MICROBIAL PRODUCTION OF CELLULASES FOR BIOCONVERSION OF LIGNOCELLULOSIC WASTES

A THESIS

Submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOSCIENCES AND BIOTECHNOLOGY

By

RAJ KUMAR

DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
UNIVERSITY OF ROORKEE
ROORKEE–247 667 (INDIA)

SEPTEMBER, 2000

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"MICROBIAL PRODUCTION OF CELLULASES FOR BIOCONVERSION OF LIGNOCELLULOSIC WASTES"** in the fulfilment of the requirement for the award of the degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology** of the University, is an authentic record of my own work carried out during a period from August 1995 to September 2000 under the supervision of **Dr. R.P. Singh,** Assistant Professor, Department of Biosciences and Biotechnology.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

Raj Kumar

Dated: 23-06-2001 **(RAJ KUMAR)**

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

23.6.2001

Dr. R.P. Singh
Assistant Professor
Department of Biosciences
and Biotechnology,
University of Roorkee,
Roorkee- 247 667, INDIA

The Ph.D. viva-voce examination of **Mr. Raj Kumar**, Research Scholar, has been held on 7.8.2001

Signature of Supervisor(s)

Signature of H. O. D.

Head
Deptt. of Biosciences and

Signature of External Examiner

ABSTRACT

The prime emphasis of the present investigation was to define the factors allowing maximum bioconversion of the major, high cellulose containing lignocellulosic waste materials, sugarcane bagasse and water hyacinth (*Eicchornia crassipes*) biomass into fermentable sugars. Sugarcane bagasse is generated in large amounts from the sugarcane industries, has no further application and is burnt; whereas, water hyacinth (*Eicchornia crassipes*) an aquatic biomass, found in ponds, lakes and rivers is a serious concern for the water resources. In an attempt to isolate a strain with potential bioconversion ability, twenty two (fungal and bacterial) strains were isolated from decomposing substrates, among them *Aspergillus niger* RK-3 was found to have maximum cellulase producing ability. To further ascertain its bioconversion capability, the levels of cellulases produced were evaluated using the commercially available synthetic cellulosic substrates under submerged state fermentation. Production of higher cellulase levels was detected with these substrates. The wild type isolate *Aspergillus niger* RK-3 thus appeared as an attractive choice for bioconversion of lignocellulosic substrates. Analysis of cellulase production with sugarcane bagasse and *Eicchornia crassipes* biomass showed that high pressure steaming followed by alkali treatment of the sugarcane bagasse and *Eicchornia crassipes* biomass led to significant increase in the cellulase production as compared to the untreated substrates.

Evaluation of physical and biological factors affecting the cellulase production denoted that 5% inoculum, 130 rpm as the agitation rate along with the

pH at 4.8 and a temperature of 30°C were suitable for enzymatic production, however, temperature required for measuring the activity was 50°C. Among the medium nutrients and other chemical factors derived, use of 1% cellulosic substrates, ammonium sulfate, 2.1 gl^{-1} along with ammonium chloride, 0.4 gl^{-1}; potassium dihydrogen phosphate, 2.45 gl^{-1}; vanillin, 0.5 gl^{-1} ; succinic acid, 1.0 gl^{-1} and cellobiose 2.0 gl^{-1} led to maximum production of the cellulases. The production medium denoted as R_6m containing the sugarcane bagasse and the constituents as defined above led to significant increase in CMCase (83%), FPase (59%) and β-glucosidase (288%) levels with respect to the enzymatic production with Mandels and Weber's medium. Similar induction in activities of CMCase (112%), FPase (79%) and β-glucosidase (316%) were observed with *Eicchornia crassipes* biomass as the substrate.

A further improvement in cellulolytic activities of wild type *Aspergillus niger* RK-3 was attempted by genetic manipulation using physical (UV), chemical (NTG, sodium azide and colchicine) and mixed mutagenic treatments. A high yielding and end product resistant mutant UNSC-442 was finally selected having substantially higher cellulase production abilities. The mutant UNSC-442 resulted into CMCase (7.18 IU ml^{-1}), FPase (6.0 IU ml^{-1}) and β-glucosidase (2.55 IU ml^{-1}) activities with treated sugarcane bagasse as the substrate and the activities respectively were 89%, 130% and 54.5% higher as compared to the cellulase production with wild type *Aspergillus niger* RK-3 strain under similar conditions. A similar level increase in cellulases was also observed with *Eicchornia crassipes* biomass. The extent of cellulose degradation observed was 61.5% - 67% higher with the mutant strain. The

protein profile alongwith the zymogram analysis indicated increasing levels of CMCase (91.2 kD), FPase (68 kD) and β-glucosidase (52.4 kD) components in the mutant UNSC-442 strain as compared to the activities detected in the *Aspergillus niger* RK-3 wild type strain. Scanning electron microscopy demonstrated entirely distinct morphological features of the mutant UNSC-442 strain than its parental strain.

Incubation under co-cultured condition using *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 resulted into 23-33% increase in CMCase and FPase activities and 13% increase in β-glucosidase activity over the respective maximum activities observed under single culture condition with sugarcane bagasse as the substrate. Similarly, a 20-24% increase in CMCase and FPase and a 13% increase in β-glucosidase activity were observed with *Eicchornia crassipes* biomass as the substrate. On the other hand the co-cultivation of mutant UNSC-442 along with *Trichoderma reesei* MTCC-164 showed a further increase (8-23%) in CMCase and FPase activities and around 24% increase in β-glucosidase activity as compared to the activities obtained with mutant UNSC-442 strain with sugarcane bagasse in single culture conditions. Similar results were obtained with *Eicchornia crassipes* biomass under the conditions as referred for the sugarcane bagasse substrate. Further, the sugars generated from the enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass was found to be a cheaper and excellent substrate for the single cell protein, gluconic acid and citric acid production.

CONTENTS

	Page No.
CANDIDATE'S DECLARATION	(i)
ABSTRACT	(ii)
CONTENTS	(v)
ACKNOWLEDGEMENT	(x)
LIST OF FIGURES	(xii)
LIST OF TABLES	(xvi)
LIST OF ABBREATIONS	(xviii)
CHAPTERS	
CHAPTER 1 INTRODUCTION	1
CHAPTER 2 REVIEW OF LITERATURE	5
2.1 NATURAL OCCURRENCE OF LIGNOCELLULOSIC MATERIALS	5
2.2 MOLECULAR STRUCTURE OF CELLULOSE	7
2.3 CRYSTALLINITY OF CELLULOSE	11
2.4 PRETREATMENT OF LIGNOCELLULOSIC MATERIALS	12
2.4.1 Chemical Treatment	12
2.4.2 Physical Treatment	13
2.4.3 Biological Delignification	14
2.5 SOURCES OF CELLULOLYTIC ENZYMES	15
2.5.1 Fungal Origin	16
2.5.2 Bacterial Origin	18
2.5.2.1 Novel bacterial cellulase system: cellulosome	18
2.6 MOLECULAR STRUCTURE OF CELLULASES	20
2.7 MECHANISM OF ACTION	21
2.8 ADSORPTION-DESORPTION OF CELLULASES	23
2.9 EVALUATION OF FACTORS AFFECTING ENZYME PRODUCTION	25
2.9.1 Physical Factors	25
2.9.1.1 pH	25
2.9.1.2 Temperature	26
2.9.2 Chemical Factors	28
2.9.2.1 Carbon source	28

2.9.2.2 Nitrogen source 29
2.9.2.3 Phosphorus source 30
2.9.2.4 Phenolic compounds 31
2.9.2.5 Sugars 32

2.10 MUTAGENESIS 33
2.11 CO-CULTIVATION 34
2.12 REGULATION OF CELLULASE BIOSYNTHESIS 36
2.13 CELLULASE FAMILY 38
2.14 GENE CLONING 39

CHAPTER 3 MATERIALS AND METHODS 41

3.1 MATERIALS 41
3.1.1 Microorganisms 41

3.2 METHODS 42
3.2.1 Isolation, Purification and Screening of Strains 42
3.2.3 Pretreatment of Lignocellulosic Substrates 42

3.2.3.1 High pressure steaming and hemicellulose separation 43
3.2.3.2 Sodium hydroxide pretreatment and lignin separation 43

3.2.4 X-Ray Diffractographic Analysis of Lignocellulosic Samples 44
3.2.5 Production of Cellulases with Synthetic Cellulosic Substrates 44
3.2.6 Production of Cellulases with Natural Cellulosic Substrates 44

3.2.6.1 Preparation of inoculum 45
3.2.6.2 Composition of the production medium 45
3.2.6.3 Production of cellulases in submerged fermentation 45
3.2.6.4 Determination of dry mycelial weight 46

3.2.7 Analysis of Nutritional Factors Affecting Enzyme Production 46
3.2.8 Adsorption of Enzymatic Protein 46
3.2.9 Mutagenesis and Screening 47

3.2.9.1 Single treatment 47
3.2.9.1.1 Mutagenesis by ultraviolet radiation 47
3.2.9.1.2 Chemical mutagenesis 47

3.2.9.2 Mutagenesis by mixed chemical treatment 48
3.2.9.3 Screening of the mutant strains 48

3.2.10 Production of Cellulases During Co-Cultivation of Two Fungal Strains in Semi-solid State Fermentation (SSSF) 49

3.2.10.1 Preparation of inoculum 49
3.2.10.2 Preparation of production medium 51
3.2.10.3 Production of cellulases in semi-solid state fermentation (SSSF) 51
3.2.11 Enzymatic Hydrolysis of Cellulosic Substrates 51
3.2.12 Cultivation of Baker's Yeast on Enzymatic Hydrolyzates of Sugarcane Bagasse and *Eicchornia crassipes* Biomass 52
3.2.13 Production of Organic Acids 53
3.2.13.1 Culture conditions for organic acid production 53
3.2.13.1.1 Fermentation medium for gluconic acid production 53
3.2.13.1.2 Fermentation medium for citric acid production 53
3.3 ANALYTICAL METHODS 54
3.3.1 Estimation of Total Cellulose Content 54
3.3.2 Estimation of Total Lignin Content 54
3.3.3 Estimation of Total Reducing Sugar as Glucose 55
3.3.4 Estimation of Endoglucanase (CMCase) Activity 56
3.3.5 Estimation of Exoglucanase (FPase) Activity 56
3.3.6 Estimation of β-glucosidase Activity 57
3.3.7 Protein Estimation 57
3.3.8 Estimation of Gluconic Acid (Gluconolactone) 58
3.3.9 Estimation of Citric Acid 59
3.3.10 Enzyme Purification 59
3.3.10.1 Gel filtration 60
3.3.10.2 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis 60
3.3.10.2.1 Preparation of resolving and stacking gels 61
3.3.10.2.2 Casting of gel 62
3.3.10.2.3 Sample preparation 62
3.3.10.2.4 Electrophoresis 63
3.3.10.2.5 Zymogram analysis of cellulase components 63
3.3.11 Scanning Electron Microscopy 64
3.3.12 Statistical Analysis 65
CHAPTER 4 RESULTS **66**
4.1 ISOLATION, PURIFICATION AND SCREENING OF STRAINS 66

4.2 ANALYSIS OF CELLULASE PRODUCTION WITH SYNTHETIC SUBSTRATES 66

4.2.1 X-Ray Diffraction and Adsorption Analysis of Synthetic Cellulosic Substrates 69

4.3 EVALUATION OF THE PRETREATMENT OF LIGNOCELLULOSIC SUBSTRATES 72

4.3.1 High Pressure Steaming Followed By Alkali Pretreatment 74
4.3.1.1 X-ray diffraction analysis of lignocellulosic substrates 76

4.4 PRODUCTION OF CELLULASES IN SUBMERGED STATE FERMENTATION 76

4.4.1 Evaluation of Sugarcane Bagasse as Substrate 78
4.4.2 Evaluation of Water Hyacinth (*Eicchornia crassipes*) as Substrate 78
4.2.3 Scanning Electron Microscopic Studies of Lignocellulosic Wastes 83

4.5 ANALYTICAL EVALUATION OF FACTORS AFFECTING CELLULASE PRODUCTION BY *ASPERGILLUS NIGER* RK-3 83

4.5.1 Analysis of Physical and Biological Factors 83
4.5.2 Analysis of Chemical Factors 86

4.5.2.1 Analysis of carbon sources 86
4.5.2.2 Effect of nitrogen sources 90
4.5.2.3 Effect of phosphorus sources 90
4.5.2.4 Effect of phenolic compounds 92
4.5.2.5 Effect of organic acids 95
4.5.2.6 Effect of soluble sugars 95

4.6 PRODUCTION OF ENZYMES IN THE FINALLY DEFINED MEDIUM (R6m) 97

4.7 MUTAGENESIS OF *ASPERGILLUS NIGER* RK-3 FOR IMPROVED CELLULASE PRODUCTION 101

4.7.1 Single Step Mutagenesis 102
4.7.1.1 Ultraviolet mutagenesis 102
4.7.1.2 Chemical mutagenesis 103

4.7.1.2.1 Production of cellulases with mutants obtained after single mutagenesis 103

4.7.2 Mixed Chemical Mutagenesis 106
4.7.2.1 Production of cellulases with mutants obtained after mixed mutagenesis 109

4.7.2.2 Evaluation of cellulase production by mutant UNSC-442 with treated cellulosic waste materials 113

4.7.3 Analysis of Stability and Morphological Features of Mutant UNSC-442 116

4.8 EVALUATION OF CELLULASE PRODUCTION UNDER CO-CULTIVATION CONDITIONS WITH STRAINS *ASPERGILLUS NIGER* RK-3, MUTANT UNSC-442 AND *TRICHODERMA REESEI* MTCC-164 119

4.8.1 Analysis of Substrate Concentration for Cellulase Production 122
4.8.1.1 Evaluation of Sugarcane Bagasse 123
4.8.1.2 Evaluation of *Eicchornia crassipes* Biomass 126

4.9 APPLICATIONS OF CELLULOSE BIOCONVERSION TECHNOLOGY 129

4.9.1 Use of Cellulose Hydrolyzates for the Production of Baker's Yeast 129
4.9.2 Use of Cellulose Hydrolyzates for Gluconic Acid Production 131
4.9.3 Use of Cellulose Hydrolyzates for Citric Acid Production 131

CHAPTER 5 DISCUSSION 135

5.1 EVALUATION OF NATURAL LIGNOCELLULOSIC MATERIALS AS THE SUBSTRATES 138

5.1.1 Cellulase Production with Treated Cellulosic Materials 140

5.2 ANALYSIS OF FACTORS AFFECTING CELLULASE PRODUCTION 142
5.3 STRAIN IMPROVEMENT BY MUTAGENESIS 147

5.3.1 SDS- PAGE Analysis of Cellulase Components 149

5.4 ANALYSIS OF CO-CULTIVATION CONDITIONS FOR CELLULASE PRODUCTION 150
5.5 APPLICATIONS OF CELLULOSE BIOCONVERSION 153

CHAPTER 6 SUMMARY AND CONCLUSIONS 155

REFERENCES 161

LIST OF PUBLICATIONS

ACKNOWLEDGEMENT

In a venture like the present one so many people contribute in so many ways that it is quite impossible to list their names, not to mention the extent of their contribution.

I am short of word to express my gratefulness and sincerest regards to my supervisor **Dr. R.P. Singh**, Assistant Professor, Department of Biosciences and Biotechnology, University of Roorkee, for his immense help, keens interest, invaluable guidance and constant encouragement throughout the course of this work. He has been constant source of inspiration not only for me but also perhaps for everyone who has come in his contact. His analytical approach to my every problem has given me moral support in my moment of desperation. I consider myself quite fortunate for having the opportunity to work under his able supervision.

I take this opportunity to express my deep sense of gratitude to **Dr. Ben. M.J. Pereira**, Associate Professor and Head, Department of Biosciences and Biotechnology, University of Roorkee, for providing the necessary infrastructure facilities to carry out this work.

I also extend my sincerest thanks to all the faculty members of the Department of Biosciences and Biotechnology, University of Roorkee, for their kind support all through the duration of this work.

I also wish to express my sincere thanks to **Prof. D.K. Maheshwari**, Department of Botany, Unit of Microbiology Gurukul Kangri University, Hardwar, for his valuable suggestions with regards to the present work and his immense help throughout the course of this study.

I am highly obliged to **Dr. P. Kaushik**, Reader, Department of Botany, Unit of Microbiology, Gurukul Kangri University, Hardwar, for providing me a strain of *Trichoderma reesei* MTCC-164 as well as his timely advice and moral courage.

I am also thankful to **Prof. Ajit Varma**, School of Life Sciences, JNU, New Delhi, for providing me opportunity to learn some standard technique and protocols regarding to my work.

I have been lucky enough to have been blessed with my lab colleagues like **Mr. Omvir Singh**, **Mr. C.S.K. Reddy**, **Ms. Ruma Ganguly** and **Amit Sharma**, who

have constantly helped with their valuable suggestion and friendly guidance, without their cooperation it is quite difficult to complete this work.

I would like to extent thanks to my well-wishers specially **Sri. Daya Ram Bharti**, Department of Mechanical engineering, University of Roorkee and **Sri. Budh Singh**, Administrative Block, University of Roorkee, for their immense help and encouragement throughout the duration of this work.

My special thanks to my senior and friends specially **Dr. Jitendra Singh**, Lecturer, Department of Microbiology, S.B.S.(P.G.) Institute of Biomedical Sciences and Research, Balawala, Dehradun, **Mr. Inderjit Singh**, NPL, New Delhi, **Mr.Anand Bharti, Mrs. Chandrika Bharti, Mr. Anil Kumar, Mr. Pusham Kumar, Mr. Rajesh Kumar, Mr. Ompal Singh, Mr. Jashwant Singh Rathor, Mr. Vinod Kumar Dolkhe** and **Mr. Pradeep Kumar Verma** for their immense help, encouragement and providing me the much needed moral support. My special thanks to **Mr. Kapil** for his efficient word processing and graphics.

My wife **Mrs. Rupa** has been a constant companion for the last two year. Her interest and enthusiasm in my research work was a big support for me. Inspite for her own demanding work, she still found time to provide me the moral courage and determination to bring this work to fruition. I owe a lot to her

I also appreciate the cooperation of the official and technical staff specially, **Sri. Vedpal Singh Saini** and **Sri. Riazkalam** of the Department of Biosciences and Biotechnology, University of Roorkee.

