	45
4	Result and discussion	46
4.1	Production of mutants	46
4.1.1	Mutation induced by UV radiation	46
4.1.2	Mutation induced by chemicals	47
4.2	Selection and evaluation of mutant	47
4.2.1	Colony restriction	48
4.2.2	Selection of specific mutants by using 2-thiouric acid	50
4.2.3	Enzyme diffusion zone	54
4.2.4	Analytical test	54
4.3	Optimization of fermentation parameters by Bacillus subtilis	57
4.3.1	Effect of substrate	57
4.3.2	Effect of fermentation period	60
4.3.3	Effect of pH	62
4.3.4	Effect of temperature	64
4.3.5	Effect of Inoculum Size	66
4.3.6	Effect of nitrogen source and concentration	67
4.3.6.1	Effect of peptone	67
4.3.6.2	Effect of yeast extract	69

4.3.6.3	Effect of ammonium chloride	71
4.3.6.4	Effect of sodium nitrate	72
4.3.6.5	Comparison of different nitrogen sources	74
4.3.7	Effect of carbon source	75
4.3.7.1	Effect of maltose	75
4.3.7.2	Effect of sucrose	77
4.3.7.3	Effect of glucose	78
4.3.7.4	Effect of galactose	80
4.3.7.5	Compare the effect of carbon sources	81
4.4	Purification of urate oxidase	84
4.4.1	Purification by ammonium sulfate precipitation	84
4.4.2	Ion exchange chromatography	86
4.4.3	Gel filtration chromatography	88
4.4.4	SDS-PAGE	90
4.5	Molecular mass determination	90
4.6	Kinetic and thermodynamic studies	91
4.6.1	Optimum pH	91
4.6.2	Optimum temperature	93
4.6.3	Michaelis-Menten constant determination	95
4.6.4	Irreversible thermal denaturation	96
4.6.5	Thermodynamics of irreversible thermal inactivation	98
4.7	Determination of Stability	101
4.8	Uric acid estimation kit	104
4.8.1	Comparison with standard kit	106
5	Summary	108
6	Literature cited	110