I am highly indebted to my father **Sri. Mamraj Singh**, mother and brother **Mr. Pawan Kumar** as without their persuasion and cooperation, I might have failed to complete this work.

Financial assistance by the University Grant Commission, Government of India is gratefully acknowledged.

(RAJ KUMAR)

LIST OF FIGURES

Figure No.	Particulars	Page No.
Fig. 1	Chair configuration of cellulose molecule.	8
Fig. 2	Cross-sectional structure of cellulosic microfibrils	10
Fig. 3	Production of CMCase, FPase β-glucosidase and total soluble proteins using commercially available synthetic cellulosic substrates.	70
Fig. 4	X-ray diffractographic analysis representing the crystallinity of synthetic cellulosic substrates.	71
Fig. 5	Adsorption behavior of cellulase proteins and relative saccharification of the synthetic cellulosic substrates at 50°C.	73
Fig. 6	X- ray diffractographic analysis representing crystallinity of sugarcane bagasse , *Eicchornia crassipes* biomass.	77
Fig. 7	Production of cellulases and relative cellulose degradation by *Aspergillus niger* RK-3 with treated and untreated sugarcane bagasse.	79
Fig. 8	Production of cellulases and relative cellulose degradation by *Aspergillus niger* RK-3 with treated and untreated *Eicchornia crassipes* biomass.	80
Fig. 9	Adsorption behavior of cellulase proteins and the relative saccharification of sugarcane bagasse and *Eicchornia crassipes* biomass	82
Fig. 10	Scanning electron micrographs of lignocellulosic substrates before and after the steam-alkali treatments.	84

Fig. 11	Scanning electron micrographs representing colonization of fungal growth on treated and untreated lignocellulosic substrates.	85
Fig. 12	Production of cellulases by *Aspergillus niger* RK-3 as a function of pH with treated cellulosic substrates.	87
Fig. 13	Production of cellulases by *Aspergillus niger* RK-3 as a function of temperature and agitation with treated substrates.	88
Fig. 14	Production of cellulases by *Aspergillus niger* RK-3 as a function of inoculum level with treated cellulosic substrates.	89
Fig. 15	Production of cellulases by *Aspergillus niger* RK-3 as a function of carbon source concentration with treated cellulosic substrates.	89
Fig. 16	Effect of nitrogen sources on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass .	91
Fig. 17	Effect of phosphorus sources on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass .	93
Fig. 18	Effect of phenolic compounds (0.05%) on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass as the substrates.	94
Fig. 19	Effect of organic acids (0.1%) on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass as the substrates.	96
Fig. 20	Effect of sugars (0.2%) on cellulase production by	96

Aspergillus niger RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass as the substrates.

Fig. 21 Production of cellulases and relative cellulose degradation by wild type *Aspergillus niger* RK-3 at different time intervals with treated sugarcane bagasse and *Eicchornia crassipes* biomass as the substrates. 99

Fig. 22 Production of cellulases by the mutants derived by physical or chemical mutagenesis of the wild type *Aspergillus niger RK*-3. 107

Fig. 23 Screening of the end product resistant mutants 111

Fig.24 Production of cellulases by mutant strains derived after mixed mutagenesis. 112

Fig. 25 Production of cellulases and relative cellulose degradation by mutant UNSC-442 with treated sugarcane bagasse and *Eicchornia crassipes* biomass with the defined R_6m medium. 114

Fig. 26 SDS-PAGE analysis of proteins produced by wild type *Aspergillus niger* RK-3, mutants RKU-40 and UNSC-442 after 18 days of incubation. 117

Fig. 27 Zymogram analysis of cellulase components using the native polyacrylamide gel 118

Fig. 28 Stability analysis of the mutant UNSC-442 for enzymatic production. 120

Fig. 29 Scanning electron micrographs of 72 h old mycelia of the wild type *Aspergillus niger* RK-3 and the mutant UNSC-442 and the pattern of sporulation from the wild type *Aspergillus* 121

niger RK-3 and the mutant UNSC-442 strain.

Fig. 30 Effect of substrate concentration on cellulase production in Semi-solid state fermentation using treated sugarcane bagasse and *Eicchornia crassipes* biomass as the substrates after 18 days of incubation at 32°C. 124

Fig. 31 Effect of substrate concentration on total soluble proteins and dry mycelial mass in semi-solid state fermentation using sugarcane bagasse and *Eicchornia crassipes* biomass after 18 days at 30°C. 125

Fig. 32a Production of CMCase, FPase and β-glucosidase by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi-solid state fermentation using treated sugarcane bagasse. 127

Fig. 32b Production of soluble proteins and dry mycelial mass by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi solid state fermentation using treated sugarcane bagasse. 127

Fig. 33a Production of CMCase, FPase and β-glucosidase by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi-solid state fermentation using treated *Eicchornia crassipes* biomass. 128

Fig. 33b Production of soluble proteins and dry mycelial mass by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi solid state fermentation using treated *Eicchornia crassipes* biomass. 128

LIST OF TABLES

Table No.	Particulars	Page No.
1	Major components of agrocellulosic waste materials.	7
2	Major lignocellulosic pretreatment and sugar yields.	13
3	Chemical pretreatments of lignocellulosic materials.	15
4	Screening of fungal strains isolated from decomposed bagasse (sugarcane industry waste).	67
5	Screening of bacterial strains isolated from decomposed bagasse (sugarcane industry waste) and the paper waste.	67
6	Analysis of enzymatic production by selected strains isolated from decomposing waste materials.	68
7	Composition of sugarcane bagasse and *Eicchornia crassipes* biomass lignocellulosic waste materials.	75
8	Defined set of conditions and factors for the medium designated as R_6m.	98
9	Analysis of variance for cellulase, protein production and relative cellulose degradation before and after optimization of production medium.	100
10	Single step mutagenesis of *Aspergillus niger* RK-3 by ultraviolet radiation.	104
11	Single step mutagenesis of *Aspergillus niger* RK-3 by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) treatment.	104

12	Single step mutagenesis of *Aspergillus niger* RK-3 by sodium azide treatment.	105
13	Single step mutagenesis of *Aspergillus niger* RK-3 by colchicine treatment.	105
14	NTG mutagenesis of RKU-40 and RKU-45 mutant (UV treated) strains.	108
15	Sodium azide mutagenesis of UN-440 and UN-445 mutant (UV-NTG treated) strains.	108
16	Colchicine mutagenesis of UNS-442 and UNS-443 mutant (UV-NTG-Sodium azide treated) strains.	110
17.	Analysis of variance for cellulase, protein production and relative cellulose degradation by wild type *Aspergillus niger* RK-3 and its mutant UNSC-442.	115
18	Cultivation of *Saccharomyces cerevisiae* (baker's yeast) on enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass.	130
19	Production of gluconic acid by enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass by *Aspergillus niger* ORS-4.	132
20	Production of citric acid by enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass by *Aspergillus niger* KCU-5.	133

LIST OF ABBREVIATIONS

Å	-	Angstrom
°C	-	Degree Centigrade
CBDs	-	Cellulose – Binding-Domains
CBH-I	-	Cellobiohydrolase - I
CBH-II	-	Cellobiohydrolase-II
CMC	-	Carboxymethylcellulose
CMCase	-	Carboxymethylcellulase
CD	-	Catalytic-Domain
CAM	-	Cellulase Activator Molecule
CQOase	-	Cellobiose-Quinone-Oxidoreductase
DNS	-	Dinitrosalicylic acid
DEAE-Cellulose-		Diethylaminoethyl-cellulose
EG	-	Endoglucanase
FAO	-	Food and Agriculture Organization
FPase	-	Filter Paper Cellulase
g	-	gram
Jm^{-2}	-	Joule per Meter Square
kD	-	Kilo Dalton
LMM	-	Low Molecular Mass
MUC	-	4-Methylumbelliferyl β-D-cellobioside
MUG	-	4-Methylumbelliferyl β-D-glucoside
MTCC	-	Microbial Type Culture Collection
NTG	-	N-methyl-N'-nitro-N-nitrosoguanidine
p-NPG	-	p-Nitrophenyl β-D-glucopyranoside
PDA	-	Potato Dextrose Agar
p.s.i.	-	Per Square Inch
PAGE	-	Polyacrylamide Gel Electrophoresis

SSF	-	Solid-state Fermentation
SSSF	-	Semi-Solid Sate Fermentation
SDS	-	Sodium -Dodecyl Sulfate.
UV	-	Ultra Violet
V	-	Volt
W	-	Watt

Chapter 1

INTRODUCTION

INTRODUCTION

The growing need for energy, food, chemicals, coupled with shortage of fossil fuel and due to the ever present problems related to waste disposals, attention has been focused towards the renewable lignocellulosic waste materials. Lignocellulosic wastes can be used for generation of fermentable sugars, may prove to be a potential replacement for fossil hydrocarbons, thus may be a promising source for fuel and industrially significant chemicals such as organic acids, alcohol, feed stock and many others. (Monem et al. 1984; Vatsala, 1989; Kuhad and Singh, 1993). Lignocelluloses as the agricultural, industrial and forest residues account for the majority of the total biomass present in the world. Around 10 to $50x10^9$ tons of this potential biomass accumulate every year which remain largely unutilized thus causing a loss of this potentially valuable resource material (Bisaria and Ghose, 1981; Singh et al. 1988a; Kuhad and Singh, 1993).

Cellulose is a linear homopolymer consisting of the β-1,4 D-glucopyranosyl units assembled with varying degree of polymerization. These thread like bundles of the molecules are established laterally by hydrogen bonding between hydroxyl group of the adjacent molecules (Stone et al. 1969). Cellulose molecules are linked together to form elementary fibrils or protofibrils about $40A^0$ wide, $30A^0$ thick and $100A^0$ long. These elementary fibrils are aggregated into long slender bundles called microfiblrils (Lakhani, 1990).

The major obstacle in the lignocellulose utilization is, its chemically unreactive nature and in particular its resistance to hydrolysis due to the crystallinity of cellulose, lignin content, pore size, moisture content and the

availability of surface area all of which critically affect the susceptibility of the substrate for enzymatic hydrolysis. A wide spectra of pretreatments employing steam or chemicals e.g. acids, alkali or organic solvents have been investigated for effective hydrolysis (Weil et al. 1994). Very few of these have been developed sufficiently to be called technologies and most are at the laboratory scale only. The variety of conversion is truly enormous, its likely that pretreatment which is effective for one raw material may not be equally effective or appropriate for the another. Treated lignocellulosic materials are next subjected for enzymatic hydrolysis by cellulases produced from different microbial sources. A variety of microorganisms including bacteria and fungi have the ability to degrade cellulose to sugar monomers but in limited quantities (Lakhani, 1990). *Trichoderma* strains among the cellulolytic organisms are well studied, apart from these species many other fungal strains e.g. *Fusarium oxysporium, Neurospora crassa, Chaetomium globosum, Penicillium purpurogenum, Aspergillus niger* etc. produce cellulases (Steiner et al. 1993; Vipan et al. 1994; Menon et al. 1994; Kuhad et al. 1994). The cellulases from *Aspergillus* usually have high β-glucosidase but lower endoglucanase activity, whereas, *Trichoderma* has higher endo/exoglucanase but lower β-glucosidase and hence is limited in its ability for cellulose bioconversion (Madamwar and Patel, 1992; Kuhad et al. 1994; Takashima et al. 1998).

The available lignocellulosic raw materials in India include wheat husk, wheat straw, rice husk, rice straw, sugarcane bagasse, coconut coir pith, wood dust, corncobs and groundnut shells (Rajendran et al. 1994). In addition to these many of the naturally occurring plants, grasses, growing wildly are of considerable

significance. Two major lignocellulosic waste materials with high cellulose content that are available in abundance in and around this region are sugarcane bagasse and the water hyacinth (*Eicchornia crassipes*) biomass. The sugarcane bagasse is a major waste material generated from the sugarcane processing that remains unutilized and mostly burnt causing environmental pollution, *Eicchornia crassipes* is a water born aquatic biomass found in large quantities in the ponds, lakes and rivers. This biomass with no potential application, has a continuous and unabated growth in the aquatic system causing therefore a serious threat to the water resources. These two biomass materials have primarily been selected for bioconversion studies. The present work embodies the details of the approaches undertaken for bioconversion of sugarcane bagasse and the *Eicchornia crassipes* biomass into fermentable sugars. The major outlines of the present investigation are defined as below:

* Microbial strains from decomposing substrates were isolated and evaluated for cellulase production using the synthetic cellulosic substrates. The strain producing maximum level of cellulases was selected and identified as *Aspergillus niger* RK-3.
* Two major lignocellulosic materials i.e. sugarcane bagasse and *Eicchornia crassipes* biomass were analyzed as substrates for cellulase production. Substrates were steam-alkali treated and various physico-chemical, biological factors, nutrients and other regulators were studied to derive the conditions for maximum cellulase production.

* In an attempt to improve the bioconversion the naturally occurring wild type isolate was genetically manipulated by physical, chemical and mixed mutagenic treatments for obtaining a high cellulase producing mutant capable of utilizing the lignocellulosic waste materials.
* Analysis of the morphological features of the wild type and the best cellulase producing mutant strain by scanning electron microscopy.
* Evaluation of co-cultivation conditions using the wild type and mutant strains along with *Trichoderma reesei* for cellulase production under semi-solid state fermentation
* Use of cellulose hydrolyzates for the production of materials of commercial significance.

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The cost of many important fermentation products depends mainly on the cost of the carbohydrate raw materials. The future scope of the fermentation biotechnology industry therefore depends largely on the cost of its carbohydrate raw materials. Lignocellulosic bioconversion is a potential process for the production of less expensive fermentable sugars (Dale, 1987). Generally, fermentable sugar such as glucose is the precursor for most of the organic acids e.g. gluconic, citric, itaconic acid etc. and also play a key role in the production of alcohol, solvents and other products of commercial significance. Lignocellulosic residues mainly from the agricultural and forestry sectors are the major carbohydrate sources and have potential for its bioconversion to fuel ethanol and to a variety of other commodity products (Singh et al. 1991).

2.1 NATURAL OCCURRENCE OF LIGNOCELLULOSIC MATERIALS

Cellulose is primarily synthesized by all higher plants in enormous amounts thus making it as a most abundant biopolymer on the earth. This has focused further attention on cellulose as a potential replacement for fossil hydrocarbon, as a source of fuel and the industrially significant products (Monem et al. 1984; Vatsala, 1989; Kuhad and Singh, 1993).

Lignocellulosic biomass as wastes accumulate every year in large quantities that remain largely unutilized thus causing a loss of this valuable resource material (Singh et al. 1988b). The biomass in the form of cellulose, hemicellulose and lignin provides a means of collecting and storing the solar energy and hence represent as

an important energy and the material resource. Lignocelluloses as agricultural, industrial and forest residues account for the majority of the total biomass present in the world and around 10 to 50 x 10^9 tons of this potential biomass accumulate per year. Agricultural resources of the lignocellulosic wastes are quite abundant. As estimated by Food and Agriculture Organisation (FAO), around 2.9 x 10^3 million tons from cereal crops, 1.6 x 10^2 million tons from pulse crops, 1.4 x 10^2 million tons from oil seed crops and 5.4 x 10^2 million tons from plantation crops of this lignocellulosic materials are produced annually world wide and of these, the major amount is generated in Asia (Rajaram and Verma, 1990). A major share (around 60%) of the plant materials occur in forests, whereas, 15% of these are generated from the cultivated grasslands. The various types of lignocellulosic raw materials generated include wheat straw, rice straw, corncobs, cornstems and pea husk etc. have varying amounts of cellulosic components (Table 1). In addition to this, many of the naturally occurring plants and grasses are of considerable significance. A number of pulse crops, sugarcane bagasse, distillery grapes stock, city piles and the wastes generated from the paper industry contain substantial amount of cellulose.

Table 1 Major components of agrocellulosic waste materials (Monem et al. 1984)

Wastes	Component (%)			
	Moisture	Hemicellulose	Cellulose	Protein
Artrichoke waste	3.56	36.46	22.70	4.20
Corncobs	2.80	32.90	27.15	3.91
Cornstems	3.98	14.70	53.16	3.68
Cottonstems	3.69	11.60	40.22	2.43
Pea husk	2.45	19.30	45.33	2.45
Ricestraw	2.99	40.61	26.39	3.69
Sugar beet pulp	9.25	16.20	55.70	6.25
Wheat straw	2.96	25.92	38.40	3.32

2.2 MOLECULAR STRUCTURE OF CELLULOSE

The observations of Cowling and Kirk (1976) revealed that cellulose is a linear homopolymer composed of β-1,4 D-glucopyranosyl units, long chains of these units assemble with varying degree of polymerization. The threads like bundles of these molecules are held laterally by hydrogen bonding between hydroxyl group of adjacent molecules. The cellulose chains adopt a chair configuration with hydroxyl groups in the equatorial and the hydrogen atoms in the axial positions (Fig. 1). Liang and Marchessault, (1959) proposed that every chain unit is rotated at 180° around the main axis resulting in an unstrained linear configuration with minimum steric hinderance. The hydroxyl group on the 3rd position is bound by an intramolecular hydrogen bonding to the ring oxygen atom of the next chain unit. The glycosidic linkage acts as a functional group and that

Fig. 1 Chair configuration of cellulose molecule

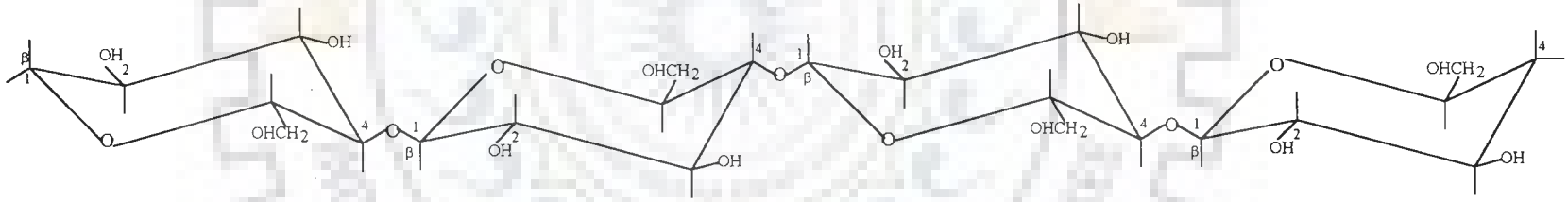


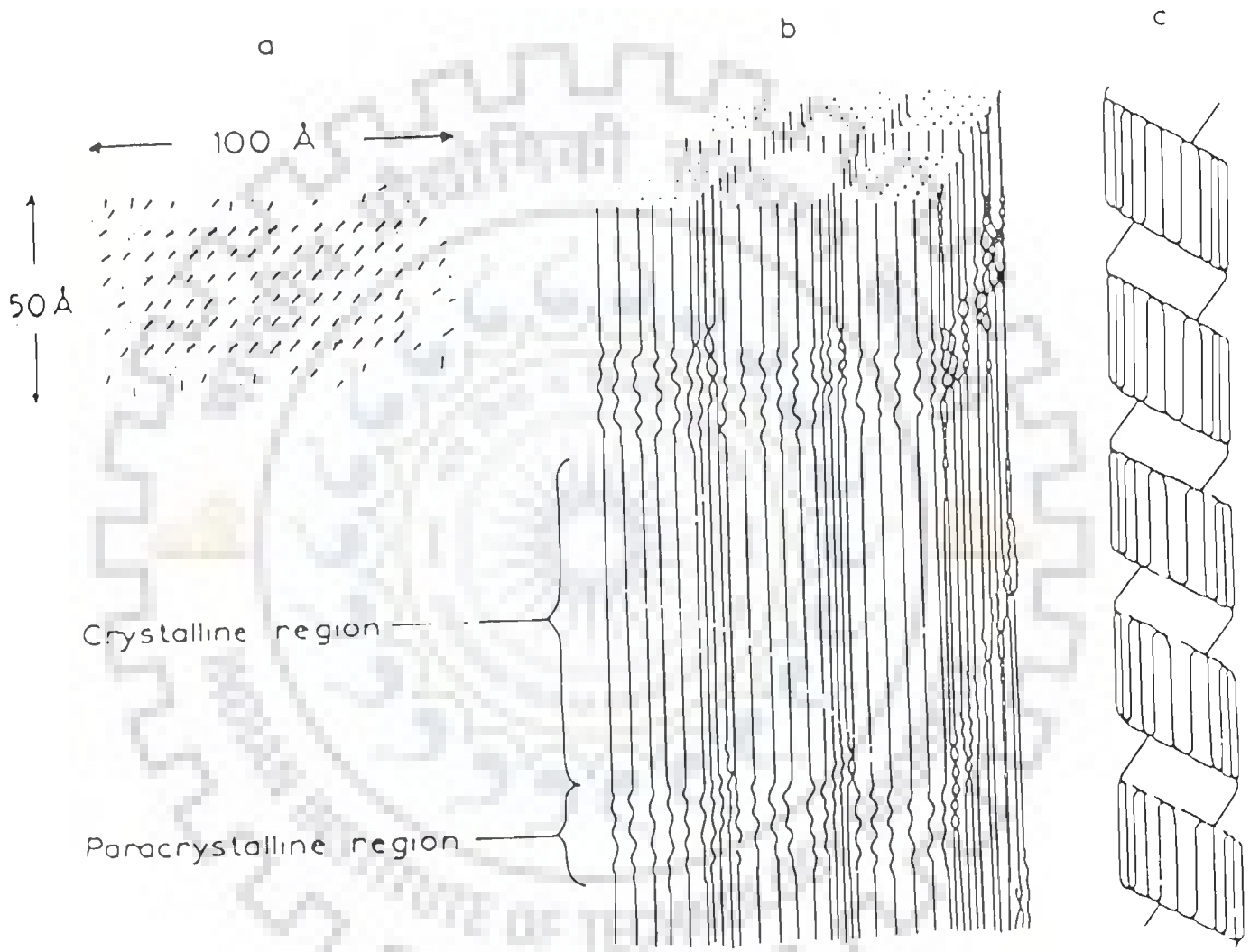
Fig. 1

along with the hydrogen group are the main determinants for the chemical properties of cellulose.

The study towards cross-sectional substructure of cellulose microfibrils has not been classified for all the organisms. One of the proposed models suggests that microfibrils are composed of thinner elementary fibrils and the crystalline portion of the fibrils is separated by para-crystalline regions. Further studies have revealed that cellulose molecules are linked together to form elementary fibrils or proto fibrils about 40 Å wide, 30 Å thick and 100 Å long, in which the polymer chains are oriented in a parallel alignment and firmly bound together by numerous hydrogen bonds. These elementary fibrils are aggregated into long slender bundles called microfibrils. Cowling (1975) has also proposed and reviewed the microfibrillar structure of the cellulose and concluded that the microfibril is about 50 x 100 Å in cross section and consists of a crystalline core of highly ordered sheath. Rowland and Roberts (1972) had described that microfibrils at certain length contain strain–distorted tilt and twisted region that are easily accessible for hydrolysis. Gardener and Blackwell (1974) had stated that cellulose molecules are linear and form intramolecular and intermolecular hydrogen bonds, the intermolecular bonds help to maintain the rigidity of the cellulose chain, whereas intramolecular bonds keep the cellulose chains in a tight and closely packed arrangement. This type of arrangement particularly refers to the crystalline region of the cellulose (Fig. 2). The less ordered region is called as the para-crystalline or

Fig. 2 **Cross-sectional structure of elementary fibrils of cellulosic materials**

a, size of microfibrils
b, crystalline and paracrystalline regions of microfibrils
c, protofibrils


a
b
c
100 Å
50 Å
Crystalline region
Paracrystalline region

amorphous region and the amorphous region of microfibrils is more susceptible for hydrolysis (Lakhani, 1990).

2.3 CRYSTALLINITY OF CELLULOSE

Cellulose exists in several crystalline forms (cellulose-I-IV) with different X-ray diffraction patterns and spectra (Blackwell, 1982). Of these four forms of celluloses, the cellulose I is the native form and occurs in plant cell walls, cellulose II is a regenerated form of cellulose obtained by mercerization in solid state or by dissolution. The crystalline form is highly resistant to microbial and enzymatic degradation while amorphous cellulose is hydrolyzed much faster.

The rate of enzymatic hydrolysis of cellulose is greatly affected by its degree of crystallinity. The crystallinity of cellulosic materials can be studied by X-ray diffraction pattern (Segal et al. 1559). The intensity of the 002 interference and the amorphous scatter at 2θ = 21-23^{o} was measured and defined as X-ray crystallinity index. Dunlap et al. (1976) had analyzed the relationship between the cellulose crystallinity and it's digestibility by cellulases. Cellulases degrade readily the accessible amorphous regions of regenerated cellulose but are unable to attack the less accessible crystalline regions (Norkrans, 1950; Walseth, 1952). Caulfied and Moore (1974) measured the degree of crystallinity of the ball milled cellulose before and after partial hydrolysis and observed that mechanical action (ball milling) increases the susceptibility of both the amorphous and crystalline components of cellulose.

2.4 PRETREATMENT OF LIGNOCELLULOSIC MATERIALS

The major obstacle in the effective lignocellulose utilization is its crystalline unreactive nature and in particular its resistance to hydrolysis. A wide spectra of pretreatments have been investigated for hydrolysis and only few of these have been developed sufficiently to be called as technologies (Bisaria and Gosh, 1981; Arora and Sandhu, 1986 and Kim et al. 1988). Among the various barriers that exist in effective hydrolysis of lignocelluloses, the lignin-hemicellulose matrix, physico-chemical nature of cellulose, crystallinity and its surface area are particularly important. Although a particular factor may predominant in a given lignocellulosic material, all of the factors mentioned are likely to exert some effect. A variety of pretreatment procedures have been evaluated for their effectiveness towards cellulose degradation (Table 2) and possibly the suitability of pretreatment procedure may vary depending on the raw material selected. It is therefore appropriate to evaluate the effect of a given pretreatment procedure on all the constituents of the material so as to maximize the production levels.

2.4.1 Chemical Treatment

Different chemical pretreatments that are generally practiced (Table 3) use sodium hydroxide, perchloric acid, paracetic acid, acid hydrolysis using sulfuric and formic acids, ammonia freeze explosion and organic solvents e.g. n-propylamine, ethylenediamine, n-butylamine etc. are also used (Tanaka et al. 1985; Singh et al. 1988c; Singh et al. 1989a; Singh et al. 1990; Maheshwari et al. 1992;

Weil et al. 1994). Besides the above the steam or acid/alkali-steam pretreatments had also been found suitable.

Table 2 Major lignocellulosic pretreatments and sugar yields (Dale, 1987).

Pretreatment	Raw material	Maximum sugar yield ($gm.\ gm^{-1}$ dry solids)
Ball milling	Wheat straw	0.26
Compression milling	Poplar	0.50
Cryomilling	Newspaper	0.44
Auto-hydrolysis	Monterey pine	0.14
Steam explosion	Aspen	0.44
Ammonia freeze explosion	Aspen	0.45
Ozone	Cotton straw	0.31
Paracetic acid	Wheat straw	0.25
Sodium hydroxide	Wheat straw	0.29
Sulfuric acid	Ryegrass straw	0.30

2.4.2 Physical Treatment

Physical methods of pretreatments like ball milling, compression milling, cryomilling or attrition milling and steam treatment using poplar, wheat straw, newspaper, oat straw etc. (Forsberg et al. 1986; Dale, 1987; Rivers and Emert, 1987; Smith and Wood, 1991; Weil et al. 1994) reduce particle size thereby

increasing the available surface area and make cellulosic substrates susceptible to enzymatic attack. Steam explosion loosen the cellulose-hemicellulose-lignin complex in lignocellulosic substrates and also removes the pentoses as well as increases the surface area. However, the draw back of the process is due to the generation of certain inhibitor during enzymatic hydrolysis (Hartree et al. 1987).

2.4.3 Biological Delignification

Biological delignification is another interesting and alternative pretreatment, which utilizes white rot fungi that selectively, degrades lignin, leaving behind the carbohydrates. The biological delignification of paddy straw, corn (*Zea mays*), sugarcane bagasse and aspen wood has been attempted by *Cyathus* sp, *Streptomyces viridosporus*, *Phelebia tremellosus*, *Pleurotus florida* and *Peurotus cornucopiae* strains respectively (Hartree et al. 1987; Deobald and Crawford, 1987; Kuhad and Johri, 1992; Chaudhary et al. 1994). Such processes have potential advantages such as require low capital cost, low energy input and lead to high yields without generating polluting byproducts. However, the long treatment time required and degradation of the residual carbohydrates are some of the draw backs of such processes.

Table 3 Chemical pretreatments of lignocellulosic materials.

Chemical treatment	Lignocellulosic substrate	Reference
n-Butylamine, Sulphuric acid	Rice straw	Tanaka et al. (1985) Abbi et al. (1996)
Ozone	Wood pulp	Neely, (1984)
Sodium hydroxide	Sulfite pulp, printed news paper, mixed waste paper, BRAM (municipal solid wastes)	Doppelbauer et al. (1987).
Sodium hydroxide, paracetic acid, sodium chlorate	Wheat straw	Dale, (1987); Doppelbauer et al.(1987); Maheshwari et al. (1992); Singh et al. (1989a, 1990)
Sodium hydroxide, sodium chlorate, paracetic acid, sulphuric acid.	Sugarcane bagasse, corncobs and groundnut shells	Olivier and Toit, (1986). Singh et al. (1988c); Singh et al. (1990); Smith and Wood, (1991).
Sulphuric acid	Rice husk, sawdust	Sharma and Sahgal, (1982)
Sodium hydroxide	Corncobs	Singh et al., (1989b)
Sulphuric acid, ammonia freeze	Aspen wood , rye grass straw	Dale, (1987)

2.5 SOURCES OF CELLULOLYTIC ENZYMES

The search for potential sources of cellulolytic enzymes is the basis for successful bioconversion of the lignocellulosic materials. A variety of microorganisms of bacterial and fungal origin may have ability to degrade the cellulosic substrate to glucose monomers but only few microorganisms can produce the cellulases in appreciably high levels (Lakhani et al.1990). In addition, some microorganisms secrete either endoglucanase or the β-glucosidase (components of cellulase complex). Only those organisms, which produce notable levels of

endo/exoglucanase and β–glucosidase, would effectively be able to degrade the native cellulose. It was observed earlier that few strains of *Trichoderma* produced an extracellular cellulase complex degrading the native cellulose (Wojtczak et al. 1987) since then, many microorganisms have been isolated but only a few have been shown to produce adequate levels for their meaningful utilization (Lakhani et al. 1990). As observed, all components of extracellular cellulase complex (endoglucanase, exoglucanase and β-glucosidase) are essential for cellulose hydrolysis and in general β-glucosidase that catalyses cellobiose hydrolysis, a reaction product of cellulose degradation by endo and exoglucanase, is either lacking or present in relatively small proportions in the extracellular cellulase complex. Thus, the sugars that are the end product of hydrolysis decrease, since, cellobiose inhibits the endo and exoglucanases (Bisaria and Ghosh, 1981). One of the ways to meet this deficiency is to add β-glucosidase to the reaction mixture containing other cellulase components. Another approach can be the design of a suitable bioreactor in which cellobiose is removed from the reaction mixture and treated in a separate reactor to yield glucose.

2.5.1 Fungal Origin

The decay of lignocellulosic material by cellulolytic fungi is of great significance in our ecosystem. Not surprisingly the fungal cellulases have been the subject of major investigations over the years. The major interest in fungal cellulases stem from the fact that several fungi produce extracellular cellulases in significant amounts. Like bacterial cellulases, fungal cellulase too have synergistic

action involving endoglucanase, exoglucanase and β-glucosidase for cellulosic hydrolysis (Wojtczak, 1987).

Apart from the cellulolytic fungus *Trichoderma viride*, many other fungi produce cellulases and degrade the treated cellulosic materials or the soluble cellulose derivatives e.g. carboxymethylcellulose but are not effective to the crystalline cellulosic substrates. Besides the *Trichoderma viride* the other mesophilic strains producing cellulases are *Trichoderma reesei*, *Fusarium oxysporium*, *Bortryodiplodia theobromal*, *Geotrichum candidum*, *Pellicularia filamentosa*, *Penicillium funiculosum*, *Penicillium rubrum*, *Penicillium purpurogenum*, *Neurospora crassa*, *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Alternaria alternata*, *Chaetomium globosum*, *Sclerotium rolfsii*-1644, *Myrothecium verrucaria*, *Mucor recemosus* and *Dictyostelium discoideum* have also been reported (Borgia and Sypherd, 1977; Parish, 1977; Eberhart et al. 1977; Yadav et al. 1988; Aikane and Patil, 1989; Macris et al. 1989; Steiner et al. 1993; Vipan et al. 1994; Menon et al. 1994; Kuhad et al. 1994; Kim et al. 1997; Sineiro et al. 1997; Takashima et al. 1998; Ju and Afolabi, 1999, Breen and Singlton, 1999). The cellulases from *Aspergillus* usually have high β-glucosidase but lower endoglucanase levels, whereas, *Trichoderma* has higher endo and exoglucanase components but lower β-glucosidase levels (Ryu and Mandels, 1980; Duff et al. 1986) hence has limited ability towards cellulose hydrolysis. Among the thermophillic fungi such as *Sporotrichum thermophile*, *Thielavia terrestris*, *Thermoascus aurantiacus*, *Myceliophthora fergusii*, *Actinomycetes*, *Chaetomium thermophile*, *Humicola fuscoatra*, *Humicola*

lanuginosa and *Thermonospora curvata* also produce the cellulase complex and can degrade native cellulose (Tanaka et al. 1985; Wojtczak et al. 1987; Stutzenberger, 1988; Anand and Vithayuthil, 1990; Gunju et al. 1990). Such thermophilic organisms may be the valuable sources of thermostable cellulases like that of cellulases from mesophilic strains.

2.5.2 Bacterial Origin

Bacteria also have the ability to produce cellulase complex aerobically as well as anaerobically. Some of the bacterial strains producing cellulases are *Rhodospirillum rubrum*, *Cellulomonas flavigena*, *Cellulomonas fimi*, *Clostridium stercorarium*, *Xanthomonas campestris*, *Clostridium thermocellum*, *Bacillus polymyxa*, *Bacillus licheniformis*, *Pyrococcus furiosus*, *Acidothermus cellulolyticus*, *Micrococus Sp* and *Erwina carotovora*. (Stewart and Leatherwood, 1976; Akhtar et al. 1988; Bronnenmeier and Staudenbauer, 1988; Whelan and Prembroke, 1989; Petit et al. 1990; Gough et al. 1990; Din et al. 1990; Baik and Pack, 1990; Saarilahti et al. 1990; Sexena et al. 1991; Shiang et al. 1991; Aparicio et al. 1994; Pons et al. 1995; Sakon et al. 1996; Fischer et al. 1996).

2.5.2.1 Novel bacterial cellulase system: cellulosome

Bacterial cellulases exist as discrete multi-enzyme complexes, called cellulosomes, that consist of multiple subunits that interact with each other and with the cellulosic substrates. One of these subunits represents a distinctive new class of non-catalytic scaffolding polypeptide that selectively integrates the various

cellulase or xylanase subunits into the cohesive complex. The cellulase system of the anaerobic cellulolytic bacterium *Clostridium thermocellum* was shown to consist of a discrete multifunctional, multienzyme complex (Lamed, 1983), which appeared to account for cellulose solubilization, hence was referred to as the cellulosome complex and is considered as one of the major molecular paradigms of bacterial cellulolysis.

The major components of cellulosome structure include: Cellulose-Binding Domain (CBD), that is associated with scaffoldin, which is responsible for integrating catalytic subunits into the cellulosome complex and catalytic components of the cellulosome: Cohesin, a subunit-binding domain of scaffoldin; Dockerin, docking domain of the catalytic subunit that interacts with a cohesin domain of the scaffoldin subunit; Linker, relatively short sequence that links the various domains of the cellulosomal subunits; Protubozyme, protuberance like cell surface organelle which contains multiple copies of the cellulosome and fibrous connective matrix. The genes responsible for the noncatalytic subunits of the cellulase systems of *Clostridium thermocellum* and *Clostridium cellulovorans* have recently been sequenced. Both genes encode large polypeptides of about similar size (1800 amino acid residues) and both the proteins have a single cellulose binding domain having striking homology with that of the cellulose binding domains found in other bacterial cellulases. Both proteins have nine distinct, but closely related domains that evidently interact with the other (catalytic) subunits to form the cohesive cellulosome structure. These characteristics indicate that both polypeptides may be classified as scaffoldins (Bayer, 1994).

2.6 MOLECULAR STRUCTURE OF CELLULASES

Cellulolytic enzyme system from the filamentous fungi especially *Trichoderma reesei* contains two exoglucanases or cellobiohydrolases (CBH_1 and CBH_2) and at least four endoglucanases (EG-1, EG-2, EG -3 and EG - 5) and one β-glucosidase. These enzymes act synergistically to degrade crystalline cellulose efficiently (Giorda et al. 1991) and have been shown to have two distinct domains: the cellulose binding domain (CBD) and catalytic domain (CD). This bifunctional organization with separate domains is a general feature of cellulose degrading enzymes produced by a number of cellulolytic microorganisms belonging to both bacteria and fungi. Since, CBD itself is unable to produce any reducing sugar from cellulose, it is believed that its role in cellulose degradation is to "target" the catalytic domain of the enzyme on cellulose to break β-1,4-glucosidic bonds. Some observations have indicated that the CBD is infect capable of causing non-hydrolytic disruption of cellulose fibers (Bisaria and Ghose, 1981). The main portion of the enzyme, the catalytic core, is linked through a heavily glycosylated linker region to a cellulose binding domain (CBD). CBH-I is probably the key enzyme in fungal cellulose degradation. The cellulolytic domains of CBH-I and EG-I show significant amino acid homology with 45% identity and have similar 3-D structures. The EG-I catalytic domain (371 residues) has the similar dimension as CBH-I and also has the two large antiparallel β-sheets forming a β-sandwhich (Pentilla et al. 1986). The sequence alignment of CBH-I and EG-I showed that four deletions in EG-I mapped to the tunnel forming loops in CBH-I, as a result, EG-I has its active site in a deep but open groove rather than a tunnel. The cellulose

binding domain in CBH-I, EG-I and EG-V is at the C-terminus, whereas in CBH-II and EG-II is at the N-terminus. The precise function of the CBD is not yet known but domains with similar function are also found in bacterial cellulases and in at least one mannase. The three dimensional structure of the cellulose-binding domain of *Trichoderma reesei* CBH-I has been determined by NMR spectroscopy, revealing a wedge-shaped molecule of 30 Å x 30 Å x 18 Å with triple-stranded antiparallel β-sheet as the major secondary structure (Tomme et al. 1996). The structural features include two distinct faces: rougher less hydrophilic face and a flat more hydrophilic face. Chemical modification of tyrosines in intact CBH-I causes almost complete loss of cellulose binding activity. Results from site-directed mutagenesis of intact CBH-I and from chemically synthesized CBD peptides attribute this effect to the three tyrosines in the flat face of the CBH-I cellulose binding domain.

2.7 MECHANISM OF ACTION

The cellulolytic enzymes are generally induced as the multienzyme systems consisting of five or more enzymes and have traditionally been divided into three classes: endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Knowles et al. 1987). The enzymatic degradation of cellulose into glucose by cellulolytic enzymes typically involves concerted and synergistic action of the following enzymes (Medve et al. 1998) :

(a) endoglucanases (EG) (endo β-1, 4 D-glucan-4 glucanohydrolase, EC 3.2.1.4), split internal β-1,4-glucosidic linkages,

(b) exoglucanases (cellobiohydrolases, CBH) (exo-β-1,4 D-glucan 4-cellobio-hydrolases, EC 3.2.1.91) which cleave cellobiose units from the ends of cellulose chain and

(c) β-glucosidase (EC 3.2.1.21) that hydrolyze cellobiose and cellodextrins, their action on cellodextrins, however, decreases as chain length increase.

The action of cellulases and the participating enzyme components have been the subject of numerous investigations. Studies done so far have indicated that endo β-1, 4 glucan glucanases attack at different sites along the chain causing rapid increase in the fluidity and enables generation of reducing groups during the reaction (Yadav. et al. 1988). The rate of cello-oligosaccharide hydrolysis increases with degree of polymerization within the limits of substrate solubility. The major end products of endoglucanases are mixture of transient cello-oligosaccharides, cellotriose, cellobiose and small quantities of glucose (Lakhani et al. 1990). Endoglucanases act with retention of configuration. Different endoglucanases exhibit different modes of attack on cellodextrins. Cellobiose is not hydrolyzed while cellotriose is hydrolyzed extremely slowly by most of the endoglucanases. Most endoglucanases preferably cleave second bond from the non reducing end of cellotetrose through bond one and bond three are also cleaved slowly (Kuhad and Singh, 1993). With cellopentose, the second and or third glucosidic bonds are cleaved yielding cellobiose and cellotriose, almost in equal amounts as principal products, indicating cleavage at bond two or bond three of the molecule. Alternatively the endoglucanase produced cellobiose and cellotriose from the reducing or non reducing end with equal ease (Gong et al.1977). Exo β-1,4

glucan glucanases had been identified to be having two major forms, (a) β-1,4 cellobiohydrolase-I (CBH-I), (b) β-1,4 glucan glucohydrolase-II/β-1,4 cellobio-hydrolase-II (CBH-II). CBH-I has the highest affinity for cellulose and show a synergistic effect when acting in concert with endoglucanases to solubilize the crystalline cellulose. The CBH-II removes the terminal D-glucopyranosyl units from the non reducing end of the chain and has the greater affinity to the cello-oligosaccharide of four to seven units. This enzyme does not act synergistically with endoglucanases in solubilization of cotton (Wood and McCrae,1982; Lakhani,1990). The β-1,4 glucosidase or cellobiase converts cellodextrins and cellobiose to fermentable sugar (i.e. glucose). It is suggested that endoglucanases cause random nicks in the amorphous regions of cellulose long chain polymer, releasing new chain ends followed by the removal of cellobiose by cellobiohydrolase. Some kind of synergism has strongly been indicated between endo and exoglucanases resulting in production of cellulose oligomers and cellobiose (Lakhani, 1990). In the last and the final step of cellulose hydrolysis the β-glucosidase brings about the degradation of cellodextrins and cellobiose to glucose.

2.8 ADSORPTION-DESORPTION OF CELLULASES

One of the significant aspect of cellulose hydrolysis is the adsorption of cellulolytic enzymes by the active components of the cellulosic substrates. A correlation between the adsorption of cellulases and the relative enzymatic hydrolysis of cellulose was observed by Klyosov et al (1986). It has been shown

that the available surface area of the cellulose polymer plays an important role for the interaction between the cellulases and the cellulose and is an essential step for the hydrolysis to proceed. Large surface area of the cellulose would render accessibility of the substrate for enzymatic adsorption and consequently its hydrolysis (Puri and Mamers, 1983). It has also been demonstrated that cellulose strongly adsorbs cellulases under optimal conditions for enzyme actions and the extent of adsorption is proportional to the initial cellulose concentration. It was observed that about 70% of the enzyme got adsorbed from the concentrated enzyme preparation (Halliwel, 1961; Mandels et al. 1971). As detected 50% of endo and exoglucanase and 80% of β-glucosidase was adsorbed on delignified bagasse and rice straw within 15 minutes of exposure of the substrates (Ghose and Bisaria, 1979; Goel and Ramachandran, 1983).

Analysis of various factors e.g. pH, ionic strength, temperature and surface area have indicated that maximum adsorption of cellulases on microcrystalline cellulose occurred at pH 4.8 at 50°C and Vanderwall's interaction is responsible for the adsorption phenomenon (Kim et al. 1988). However, Reinikainen et al. (1995) had reported that the maximum adsorption occurred at pH 6.5 and suggested that electrostatic repulsion between the bound proteins may regulate the levels of adsorption. The binding of enzyme with cellulose was significantly affected by high ionic strength suggesting that hydrophobic interaction may also contribute towards adsorption. Van-Wyk, (1997) had demonstrated that relative rate of adsorption and saccharification increases with increasing temperature and showed the increased adsorption at 60°C while enzyme activity decreased.

Analysis of the adsorption behaviour of cellobiohydrolases indicated that cleavage of the cellulose binding domain of cellobiohydrolase-I led to a 76.5% decrease in the adsorption affinity at 25°C and similarly a 20.7% decrease in the adsorption affinity for cellobiohydrolase-II (Kim et al. 1997). The synergism between these two cellobiohydrolases may be due to formation of a partial complex between binding domain of CBH-I and core protein of CBH-II, which have higher adsorption affinity and tightness than those of individual components. Recently, a linear relationship between the production of soluble sugar and adsorption was observed for CBH, (Medve et al. 1998) and also the adsorption of EG-II decreased in presence of CBH-I due to competition for common binding sites.

2.9 EVALUATION OF FACTORS AFFECTING ENZYME PRODUCTION

2.9.1 Physical Factors

2.9.1.1 pH

Different physical parameters influence the cellulose bioconversion and pH is an imported factor affecting cellulase production. Different pH conditions are required by the components of cellulase complex (Macris et al. 1989), such as pH 4.5 for cellobiohydrolase, 4.0 for endoglucanase and 5.0 for β-glucosidase resulted into maximum activity. Singh et al., (1989b) had analyzed the effect of pH on cellulase production using *Aspergillus niger* and observed that pH 5.5 was optimal for maximum cellulase production. On the other hand the pH range of 5.5 - 6.5 was optimal for β-glucosidase production from *Penicillium rubrum* O Stoll (Menon et al. 1994). Eberhart et al. (1977) had reported that induction and release of

cellulases depended on the pH of the medium. His observations indicated that extracellular release of cellulase from *Neurospora crass* occurred at pH 7, whereas the enzyme remained accumulated in the cell at pH 7.5. Similarly pH 7 was suitable for extracellular production of cellulase from the *Humicola fuscoatra* (Rajendaran et al. 1994). Further the adsorption behavior of cellulases was also affected by the pH of the medium. Bailey (1981) and Kim et al., (1988) had reported that maximum adsorption of cellulase from *Aspergillus phoenicus* occurred at a pH of 4.8-5.5. The pH range 4.6-5.0 was found suitable for CMCase, FPase and β-glucosidase production with *Aspergillus ornatus* and *Trichoderma reesei* ATCC-26921 (Yeoh et al. 1986; Mukhopadhyey and Nandi, 1999) whereas, a broad pH range from 5 to 7 was observed suitable for extracellular as well as cell associated β-glucosidase production and the pH of 5.5 was found to yield maximum production (Bronnenmeier and Staudenbauer, 1988).

2.9.1.2 Temperature

Temperature imposes a profound effect on lignocellulosic bioconversion. The temperature for assaying cellulase activities are generally within 50-65°C for a variety of microbial strains e.g. *Thielavia terrestris* -255B, *Myceliophthora fergussi*- 246C, *Aspergillus wentii*, *Penicillium rubrum* O Stoll, *Aspergillus niger*, *Aspergillus ornatus*, (Yeoh et al. 1986; Wojtczak et al. 1987; Srivastava et al. 1987; Singh et al. 1990; Rajendran et al. 1994; Menon et al. 1994). The temperature required for measuring endoglucanase, cellobiohydrolase and β-glucosidase from *Neurospora crassa* was observed to be as 50°C, 55°C and 60°C respectively

whereas, growth temperature was found to be 25°C (Bronnenmeier and Staudenbauer, 1988; Macris et al. 1989). Similarly, a native strain of *Penicillium purpurogenum* showed higher growth at 28°C but maximum cellulase activities at 50°C (Steiner et al. 1994) and about 98%, 59% and 76% of the CMCase, FPase and β-glucosidase activities retained after 48 h at 40°C respectively. Further *Pleurotus florida* and *Pleurotus cornucopiae* showed maximum growth at 27°C with sugarcane bagasse in solid substrate fermentation system (Chaudhary et al. 1994). The effect of temperature on saccharification by supplemented cellulases was investigated by Vipan et al., (1994). His observations had indicated that optimum saccharification temperature for *Trichoderma viride* QM 9414 and *S. rolfsii* NG_2 was 45°C. Whereas *Trichoderma viride* QM 9123, *S. rolfsii* 1644 alongwith *M. verrucaria* supplemented cellulases required 50°C; the saccharification process sharply decreased at temperatures higher or lower than optimum. Temperature also has been shown to influence the cellulase adsorption. A positive relationship between enzymatic adsorption and saccharification of cellulosic substrates was observed at temperature below 60°C, the activity beyond this temperature decreased possibly because of the loss of enzyme configuration leading to denaturation of the enzyme activity (Van-Wyk, 1997). Bronnenmeier and Staudenbauer (1988) reported that extracellular as well as cell bound β-glucosidase from *Clostridium stercorarium* required an identical temperature of 65°C for activity. Further increase in the temperature led to a sharp decrease in the enzyme activity. His observations further indicated that crude enzyme was markedly thermostable compared to the purified extracellular enzyme. Further, a temperature of 60°C was

also required for maximum β-glucosidase production by *Aspergillus ornatus* (Yeoh et al. 1986). Some of the thermophilic fungi, having maximum growth at or above 45-50°C had produced cellulases with maximum activity at 50-78°C and the extracellular β-glucosidase component of the thermophilic cellulases appeared to be highly thermolabile (Wojtczak et al. 1987).

2.9.2 Chemical Factors

2.9.2.1 Carbon source

Many different substrates that are agro or industrial wastes, synthetic or naturally occurring have been analyzed as the carbon sources for the process. Among the cellulosic materials the sulfite pulp, printed papers, mixed waste paper, wheat straw, paddy straw, sugarcane bagasse, jute stick, carboxymethylcellulose, corncobs, groundnut shells, cotton, ball milled oat straw, ball milled barley straw, delignified ball milled oat spelt xylan, larch wood xylan etc had been used as the substrates for cellulase production (Arora and Sandhu, 1986; Doppelbauer et al. 1987; Singh et al. 1990; Gunju et al. 1990; Smith and Wood, 1991; Singh et al. 1991).

The observations of Singh et al. (1989b) indicated that the production of cellulases increased with increase in substrate concentration upto 12% during solid state fermentation using *Aspergillus niger*. Further increase in substrate concentration resulted into decreased production levels. This might be due to limitation of oxygen in central biomass of pellets, and exhaustion of nutrients other than energy sources. Aikane and Patil (1989) demonstrated that carboxy

methylcellulose (1% w/w) would be the best carbon source compared to saw dust for CMCase and β-glucosidase production using *Chaectomium globosum* as cellulolytic agent. Similarly 1% (w/v) of either cellulose or cereal straw were found suitable for cellulase production from *Penicillium rubrum* O Stoll and *Penicillium purpurogenum* (Menon et al. 1994; Steiner et al. 1994) and from *Sporotrichum thermophile* (Sugden and Bhat, 1994).

Srivastava et al., (1987) and Mukhopadhyey and Nandi (1999) had indicated that 3% malt extract or water hyacinth was optimum for CMCase, FPase and β-glucosidase production, whereas lower levels of β-glucosidase was observed with lactose and cellulose as carbon sources and may be accounted for the differential biomass production with these substrates. The saccharification of alkali treated bagasse was attempted at higher substrate level upto 4% (w/v) and observed higher rate of saccharification at 0.5% (w/v) substrate concentration, followed by a decrease with increased substrate concentration (Singh et al. 1990; Rajendran et al. 1994). Interestingly, higher concentration (2.5 - 6.2% w/v) of carbon source was observed to be suitable for maximum saccharification when cellobiase was supplemented into the medium containing delignified rice straw, news print or other paper wastes as substrates. (Vipan et al. 1994; Wu and Ju, 1998; Ju and Afolabi, 1999).

2.9.2.2 Nitrogen source

The effect of different nitrogen sources, such as ammonium sulfate, ammonium nitrate, ammonium ferrous sulfate, ammonium chloride and sodium

nitrate had been studied (Singh et al. 1991) and among these, ammonium sulfate (0.5 gl^{-1}) led to maximum production of cellulases (Singh et al. 1991; Gutierrez - correa and Tengerdy, 1998). In contrast to this finding Menon et al., (1994) observed a significant reduction in enzymatic levels in the presence of ammonium salts as the nitrogen source. However, increase in the level of β-glucosidase was reported when, corn steep liquor (0.8% v/v) was added. Corn steep liquor also resulted into a 3-5 fold induction into endoglucanase and exoglucanase levels with cellulose (Sigma cell type-20), wheat straw and wheat bran as the substrates (Steiner et al. 1994). Enzyme production was sensitive to corn steep liquor (0.88 gl^{-1}) and production increased significantly when mixed nitrogen sources (corn steep liquor and ammonium nitrate) were used (Steiner et al. 1994). Addition of urea in the medium was effective for increased production of xylanase (Smith and Wood, 1991).

2.9.2.3 Phosphorus source

Phosphorus is an essential requirement for fungal growth and metabolism. It's an important constituent of phospholipids involved in the formation of cell membranes. Besides it's role in effecting the linkage between nucleotides forming the nucleic acid strands, it's also involved in the formation of numerous intermediates, enzymes and coenzymes that are essential to the metabolism of carbohydrates, as well as for many oxidative reactions and other intracellular processes (Palczar et al. 1986; Singh et al. 1990; Singh et al. 1991). Different phosphate sources, such as potassium dihydrogen phosphate, tetra-sodium

pyrophosphate, sodium β-glycerophosphate and dipotassium hydrogen phosphate have been added to analyze their effect on enzyme production (Garg and Neelkantan, 1982 and Singh et al. 1991). It has been widely demonstrated that potassium dihydrogen phosphate is the most favourable phosphorus source for cellulase production.

2.9.2.4 Phenolic compounds

The phenolic compounds have the ability to induce laccase that in turn stimulates the cellobiose-quinone-oxidoreductase enzyme, this enzyme possibly is involved in cellobiose (CMCase and FPase inhibitor) oxidation to cellobionic acid thus affecting indirectly an increase in the cellulase levels (Arora and Sandhu, 1986). Among various phenolics e.g. gallic acid, tannic acid, maleic acid, salicylic acid and β-nepthnol used, salicylic acid was observed to be a better inducer (Singh et al. 1988d), other phenolic compounds however had shown the inhibitory effect. Muller et al., (1988) had also proposed a similar mechanism of cellulase regulation in *Trametes versicolor* in the presence of phenolics. His observations indicated the vanillin had a stimulatory effect on the cellulase biosynthesis and its regulation was possibly due to cellobiono-lactone formed by the interactions of laccase, phenol, cellobiose and cellobiose-quinone-oxidoreductase. This lactone seemed to influence the cellulase production. Thus, cellobiose formed during cellulose hydrolysis was continuously withdrawn from the system by oxidation.

2.9.2.5 Sugars

Several investigations so far have indicated that cellulases are inducible enzymes and different carbon sources have been analyzed to find their role in effecting the enzymatic levels. Cellobiose (1mM) may act as an effective inducer of cellulase in fungi and also induces cellobiase and aryl-β-glucosidase in *Neurospora* (Mandels and Reese, 1960; Myers et al. 1966 and Eberhart et al. 1977). Increased rate of endoglucanase biosynthesis in *Bacillus* sp was reported in presence of cellobiose or glucose (0.2%) added to the culture medium (Paul and Varma, 1990). Xylanase biosynthesis was also induced by xylose or cellobiose added to the culture medium during growth (Paul and Varma, 1990). Ryu and Mandels (1980) have reviewed the cellulase biosynthesis and shown that, cellulose, cellobiose, sophorose and lactose are effective inducers. Glucose among the sugars has been found to be a negative regulator of cellulase biosynthesis (Ryu and Mandels, 1980). The catabolite repression of cellulase biosynthesis occurred at the translation level (Kubicek et al. 1990). Higher concentrations of the sugars were inhibitory for cellulase production. Yeoh et al., (1986) had reported that cellobiose, gentibiose at higher concentration inhibited about 80% of the β-glucosidase activity, similarly, laminaribiose and glucose also led to a 55-60% inhibition in the enzymatic activity Shiang et al (1991) have described a possible regulation mechanism of cellulase biosynthesis, it was proposed that sugar alcohols, sugar analogues, xylose, glucose, sucrose, sorbose, cellobiose, methylglucoside etc at a particular concentration may induce a regulatory protein called cellulase activator molecule (CAM). The levels

and yields of CAM is possibly affected due to substrate concentration and some unknown factors imparted by moderators.

2.10 MUTAGENESIS

Major producers of fermentation products extensively undertake mutation selection programs for developing strains of commercial significance. The selected strains are vigorously analyzed mainly for its higher production ability and stability before its usage for industrial applications. Mutagenesis of strain is normally accomplished by employing physical or the chemical reagents. The production of cellulases by the microbial cell is under controlled genetic and biochemical programs that include, induction, catabolite repression or the end product inhibition. These controls are operative under cellulase production conditions, thus resulting into limited yields of the enzymatic constituents. Mutagenic treatments of *Trichoderma reesei* Qm 6a, a wild type strain isolated at US Army Natick Research and Development Command, Natick, USA led to the development of mutants with higher cellulolytic activity (Bisaria and Ghose, 1981). Bailey and Nevalainen (1981) mutageneized *Trichoderma reesei* Qm 6a mutant VTT-D 78085 through N-methyl-N'-nitro-N-nitrosoguanidine (NTG) followed by gamma irradiation (100 K rad h^{-1}) and obtained the mutants VTT-D 80132 and VTT-D 80133 with improved cellulolytic activity. Exposure of *Aspergillus awamori* using NTG resulted into a mutant AANTG-43 with improved enzyme production (Smith and Wood, 1991). A hypercellulolytic mutant NTG-19 from *Fusarium oxysporum* was developed by Kuhad et al., (1994) by ultraviolet treatment followed by chemical mutagenesis

using NTG (100 μg ml^{-1}). The resultant mutant strain had substantially higher (80%) cellulolytic activity than its parental strain. NTG treatment of *Cellulomonas flavigena* also produced four mutant strains (M4, M9, M11 and M12) with improved xylanolytic activities (Reyes and Noyola, 1998). A mutant $creA^{d}30$ having resistance to end product inhibition was constructed from *Aspergillus nidulans*, the mutant showed improved levels of D-glucose metabolism and its utilization (Veen et al. 1995). Little information is also available on strain improvement by heterokaryon development for lignin degradation by *Phanerochaete chrysosporium* (Wyatt and Broda, 1995). Besides NTG, other mutagens e.g. colchicine, sodium azide had also been used for strain improvements (Lotfi and Santelli, 1996; Zohrer et al. 1996). Site directed mutagenesis, employing a 25 mer oligonucleotide primer, of the *Cellulomonas fimi* Cex gene active site led to decreased exoglucanase activity (MacLeod et al. 1996). Cappa et al., (1997) constructed a novel *Ruminococcus albus* mutant strain with improved cellulase activity by cloning the *Streptomyces rochei* endoglucanase gene using a pCRB1 plasmid vector and reported that the *R. albus* strains harbouring the heterologous cellulase gene showed the higher cellulase activity.

2.11 CO-CULTIVATION

Cellulosic bioconversion is a complex process and requires the synergistic action of the three enzymatic components consisting of endoglucanase, exoglucanase and β-glucosidase. The ability of major cellulolytic microbial strains identified so far remains unexploited to the derived extent due to the limiting levels

of one or the other enzymatic components. For instance, *Trichoderma reesei* strain, a major cellulolytic organism was reported to have lower levels of β-glucosidase (Madmwar and Patel, 1992) while *Aspergillus niger* have limited level of the endoglucanase component (Maheshwari et al. 1994). Thus attempts have been made to increase the levels of the enzymatic components either by genetic manipulation (Kuhad et al. 1994) or by externally supplementing the desired component (Srivastava et al. 1987). Recently, co-cultivation of the cellulolytic organisms complementing the desired cellulolytic component have been attempted for achieving increased rate of lignocellulosic bioconversion; *Trichoderma reesei* Qm 9123 and *Aspergillus niger* had been co-cultured for cellulase production using paper mill sludge as a cellulosic substrate (Maheshwari et al. 1994). Similarly Gupte and Madamwar (1997) cultivated *Aspergillus ellipticus* and *Aspergillus fumigatus* and reported improved hydrolytic activities as compared to when they were used separately in solid state fermentation system. Improved enzyme levels were also achieved by Madamwar and Patel (1992) when *Trichoderma reesei* was co-cultured with *Aspergillus niger* strain using bagasse, corncobs and saw dust, as the substrate in solid state fermentation. The judicious selection of the compatible strains and media constituents play an important role for achieving higher enzymatic production under mixed culture conditions. Gutierrez-correa and Tengerdy (1998) had reported single cultures of *Trichoderma reesei* and *Aspergillus phoenicus*, when supplemented with inorganic nitrogen produced similar xylanase levels as in mixed culture. However, supplementation of soymeal to the fermentation medium led to a 35-45% higher production of xylanase by

mixed culture in comparison to the xylanase levels produced under single culture conditions.

2.12 REGULATION OF CELLULASE BIOSYNTHESIS

The synergistic action of cellulase complex initially results into cellobiose as the major end product (Eberhart et al. 1977). This disaccharide is the first soluble compound from cellulose and its appearance in the cell indicates the presence of extracellular cellulase. It therefore, could be the "logical" inducer of further cellulase biosynthesis. Indeed there are several microorganisms known, in which cellulose fermentation can be provoked by cellobiose (Ryu and Mandels 1980). However, the regulation of cellulase biosynthesis may differ from species to species (Ryu and Mandels, 1980) and is not well defined. Borgia and Syphered (1977) had reported that cAMP not only inactivated the cellulase activity but also repressed the synthesis of new enzyme. On the other hand Robson and Chamblis (1989) have discussed cAMP as an inductive regulator for endoglucanase synthesis. Similarly Wood et al. (1989) and Shiang et al. (1991) had demonstrated higher cellulase yields with increasing levels of cAMP.

Sorbose, L-glucose, 2-deoxy glucose, glucose-1-phosphate, sephorose and sugar alcohols enhanced cellulase activity at smaller concentration (Bisaria and Ghose, 1981; Singh et al. 1988; Robson and Chambliss, 1989). All these substances may function as moderators of cellulase synthesis. Different reports available had indicated cellobiose, xylose, sophorose and unknown soluble derivatives from

cellulose as inducers (Paul and Varma 1990; Shiang et al. 1991). In a possible regulatory mechanism of cellulase synthesis, the repressor, inducer, cAMP and moderator may all be involved in regulating the rate and yield of enzyme production (Robson and Chambliss, 1989).

It was hypothesized for a long time that the initially fungi contains low, constitutive cellulase levels, which may attack cellulose and results into formation of inducers. Since the method for the assay of cellulases is not very sensitive, the existence of the lower cellulase levels lacked scientific approval for a long time. Only very recently, the availability of poly as well as monoclonal antibodies against cellulase components provided a suitable tool for the separate identification and quantification of even very low levels of individual cellulase components (Bisaria et al. 1981; Kubicek et al. 1990). However, with respect to the ecology of cellulose breakdown, it is not known, if the enzymes alone are able to bind to cellulose during the initial phase of contact. Some authors have reported the importance of adsorption on to cellulose for efficient hydrolysis (Kim et al. 1988; Kubicek et al. 1990; Reinikainen et al. 1995).

Northern blots of mRNA isolated from *Trichoderma reesei* during growth on various carbon sources had indicated cellulase gene transcription within 20 minutes of incubation and the regulation of cellulase formation occurs at the pretranslation level. At least the CBH-I, CBH-II, EG–III and β-glu 1 genes are present in single copies in the *Trichoderma reesei* genome. CBH-I, CBH-II and EG-III are located on chromosome II (Kubicek et al. 1998). The studies have shown that both, the 5' region as well as the chromosomal locus influence cellulase gene

transcription significantly. Further, the report on cellulase regulation indicated that transcription of Cel1 gene is mediated by an activator protein interacting with operator (Walter and Schrempf, 1996). Glucose along with the glycerol that exert a strong repression, possibly bind to its palindrome, as a consequence, transcription is not initiated. The report further stated that, 14 bp palindromic sequence binding with repressor are additionally supported by its relative location between the"promotor" and the translational start site. It seems probably that all cellulase genes containing the 14 bp palindrome in the upstream region have a similar mechanism of substrate induction.

2.13 CELLULASE FAMILY

The catalytic domains with respect to sequence identity of cellulases have been described (Henrissat et al. 1989; Neill et al. 1996). The sequences known so far can be grouped into nine families, which are quite distinct. Families A,B,F and H contain fungal and bacterial enzymes, family E contain bacterial enzyme and plant enzymes, thereby raising the possibility of a lateral transfer. At present, family C contains only fungal enzymes and families D and G contain only bacterial enzymes. Cellulases vary widely in the number of amino acids they contain, but their catalytic domains tend to be more uniform in size. All enzymes reported to have exoglucosidase activity fall into families, which have members with only endoglucosidase activity. In other words, enzymes with similar sequences have different specificities. This suggests that exoglucosidase versus endoglucosidase activity may be a sequence of fine catalytic domain for which the three-dimensional

structure is known e.g. cellobiohydrolase-II from *Trichoderma reesei*. The active site is in an enclosed tunnel through which a cellulose molecule threads. Two aspartyl residues located in the middle of the tunnel may be catalytic residues (Rouvigen et al. 1990). CBH II is in family B of the cellulases, which contains exoglucanases and endoglucanases. The endoglucanase Cen A has been well characterized and is a member of family 6 of cellulases and xylanases. An interesting feature of family 6 is that it contains both endoglucanase and exoglucanase, which show precise differences in amino acid sequence (Tomme et al. 1995). Family 5 (also known as cellulase family A) is the largest of the β-glucohydrolase families classified to date including over 60 bacterial and fungal enzymes which cleave with retention of configuration. The sequences of family 5 members are rather diverse and were reported to share only seven conserved residues. An extensive mutagenesis study has shown that cellulase activity is highly sensitive to the replacement of any of the seven conserved residues.

2.14 GENE CLONING

The understanding of molecular mechanism leading to biodegradation of lignocelluloses and the development of the bioprocessing potential of lignocellulolytic microorganisms can be effectively accomplished by recombinant DNA technology. In addition, the regulation and catalytic functioning of cellulases and ligninases, the nature of synergistic interactions among different enzymes and the development of economically feasible systems for the conversion of waste biomass into value added products have been the major areas under study using

genetic engineering technologies. It is expected that for industrial applications, cellulases must have high adsorption capacities and catalytic efficiencies, high thermal stabilities and lower end product inhibition. It is therefore essential that efforts should be made for cloning cellulase genes with desirable molecular properties. Cloning of genes encoding individual components of cellulase complex would allow the biosynthesis of pure components. A large number of fungal and bacterial genes have been cloned in recent years. *Escherichia coli* was found to be the most commonly used host using plasmids or phage vectors (Schwarz et al.1989; Din et al. 1990; Ferrer et al. 1996; Scheweita et al. 1996). In addition, cellulase genes have also been expressed efficiently in other microbial systems such as *Streptomyces lividans, Lactobacillus acidophilus, Bacillus subtilis, Zymomonas mobilis, Pseudomonas fluorescens, Bacillus megaterium, Azotobacter vinelandii* and in the yeast *Saccharomyces cerevisiae* (Jourin and Gramstrom, 1989; Baik and Pack, 1990; Blanco et al. 1991; Kuhad and Singh 1993). The cloning and sequencing of the various cellulolytic genes will help in characterizing the potential systems for economizing the process. Besides the cis and trans factors regulating the cellulase genes should be identified in order to develop a better understanding of gene regulation for fully exploiting the cellulose biotechnology.

Chapter 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 MATERIALS

Sugarcane bagasse was obtained from the local sugarcane industry Mahalakshmi Sugar Mill, Laksar, Uttar Pradesh. *Eicchornia crassipes* biomass was collected from the regional ponds abundant in this area. *Accacia* powder, cellulose acetate, carboxymethylcellulose, DEAE-cellulose, phosphocellulose and other media chemicals were purchased from Hi Media and Ranbaxy Laboratories, India. Glucose, sucrose, cellobiose, lactose other sugars and starch were purchased from Glaxo Laboratories, India and were of highest grade available. Tween-80 was purchased from Fluka Goldie, Germany. p-Nitro-phenyl-β-D-glucopyranoside (pNPG), 4-methylumbelliferyl β-D-cellobioside, 4-methylumbelliferyl β-D-glucoside and standard protein markers were purchased from Sigma chemical company, USA. Whatman No. 1 filter paper used as a substrate was purchased from Whatman Asia Pacific, Singapur. All other chemicals were of analytical grade and purchased from E Merck, Germany and from other prestigious commercial sources.

3.1.1 Microorganisms

Aspergillus niger RK-3 used in this study was isolated from decomposing sugarcane industry waste. *Trichoderma reesei* MTCC-164 was a generous gift from Dr. *P. Kaushik*, Gurukul Kangri University, and Hardwar, India. The strains RKU-40 and UNSC-442 were obtained by ultraviolet radiations and mixed mutagenesis of the wild type isolate *Aspergillus niger* RK-3. The microorganisms were

maintained on potato dextrose agar (PDA) slants, stored at 4°C and sub-cultured monthly to maintain their metabolic activity.

3.2 METHODS

3.2.1 Isolation, Purification and Screening of Strains

Eleven fungal and eleven bacterial strains were isolated from the decomposing wastes of sugarcane and paper industries located in and around this region. Decomposed materials were suspended in sterile distilled water, subjected to serial dilutions and plated onto potato dextrose agar (PDA) or nutrient agar medium. Single colony was isolated and purified on potato dextrose agar or nutrient agar medium. The isolated strains were transferred onto congo-red CMC-agar plate followed by incubation at 30°C for 4 days. The isolated strains were then analyzed for cellulase production ability by measuring the zone diameters formed on congo-red CMC-agar plates (Schwarz et al. 1989). The RK-3 strain showed the maximum zone diameter and after assaying the cellulase production resulted into higher enzymatic activities as compared to the other isolated strains. This strain was identified at Indian Agricultural Research Institute, New Delhi, India as *Aspergillus niger*. This culture was thus referred to as *Aspergillus niger* RK-3 strain and used for subsequent studies for cellulase production.

3.2.3 Pretreatment of Lignocellulosic Substrates

Sugarcane bagasse and *Eicchornia crassipes* biomass were cut into small pieces (1x1 cm), crushed thoroughly using a mixer-grinder and screened through

the sieves (100 mesh) to obtain the particles of uniform size (80-100 mesh). The powdered lignocellulosic substrates were washed repeatedly with sterile distilled water to remove water-soluble impurities. The other heavier insoluble impurities that settled down at the bottom were also removed. The floating cake was removed, rewashed with water and dried at 80-90°C overnight.

3.2.3.1 High pressure steaming and hemicellulose separation

The sugarcane bagasse and *Eicchornia crassipes* biomass were suspended in water so as to obtain a 15% suspension and subjected to high pressure steaming at 180-190°C for 30 to 40 minutes (Lakhani, 1990) to break the ternary complex of hemicellulose-lignin-cellulose. The hemicellulose component is rendered water soluble and removed by filtration. The residue was dried at 110°C in an hot air oven. The difference between the weights of the starting material and the dried residues was accounted as hemicellulose content in the lignocellulosic complex.

3.2.3.2 Sodium hydroxide pretreatment and lignin separation

The hemicellulose free residue of lignocellulose was added with 0.25 M NaOH (20 ml g^{-1} substrate) autoclaved at 120°C for 30-40 minutes and filtered through muslin cloth. The residue obtained was washed thoroughly and neutralized with 0.25 N hydrochloric acid (Singh et al. 1989). The substrates were finally washed with distilled water and dried at 65°C for future use. The pH of the alkaline filtrate was adjusted to 7.0 to precipitate the lignin. The dark brown precipitate of lignin was washed with water to remove alkali. The lignin was then dried

under vacuum and its weight determined (Lakhani, 1990). The average composition of hemicellulose, lignin and cellulose present in the lignocellulosic materials was determined on the basis of at least three separate determinations.

3.2.4 X-Ray Diffractographic Analysis of Lignocellulosic Samples

Untreated and treated lignocellulosic materials were vacuums dried in a dissicator to remove the moisture content from the samples. The crystallinity was measured by X-ray diffraction (Nelson and Oliver, 1971) using a X-ray diffractometer (Phillips PW-114, Holland). The samples were scanned in the range of 0 to 100° diffraction angle.

3.2.5 Production of Cellulases with Synthetic Cellulosic Substrates

The production of endoglucanase (CMCase), exoglucanase (FPase) and β-glucosidase was assayed using 1% (w/v) *Accacia* powder, carboxymethyl-cellulose, cellulose acetate, DEAE-cellulose and phosphocellulose as the cellulosic substrates, using Mandels and Weber's modified medium (Hatakka and Pirhonen, 1985; Singh et al.1989b).

3.2.6 Production of Cellulases with Natural Cellulosic Substrates

The levels of endoglucanase (CMCase), exoglucanase (FPase) and β-glucosidase were detected using 1% (w/v) of the sugarcane bagasse or *Eicchornia crassipes* biomass as cellulosic substrates using Mandels and Weber's modified medium (Singh et al. 1989a).

3.2.6.1 Preparation of inoculum

The culture on the PDA slant was first incubated at 30°C for 24 h. The activated spores were removed and suspended in Tween -80 (0.01% v/v); 2 ml of spore suspension ($2x10^6$ spores ml^{-1}) was added to the 50 ml of preculture medium (Mandels and Weber's modified medium, pH 4.8) containing 10 gl^{-1} glucose (Singh et al. 1992). Medium after inoculation was incubated at 30°C for 24 h with continuous shaking (120 rpm) in a rotary shaker.

3.2.6.2 Composition of the production medium

The Mandels and Weber's modified medium (Hatakka and Pirhonen, 1985) used for the production of cellulases contained (gl^{-1}): cellulosic substrate (synthetic or natural, 1% w/v); $(NH_4)_2SO_4$, 2.1; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.3; $CaCl_2$ 0.3; $MnSO_4.5H_2O$, 0.00156; $ZnSO_4.7H_2O$, 0.0014; $CoCl_2.6H_2O$, 0.00266; Tween-80, 2.0 ml l^{-1}. The pH of the medium was adjusted to 4.8 before sterilization.

3.2.6.3 Production of cellulases in submerged fermentation

Basal medium (100 ml) containing 1% (w/v) cellulosic substrate (synthetic or natural) was added with 5% of the inoculum followed by incubation at 30°C for 8 days on a rotary shaker (120 rpm). Samples collected at different periods were centrifuged (2000 rpm, 15 min) and the supernatant obtained was assayed for endoglucanase (CMCase), exoglucanase, (FPase), β-glucosidase, total soluble proteins and the residual mass obtained after centrifugation was analyzed for cellulose degradation.

3.2.6.4 Determination of dry mycelial weight

The cell mass was separated from the fermentation medium by filtration, washed twice by distilled water, blotted, dried at 90 °C till constant weight.

3.2.7 Analysis of Nutritional Factors Affecting Enzyme Production

Various physico-chemical, biological as well as other factors mainly sugars, phenolics and organic acids were evaluated for achieving maximal production of cellulases. The bioconversion reaction was studied under submerged conditions and supernatant from the reactions was analyzed.

3.2.7.1 Physical and biological factors

In order to maximize the production of cellulases, the effects of pH (3.0-6.0) and temperature (30-80 °C) on the production of cellulases were analyzed using Mandels and Weber's modified medium as described (Singh et al. 1991). Analysis of the optimal temperature for higher biomass accumulation was performed in the range of 10-50 °C. The effect of agitation speed (110-180 rpm) on biomass accumulation was also analyzed. Various concentrations (1-9%) of 24 h old inoculum were used in order to define the levels of inoculum for higher enzymatic production. The cellulases were estimated after 8 days of incubation period.

3.2.7.2 Chemical factors

Various concentrations of nutrients i.e. carbon, nitrogen, phosphorus and regulators such as phenolics, organic acids and soluble sugars were analyzed for

higher cellulase production as described (Singh et al. 1988d; Singh et al. 1991) and detailed as below. The physical and biological conditions derived earlier were utilized for these studies. The nutritional factor/regulator defined at every stage for achieving maximum enzyme production was included as the medium constituent and medium thus designated served as the control for next stage of analysis for the factors affecting the enzyme production.

In order to maximize the production of cellulases, effect of different levels of cellulosic substrates (0.5-2%) such as sugarcane bagasse and *Eicchornia crassipes* biomass was estimated using the physical and biological conditions as derived in the earlier studies. The medium with this defined component for maximum enzymatic production was designated as R_1 medium (R_1m).

Similarly, the influence of additional nitrogen sources (such as ammonium chloride, ammonium ferrous sulfate, ammonium nitrate, and sodium nitrate) added at varying concentrations (0.35-0.55 gl^{-1}) along with ammonium sulfate (2.1gl^{-1}) as the major nitrogen source was analyzed for maximum enzymatic production. The medium containing the constituents as derived till stage for maximum enzymatic production was designated as R_2 medium (R_2m).

The various phosphorus sources (such as tetra sodium pyrrophosphate, potassium dihydrogen phosphate, sodium β-glycerophosphate and dipotassium hydrogen phosphate) at different concentrations (0.35-0.55 gl^{-1}) along with dihydrogen phosphate (2.0 gl^{-1}) as the major phosphorus source were used to define

their efficacy for maximum cellulase production. The medium with the constituents as defined till stage was designated as R_3 medium (R_3m).

In addition to nutritional factors, the effects of other chemical regulators were studied. Various phenolic compounds (0.1%) i.e. gallic, tannic, maleic, salicylic and vanillinic acids, β-naphthol, guaicol and vanillin were used and the medium thus defined yielding higher enzyme production was designated as R_4 medium (R_4m). Similarly, organic acids (0.1%) i.e. acetic, ascorbic, citric, oxoglutaric, propionic and succinic acids were added and the medium thus derived for higher enzyme production was designated as R_5 medium (R_5m). Different sugars (0.2%) i.e. lactose, mannitol, fructose, sucrose, glucose, galactose, cellobiose and xylose were analyzed using the conditions as derived from the earlier studies. The medium composition yielding to maximum cellulase production was designated as R_6 medium (R_6m).

3.2.8 Adsorption of Enzymatic Protein

Adsorption pattern of the enzymatic protein on the surface of the cellulosic substrates was studied as described by Van-Wyk (1997). Briefly, 0.05 gm of cellulosic substrate (synthetic or natural) mixed with 10 ml of citrate buffer (50 mM, pH 4.8) and was then added with 50 mg of partially purified enzymatic protein. The reaction mixture was then sealed and incubated for different periods at 50 °C. Samples from incubation mixture were analyzed for glucose and non adsorbed protein content. Enzyme adsorption was calculated as the difference between the control and the test incubation.

3.2.9 Mutagenesis and Screening

3.2.9.1 Single treatment

3.2.9.1.1 Mutagenesis by ultraviolet radiation

Ultraviolet mutagenesis was performed as described (Kuhad et al. 1994). Briefly, a 2-4 day old spore suspension (10^6 spores ml^{-1}) of *Aspergillus niger* RK-3 was prepared in saline (0.85%) solution containing Tween-80 (0.01%). Mutagenesis with ultraviolet (UV) radiation was carried out using UV tube (15W) at a distance of 70 cm for 5-30 minutes (dose limit 12-168 Jm^{-2} min^{-1}). Treated spores were wrapped with a black paper and incubated for 48 h at 30°C in the dark. Spores were then plated on the congo-red CMC- agar plates for screening (Schwarz et al. 1989; Reyes and Noyola, 1998).

3.2.9.1.2 Chemical mutagenesis

The chemical mutagenesis was performed by using N-methyl-N'-nitro-N-nitrosoguanidine (NTG), sodium azide and colchicine according to the methods as described for NTG (Kuhad et al. 1994), sodium azide (Zohrer et al. 1996) and colchicine (Lotfi et al. 1996). Briefly, for NTG mutagenesis the spores suspension of *Aspergillus niger* RK-3 (~10^6 -10^7 spores ml^{-1}) was prepared in 0.85% saline. The spore suspension was treated with NTG (100 µg ml^{-1}) for 30-60 minutes with an interval of 5-10 minutes, whereas for sodium azide and colchicine treatments, spore suspensions were treated with sodium azide (1-4 µg ml^{-1}) for 24 h at 30°C and with colchicine (0.01-2.0 µg ml^{-1}) for 24 h at 30°C. Spores after treatments were washed twice with saline and collected by centrifugation (5000 rpm, 10 min.). Treated spores were transferred onto congo-red CMC-agar plates for screening.

3.2.9.2 Mutagenesis by mixed chemical treatment

Analysis of the mutants after single treatments involving ultraviolet (UV) or chemical mutagenesis resulted into the mutants RKU-40 and RKU-45 with maximum zone diameters as obtained after UV mutagenesis, hence the same were chosen for mixed mutagenesis. The mutants RKU-40 and RKU-45 (selected after UV mutagenesis) were further treated with 100 µg ml^{-1} of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Kuhad et al. 1994) for 45 minutes. The strains showing higher zone diameters on congo-red CMC-agar plate as compared to UV treated mutant strains were selected. The mutants thus obtained after UV-NTG mixed treatment were further subjected to sodium azide treatment (1-4 µg ml^{-1}) and the resulting mutants with higher zone diameters were further treated with colchicine (0.01-2.0 µg ml^{-1}). The mutants thus obtained with higher zone diameters on congo-red CMC-agar plate were finally selected.

3.2.9.3 Screening of the mutant strains

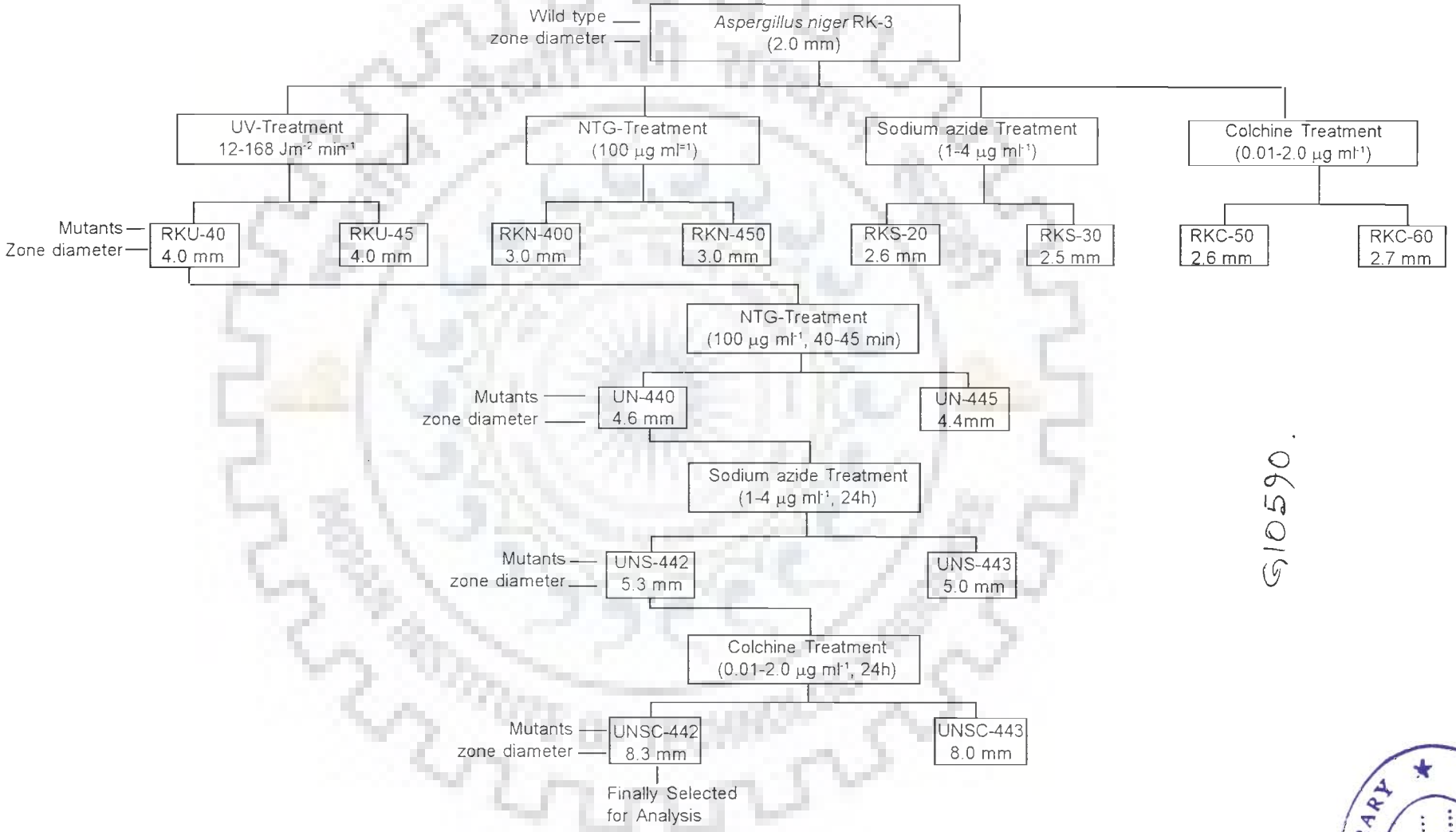
The screening of the mutant strains was carried out as described (Schwarz, 1989; Reyes and Noyola, 1998). Briefly, the treated spores were transferred on CMC-agar medium (defined R_6m medium with 1% CMC and 1% D-glucose). The plates were then incubated at 30°C for 2-4 days. Colonies appeared following incubation were treated with 0.5% congo-red solution for 5-10 min. Plates were then washed with 5% NaCl to remove the excess stain. The mutants that were able to produce cellulases showed a clear white zone around the colonies. The potential

mutant strains were selected on the basis of maximum zone diameters appeared after treatment on congo-red CMC-agar plates as compared to the zone diameters obtained either by the wild type or with the previously selected mutant strains under similar conditions. The enzymatic activities of mutants were also observed in Raj production medium containing 1% CMC alongwith 1% D-glucose in submerged state fermentation conditions. Mutant UNSC-442 thus obtained had the maximum zone diameter as well as higher cellulase production and was finally selected for further studies.

3.2.10 Production of Cellulases During Co-Cultivation of Two Fungal Strains in Semi-solid State Fermentation (SSSF)

3.2.10.1 Preparation of inoculum

The cultures on the PDA slants were incubated at 30°C for 24 h. The activated spores from *Aspergillus niger* RK-3 or mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in the ratio of 3:1 were collected and suspended in 5 ml sterile solution of 0.01% Tween-80. 4 ml of the spore suspension (10^8 spores ml^{-1}) was added to the fermentation flask containing 100 ml of the production medium (Gupte and Madamwar, 1997).



Flow chart representing mutants selection plan

3.2.10.2 Preparation of production medium

The derived R_6m medium that was a modification of Mandels and Weber's modified medium (Singh et al.1989) was used having 10% (w/v) of cellulosic substrate (sugarcane bagasse/*Eicchornia crassipes* biomass) and contained (gl^{-1}): $(NH_4)SO_4$, 2.5; NH_4Cl, 0.4; KH_2PO_4, 2.45; $MgSO_4.7H_2O$, 0.3; $CaCl_2.6H_2O$, 0.3; $MnS0_4.5H_2O$, 0.00156; $ZnSO_4.7H_2O$, 0.0014; $CoCl_2.6H_2O$, 0.00266; vanillin, 0.50; Succinic acid, 1.0; cellobiose, 2.0 alongwith Tween-80, 2.0 ml l^{-1} pH of the medium was adjusted to 4.8 before sterilization.

3.2.10.3 Production of cellulases in semi-solid state fermentation (SSSF)

Production medium (100 ml) containing alkali treated sugarcane bagasse or *Eicchornia crassipes* biomass as the cellulosic substrate was added with 5% of inoculum and incubated at 30°C for 18 days. The samples were collected from the semi-solid paste, filtered through muslin cloth and centrifuged (2000 rpm, 15 min). The supernatant obtained was analyzed for endoglucanase (CMCase), exoglucanase (FPase), β-glucosidase and for the total soluble proteins. The total dry mycelial mass obtained after 18 days of incubation was also estimated. (Gupte and Madamwar, 1997).

3.2.11 Enzymatic Hydrolysis of Cellulosic Substrates

5 gm of cellulosic materials (alkali treated sugarcane bagasse or *Eicchornia crassipes* biomass) were suspended in 100 ml of 50 mM citrate buffer, pH 4.8

containing 100 units of partially purified cellulases obtained from *Aspergillus niger* UNSC-442 mutant. The suspension was incubated for varying time intervals from 0-48 h at 50°C with constant shaking (120 rpm). Toluene 0.01% (v/v) was added in order to prevent the bacterial contamination. Samples drawn at indicated time intervals were immersed in a boiling water bath for 3-4 minutes to stop the reaction (Lakhani, 1990; Kuhad et al. 1994). The residual material was removed by centrifugation (5000 rpm, 10 min.). The supernatant was decanted and glucose as the reducing sugar was estimated by DNS method (Miller, 1959).

$$\% \text{ Hydrolysis} = \frac{\text{Reducing sugars } (gl^{-1}) \times 0.9}{\text{Substrate concentration } (gl^{-1})}$$

3.2.11 Cultivation of Baker's Yeast on Enzymatic Hydrolyzates of Sugarcane Bagasse and *Eicchornia crassipes* Biomass

Czapex's dox medium containing (gl^{-1}) $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01, pH 5.5, supplemented with 0.1% peptone and enzymatic hydrolyzate so as to have 2% sugar as the final concentration of the carbon source in the medium. The medium was sterilized (121°C, 30 min) and inoculated with 10% of the baker's yeast (*Saccharomyces cerevesiae*) for production of biomass. Incubation continued at 30°C for 12-48 h on a rotatory shaker (150 rpm). Cell mass was separated after incubation by centrifugation, washed with water and dried at 80°C for 10-20 h and weighed.

3.2.13 Production of Organic Acids

3.2.13.1 Culture conditions for organic acid production

Aspergillus niger KCU-5 and ORS-4 strains (from this laboratory) were used for organic acid production. Strains were allowed to grow on potato dextrose agar slants at 30°C for 5 days. The spores were aseptically harvested with 10 ml of sterile distilled water. 2 ml of well dispersed spore suspension containing about 2 x $10^5 - 10^6$ spores ml^{-1} was used as inoculum. Medium (50 ml) was inoculated with 4% of the spore suspension followed by incubation at 30°C for 10 days.

3.2.13.1.1 Fermentation medium for gluconic acid production

The medium used for gluconic acid production contained enzymatic hydrolyzate of sugarcane bagasse or *Eicchornia crassipes* biomass equivalent to 4-14% sugar concentration, $(NH_4)_2$ HPO_4, 0.1%; KH_2PO_4 ,0.05%; $MgSO_4$. $7H_2O$, 0.015%; $CaCO_3$, 4%, pH of the medium excluding $CaCO_3$ was adjusted to 5.5. The medium was autoclaved and sterilized. $CaCO_3$ was then added to the medium just before inoculation, that shifted the pH to 6.6 (Kundu and Das, 1984).

3.2.13.1.2 Fermentation medium for citric acid production

The medium for citric acid production contained sugarcane bagasse or *Eicchornia crassipes* biomass enzymatic hydrolyzate equivalent to 4-14% sugar concentration, NH_4 NO_3, 0.223%; KH_2PO_4, 0.1%; $MgSO_4$. $6H_2O$, 0.023%. The pH of the medium was adjusted to 2.2 with 1 N HCl.

3.3 ANALYTICAL METHODS

3.3.1 Estimation of Total Cellulose Content

Total cellulosic content present in sugarcane bagasse and *Eicchornia crassipes* biomass wastes was measured according to Updegraph method (1969). Dried sample (25 mg) containing cellulose was added with 2.5 ml of 72% H_2SO_4 followed by mixing and gentle heating for about 40 min for complete solubilization. The volume of the above sample was made upto 50 ml by adding distilled water and then filtered to remove the residual matter. 0.02 ml of the filtrate was made upto 1ml by distilled water and tubes placed in ice bath. 4 ml of ice cold 2% Anthrone reagent (prepared by dissolving 2 gm of Anthrone reagent in 98% sulphuric acid in a total volume of 100 ml) was added slowly with proper mixing. The tubes with marble on the top were incubated in a boiling water bath for 10 min and cooled under running tap water. The absorbance of the dark green colour developed was measured at 620 nm.

3.3.2 Estimation of Total Lignin Content

Total lignin content present in the lignocellulosic wastes (sugarcane bagasse and *Eicchornia crassipes* biomass) was estimated by the method of Morrison (1972).

Reagents

Solution A, 25% acetyl bromide in glacial acetic acid,

Solution B, 2N NaOH solution in 25 ml of acetic acid,

Solution C, 0.5M hydroxylamine hydrochloride,

Procedure

Briefly, sample (2 mg) was added with 5 ml of solution A in stoppered tubes followed by incubation at 70°C for 30 min. Samples were cooled and added with 4.5 ml of solution B. The volume of the solution was then made upto about 200 ml and 8 ml of reagent C was added, volume was then finally made upto 250 ml with glacial acetic acid. The contents were shaken well and kept at room temperature for 1 h followed by measuring the absorbance at 200 nm.

3.3.3 Estimation of Total Reducing Sugar as Glucose

Total reducing sugars were measured by the dinitrosalicylic acid (DNS) method (Miller, 1959).

DNS reagent

(i) 1gm dinitrosalicylic acid,

(ii) 0.2 gm phenol,

(iii) 0.05 gm sodium sulfite,

(iv) 18.2 gm sodium potassium tartrate (Rochell's salt).

Dissolved the above components sequentially in 100 ml of 1% sodium hydroxide solution. Mixture was shaken for 5 min and filtered through Whatman paper (No. 1). The reagent was stored in dark at 4°C for future use.

Procedure

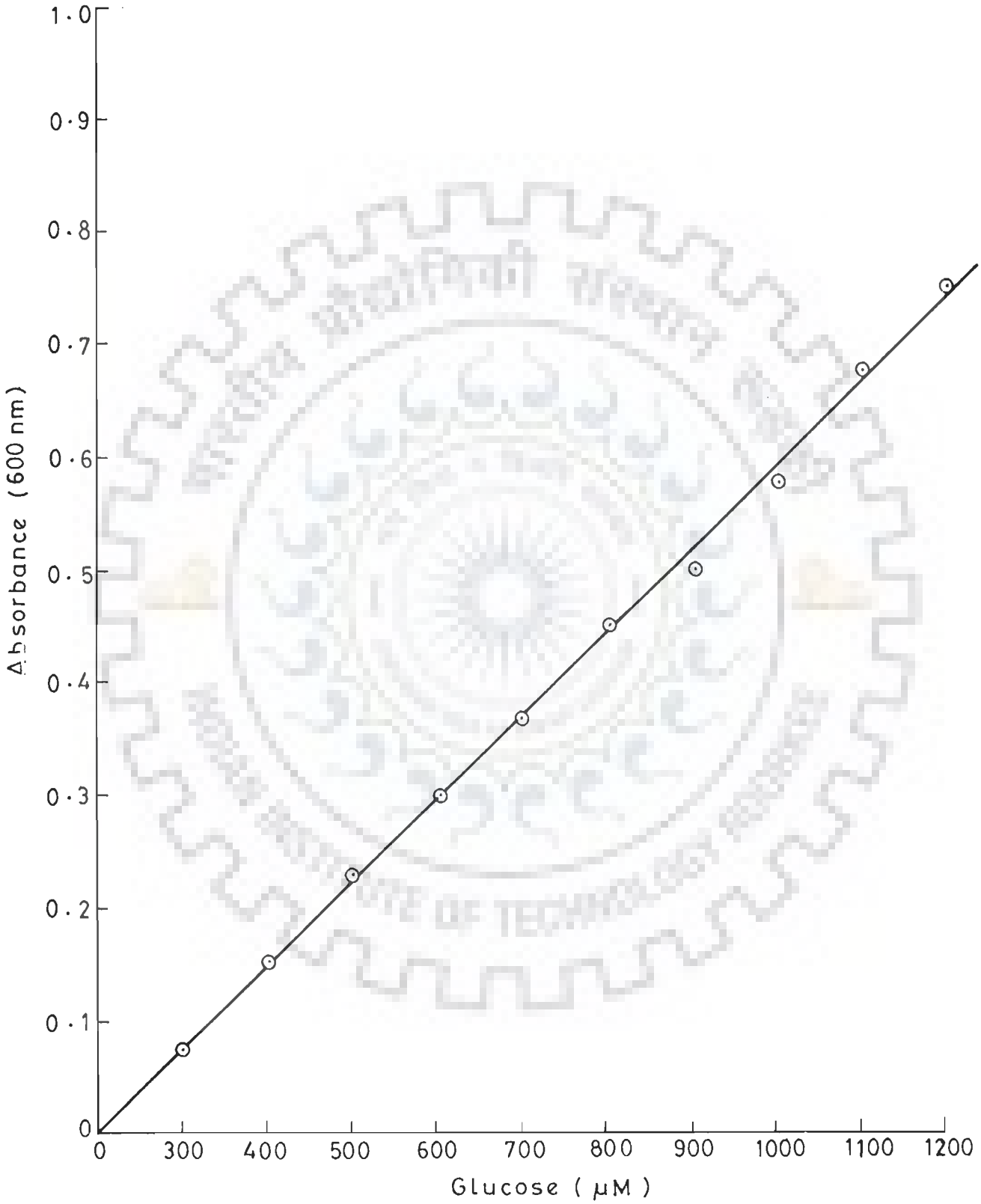
1 ml of sample (containing 0.2 to 2.0 mg of glucose) was mixed with 1 ml of 50 mM citrate buffer (pH 4.8). 3 ml of the DNS reagent was added followed by quick stirring for 15 min in the boiling water bath. Reaction was cooled to room temperature and diluted to 10 ml; the absorbance of the dark yellow colour developed was measured at 600 nm.

3.3.4 Estimation of Endoglucanase (CMCase) Activity

Carboxymethylcellulase(CMCase) activity was determined as described by Mandel (1975). Briefly, the assay mixture in a total volume of 2 ml contained 1 mM carboxymethylcellulose (CMC) in 50 mM citrate buffer (pH 4.8) and 0.5 ml of the supernatant obtained from fermentation broth as the source of enzyme. The mixture was incubated at 50°C for 30 min. The reducing sugar released was measured using DNS reagent as described (Miller,1959). The enzyme activity was expressed as μmol of glucose liberated min^{-1} ml^{-1} of the enzyme.

3.3.5 Estimation of Exoglucanase (FPase) Activity

Filter paper (FPase) activity was determined as described (Rajendran et al.1994). Briefly, the assay mixture (2 ml) contained 50 mg of Whatman No.1 strip (1 x 6 cm) in 1 ml of 50 mM citrate buffer (pH 4.8) and 0.5 ml of the supernatant obtained from fermentation broth as the source of enzyme. The reaction mixture was incubated at 50°C for 30 min, reducing sugar released was estimated as described (Miller, 1959).


STANDARD CALIBRATION CURVE OF GLUCOSE
Absorbance (600 nm)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
300
400
500
600
700
800
900
1000
1100
1200
Glucose (μM)

3.3.6 Estimation of β-glucosidase Activity

β-glucosidase activity was estimated using p-nitrophenyl β-D-glucopyranoside (p-NPG) as the substrate (Menon et al. 1994). Briefly, the assay mixture in a total volume of 1 ml contained 50 mM of substrate in 50 mM citrate buffer (pH 4.8) containing 0.1ml of supernatant from the fermentation broth. The reaction mixture was incubated at 50°C for 30 min. Following incubation 2 ml of 4% sodium carbonate solution was added and p-nitrophenol released was measured at 410 nm. The enzyme activity was expressed as μmol of p-nitrophenol liberated min^{-1} ml^{-1} of enzyme.

3.3.7 Protein Estimation

Proteins were estimated according to Lowry et al., (1951) as described below,

Reagents

Reagent A, 2% Na_2CO_3 in 0.1M NaOH,

Reagent B, 0.5% $CuSO_4$ in 1% sodium potassium tartrate (freshly prepared),

Reagent C, 50 ml of reagent A mixed with 1.0 ml of reagent B,

Reagent D, 1N Folin-Ciocalteu's phenol reagent.

Procedure

Samples (0.1 ml) were mixed with 1 ml of 0.1 M NaOH and final volume was made upto 1.5 ml. To this 1.5 ml of reagent C was added and subjected for vortexing immediately. Reaction mixture was then incubated for 15 min at room temperature and subsequently added with 0.15 ml of reagent D, mixed and incubated for 15 min at room temperature. Absorbance of the samples was measured at 690 nm.

3.3.8 Estimation of Gluconic Acid (Gluconolactone)

Gluconic acid as gluconolactone was estimated by hydroxamate method of Hestrin as modified by Lien (1959).

Reagent

Solution A, 4 M hydroxylamine hydrochloride (photographic grade),

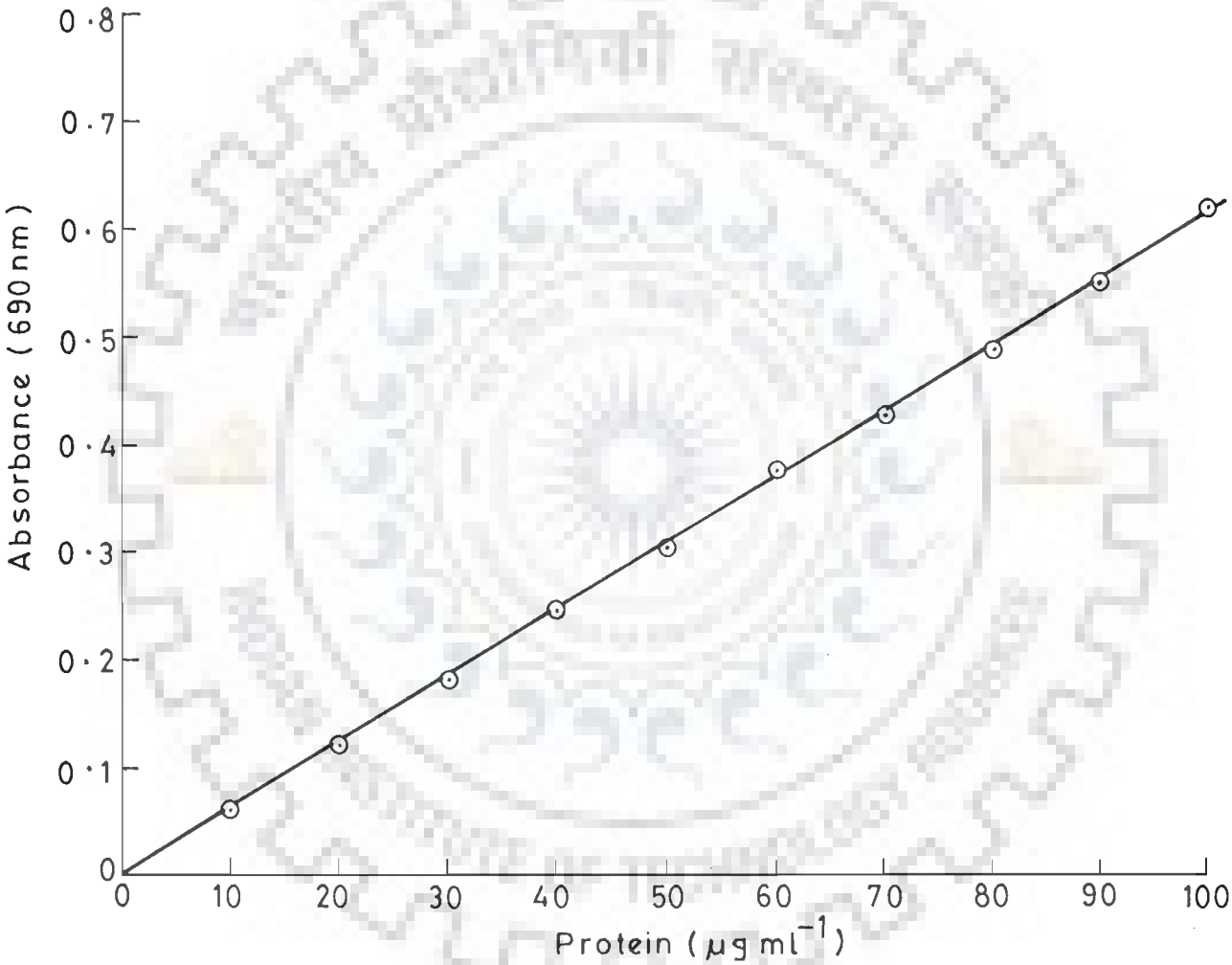
Solution B, 4 M sodium hydroxide,

Solution C, Solutions A and B were mixed in equal volumes and pH was adjusted to 8.0. This hydroxylamine reagent is stable for about 4 h,

Solution D, 4 N hydrochloric acid,

Solution E, 10% ferric chloride in 0.1N hydrochloric acid,

STANDARD CALIBRATION CURVE OF PROTEIN
(BOVINE SERUM ALBUMIN)



Procedure

1 ml of appropriately diluted sample was acidified with 4 N hydrochloric acid to a pH of 1.5-2.0 and then autoclaved (15 psi, 20 min) to convert the free gluconic acid into lactone. The samples were then cooled in a water bath and 2 ml of 4 N hydroxylamine reagent was added. This was followed by the addition of 1 ml of 4 N hydrochloric acid and 1 ml of the ferric solution (solution E). At this point the pH of the reaction mixture was 1.2. The absorbance of the blue colour developed was read in a Beckman model DU-6 spectrophotometer at 540 nm within 10 min after the colour was developed.

3.3.9 Estimation of Citric Acid

Total citric acid was measured as described by Marier and Boulet (1958). 0.1ml of the fermented sample was taken and made upto 1 ml with distilled water. This was followed by the addition of 1 ml pyridine and 5 ml of the acetic anhydride. The mixture was then incubated at 30°C for 30 min and the absorbance of the colour developed was measured at 420 nm.

3.3.10 Enzyme Purification

Eicchornia crassipes biomass was subjected for bioconversion using R_6m medium under conditions as defined for maximum enzymatic production. Broth (100 ml) after 8 days of bioconversion was collected and centrifuged (5000 rpm, 10 min). Clear supernatant was added with ammonium sulphate (80% saturation) (Yeoh et al. 1986; Singh et al.1990). Precipitate obtained by centrifugation (10000

rpm, 30 min) at 4°C was dissolved in 50 mM citrate buffer, pH 4.8 (Singh et al. 1990). Preparation was then dialysed for 14-16 h using 50 mM citrate buffer, pH 4.8 with buffer changes at appropriate intervals. Enzyme preparation was next subjected for gel filtration chromatography.

3.3.10.1 Gel filtration

5 gm Sephadex G-25 (for β-glucosidase) or Sephadex G-200 (for CMCase and FPase) was swollen overnight at room temperature in 30 ml of elution buffer (50 mM sodium citrate buffer, pH 4.8). Suspended beads were degassed and poured into vertically mounted column (1.3x15cm). Beads were allowed to settle and column was washed with three column volumes of elution buffer at a flow rate of 45 ml h^{-1}. The packed column was loaded with 1 ml of the dialysed protein samples and fractionated by passing the elution buffer at a flow rate of 40 ml h^{-1}. Fractions of 1.5 ml each were collected and the proteins present in the fractions were estimated and subjected for SDS-polyacrylamide gel electrophoresis.

3.3.10.2 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis

SDS-PAGE of the samples was performed according to Laemmli (1970). 12% polyacrylamide gels of 1.5 mm thickness were prepared for electrophoretic analysis.

Reagents

Solution A , 29.2% (w/v) acrylamide solution containing 0.8% (w/v) bisacrylamide (N, N′-methylene-bisacrylamide),

Solution B, resolving buffer, 1.5 M Tris-HCl, pH 8.8,

Solution C, stacking buffer, 0.125 M Tris - HCl, pH 6.8,

Solution D, 10% (w/v) SDS,

Solution E, 10% (w/v) ammonium persulphate (freshly prepared)

Solution F, TEMED (N,N,N`N`-tetramethylethylenediamine),

Electrophoresis buffer, 0.25M Tris, 0.192 M glycine, pH 8.3,

3.3.10.2.1 Preparation of resolving and stacking gels

Reagents	Resolving gel (12%) (ml)	Stacking gel (4%) (ml)
Solution A	12.0	2.66
Solution B	7.50	-
Solution C	–	5.00
Solution D	0.30	0.20
Solution E	0.15	0.10
Solution F	0.015	0.02
Water	10.035	12.2
	30.0	20.0

3.3.10.2.2 Casting of gel

A sandwich of glass plates (16 x 18 x 0.3 cm) was assembled by plastic spacers (1.5 mm) and the plates were held together by plastic clamps. The base of the gel mould was sealed. Resolving gel mixture was prepared by mixing all the components except ammonium persulphate and TEMED. Mixture was degassed for 15 min, ammonium persulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates, layered with a thin film of resolving gel buffer and leaving the sufficient space at the top for the stacking gel to be poured later. After polymerization of the resolving gel, the assembly was tilted to pour off the over layer, washed with sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly degassed and layered on the resolving gel, comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

3.3.10.2.3 Sample preparation

Fractions containing proteins were concentrated using Centricon (C-10) concentrators (Amicon, USA) and added with SDS-PAGE sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue. Samples were heated in a boiling water bath for 3-5 min and loaded on the gel for electrophoresis.

3.3.10.2.4 Electrophoresis

Samples containing 100 µg of the proteins were gently loaded on the gel using a micropipette. Electrophoresis was carried out at constant voltage (stacking at 60 V, resolving at 90 V) until the tracking dye reached to about 1 cm above from the bottom of the gel. Gel after electrophoresis was removed and stained overnight with gentle shaking in 0.1% coomassie brilliant blue R-250 in methanol:glacial acetic acid: water (5:2:5 v/v/v) at room temperature. Gel was then destained by washing in 10% isopropanol-7% acetic acid solution (Walker, 1996). Destaining was done so as to get the stained bands over the clear background. Gel was stored in 7% acetic acid until photographed.

3.3.10.2.5 Zymogram analysis of cellulase components

Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was performed for visualization of enzyme activities in situ as described (Schwarz et al.1989; Reyes and Noyola, 1998). Substrates to the final concentrations as indicated i.e. 0.1% carboxymethylcellulose (for CMCase activity) or 1 mM 4-methylumbelliferyl β-D-cellobioside (for FPase or MUC activity) or 1 mM 4-methylumbelliferyl β-D- glucoside (for β-glucosidase or MUG activity) were incorporated into separating gel before adding the ammonium persulphate and TEMED for polymerization. The enzyme samples were heated at 50°C for 10 min in 62.5 mM Tris-HCl buffer, pH 6.8 containing 5% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue. One set of samples was loaded in each half of the gel alongwith other standard markers. After electrophoresis, the gel

was sliced into two halves, one half was stained with coommasie brilliant blue R-250, whereas the second half was used for visualizing the enzymatic activities in situ. The second half of the gels was treated with 25% isopropanol for 25-30 min followed by incubation at 50°C for 25 min. For CMCase activity the gel was soaked with 0.1% (w/v) congo-red for 5 min with mild shaking. Excess dye was decanted and gel was washed with 5% (w/v) NaCl until excess stain was totally removed and the background was clear. On the other hand the fluorescent bands for FPase and β-glucosidase activities were visualized in the gels under ultraviolet light in the dark.

3.3.11 Scanning Electron Microscopy

The detailed morphological studies of lignocellulosic wastes (before and after treatment) and the fungal strains were carried out using scanning electron microscope (SEM, Leo 435 VP, England). Lignocellulosic substrates or the fungal mat were taken and subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with doubled distilled water. The samples were then treated with the alcohol gradients of 30%, 50%, 70%, 80%, 90% and 100% for dehydration. Samples were kept for 15 min each upto 70% alcohol gradient, there after treated for 30 min each for subsequent alcohol gradients. After passing through 100% alcohol samples were air dried and examined under SEM using gold shadowing technique (Gabriel,1982). Electron photomicrographs were taken at desired magnifications.

3.3.12 Statistical Analysis

All fermentations were carried out in triplicate and experimental results represent the mean of three identical fermentations. The statistical analysis (ANOVA) was performed as described by Somasegaran and Hoben (1994). 1% and 5% levels for ANOVA test was used for comparing the F ratio by using standard tables.

Table for Analysis of Variance (Somasegaran and Hoben, 1994)

Source of variation	Sum of square	Degree of freedom (df)	Mean Square (Ms)	F ratio
Between the Table (T)	SST	k-1	SST/(k-1)	$\frac{SST}{SSE} x \frac{bkn-k-b+1}{(k-1)}$
With in block (B)	SSB	b-1	SSB/(b-1)	$\frac{SSB}{SSE} x \frac{bkn-k-b+1}{(b-1)}$
Error (E)	SSE	Bkn-k-b+1	SSE/(bkn-k-b+1)	
Total	SS	Bkn-1		

df, degree of freedom; SST, treatment sum of squares; SSB, Block sum of squares; SSE, errors sum of squares; SS, total sum of squares; k, number of treatment; b, number of blocks (number of replicate per treatment); n, number of replicates per treatment per block.

Chapter 4

RESULTS

RESULTS

4.1 ISOLATION, PURIFICATION AND SCREENING OF STRAINS

Eleven fungal and eleven bacterial strains were isolated from the decomposing bagasse (from sugarcane industry) and paper waste materials by serial dilutions. Strains were purified by single colony isolation and screened in two phases. In phase I, the strains were initially analyzed for the growth on CMC-agar medium and also for the zone diameters formed on congo-red CMC-agar plate. The strains showing notable growth with significantly higher zone diameters were selected (Table 4,5). Based on the results observed, six (RK-2,3,7,8; SBR-3; PWR-3) of the isolated strains were selected for next stage of analysis. These strains were evaluated for extracellular production of cellulases using Mandels and Weber's modified medium with carboxymethylcellulose as the substrate under submerged state fermentation (Table 6). Among these strains, RK-3 strain, that resulted into higher zone diameter onto congo-red CMC-agar plate, was found to have maximum enzymatic activities. This strain therefore, was selected for further study. The strain was identified as *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi, India and denoted as *Aspergillus niger* RK-3. Maintenance of the culture was done by periodical transfers onto potato dextrose agar slants and was stored at 4°C for further study.

4.2 ANALYSIS OF CELLULASE PRODUCTION WITH SYNTHETIC SUBSTRATES

Five synthetic (commercially available) cellulosic substrates i.e. carboxymethylcellulose, cellulose acetate, phosphocellulose, diethylaminoethyl-cellulose and *Accacia* powder were evaluated for the production of cellulases.

Table 4 Screening of fungal strains isolated from decomposed bagasse (sugarcane industry waste) (RK-1 – RK-6) and the paper waste (RK-7 – RK-11)

Fungal strains	Growth on CMC-agar medium	Zone diameter on congo-red CMC- agar medium (mm)	Colour	
			Mycelia	Spores
RK-1	ND	ND	White	White
RK-2	++	1.8	White	Black
RK-3	+++	2.0	White	Black
RK-4	++	0.9	Yellowish White	Grey
RK-5	+	ND	Yellowish White	White
RK-6	ND	ND	Greenish White	Green
RK-7	++	1.8	Whitish yellow	Greenish
RK-8	++	1.2	Dirty white	Brown
RK-9	ND	ND	White	White
RK-10	ND	ND	White	Light grey
RK-11	ND	ND	White	Black

ND, Not detected; +, Poor growth; ++, Average growth; +++, Good growth

Table 5 Screening of bacterial strains isolated from decomposed bagasse (sugarcane industry waste) (SBR-1–SBR-6) and the paper waste (PWR-1– PWR-5)

Fungal strains	Growth on CMC-agar medium	Zone diameter on congo-red CMC- agar medium (mm)	Morphological features	
			Colour	Appearance
SBR -1	+	0.5	White	Round
SBR -2	+	0.2	White	Irregular
SBR -3	++	0.82	Yellowish	Spreading
SBR -4	ND	ND	Pink	Round
SBR -5	ND	ND	Reddish	Round
SBR -6	ND	ND	Whitish pink	Round
PWR-1	+	0.15	White	Round
PWR -2	ND	ND	Reddish	Round
PWR -3	+	0.25	Yellowish	Spreading
PWR -4	ND	ND	White	Irregular
PWR -5	ND	ND	Grey	Round

ND, Not detected; +, Poor growth; ++, Average growth; +++, Good growth.

Table 6 Analysis of enzymatic production by selected strains*[a] isolated from decomposing waste materials.

Strains	Enzymatic activity *[b] (IU ml^{-1})		
	CMCase	FPase	β– glucosidase
RK-.2	1.21 ± 0.04	1.00 ± 0.03	0.300 ± 0.001
RK-.3	1.32 ± 0.03	1.15 ± 0.04	0.405 ± 0.002
RK-7	1.10 ± 0.02	0.99 ± 0.02	0.105 ± 0.023
RK-8	0.88 ± 0.011	0.63 ± 0.02	0.066 ± 0.001
SBR-3	0.220 ± 0.012	0.068 ± 0.001	0.010 ± 0.0001
PWR-3	0.213 ± 0.013	0.0831 ± 0.001	0.011 ± 0.0001

*a, Strains were selected based on their growth on CMC-agar medium and zones formed onto congo-red CMC-agar medium.
*b, Activities were measured after 8 days of incubation.

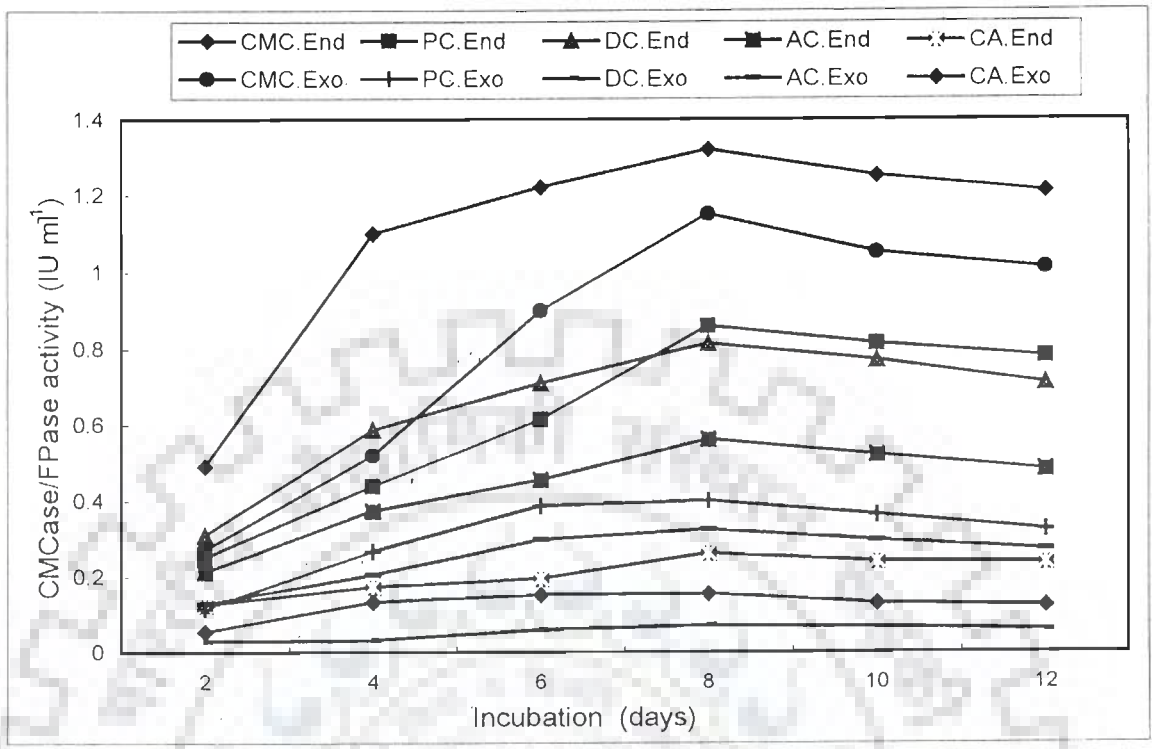
Production of cellulases was determined under submerged fermentation conditions using Mandels and Weber's modified medium supplemented with 1% of the cellulosic substrate. The extracellular enzymes i.e. endoglucanase or carboxymethylcellulase (CMCase), exoglucanase (FPase) and β-glucosidase were estimated using carboxymethylcellulose, filter paper (Whatman No.1) and para-nitrophenyl-β D-glucopyranoside (p-NPG) as the substrates respectively. The results presented in Fig. 3a,b showed that among the substrates used, carboxymethylcellulose was most suited cellulosic substrate for production of CMCase (1.32 IU), FPase (1.15 IU) and β-glucosidase (0.405 IU) after 8 days of incubation. The levels of all the three enzymatic components and soluble proteins continued increasing and showed maximum production after 8 days of incubation at 30°C, production level decreased on subsequent incubation. Among the other substrates phosphocellulose and *Accacia* powder also induced substantial levels of CMCase, FPase, and β-glucosidase, while, DEAE-cellulose induced CMCase and β-glucosidase levels significantly, whereas, FPase level remained lower with diethylaminoethyl-cellulose.

4.2.1 X-Ray Diffraction and Adsorption Analysis of Synthetic Cellulosic Substrates

Substrates were subjected for X-ray diffraction analysis to define the crystalline structure of these substrate and their suitability for enzyme production. Fig. 4a-e shows the X-ray diffractograph of the synthetic cellulosic substrate with peak at $2\theta = 21\text{-}23^{\circ}$ representing the degree of crystallinity of these substrates.

Fig. 3 **Production of CMCase, FPase (a), β-glucosidase and total soluble proteins (b) using commercially available synthetic cellulosic substrates.**

Mandels and Weber's modified medium containing 1% (w/v) of substrate was inoculated with *Aspergillus niger* RK-3. Samples at indicated intervals were assayed for cellulases and proteins levels, (CMC, Carboxymethylcellulose; PC, Phosphocellulose; DC, DEAE-Cellulose; AP, *Acaccia* powder; CA, Cellulose acetate; End, Endoglucanase (CMCase); Exo, Exoglucanase (FPase); β-glu, β-glucosidase ; Pro. Protein.


CMC.End
PC.End
DC.End
AC.End
CA.End
CMC.Exo
PC.Exo
DC.Exo
AC.Exo
CA.Exo
CMCase/FPase activity (IU ml-1)
Incubation (days)
a

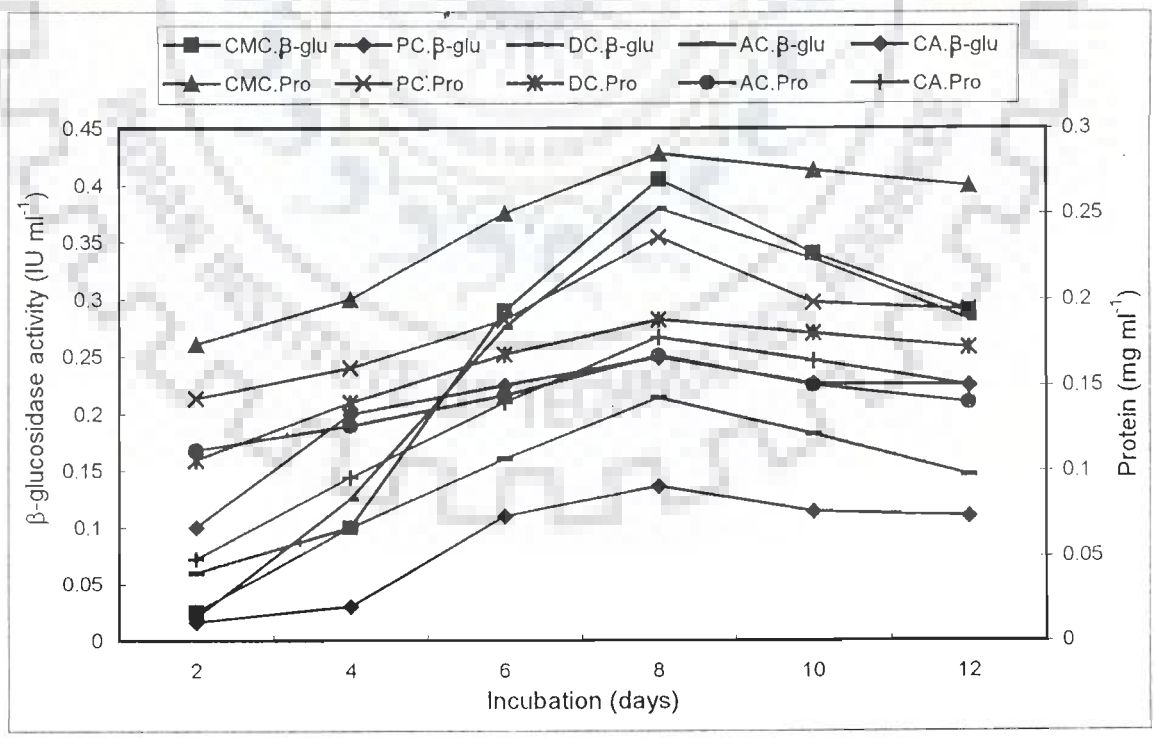

CMC.β-glu
PC.β-glu
DC.β-glu
AC.β-glu
CA.β-glu
CMC.Pro
PC.Pro
DC.Pro
AC.Pro
CA.Pro
β-glucosidase activity (IU ml-1)
Protein (mg ml-1)
Incubation (days)
b

Fig. 3

Fig. 4 **X-ray diffractographic analysis representing the crystallinity of synthetic cellulosic substrates.**
a, Carboxymethylcellulose; b, Phosphocellulose; c, DEAE-Cellulose; d, Cellulose acetate; e, *Accacia* powder

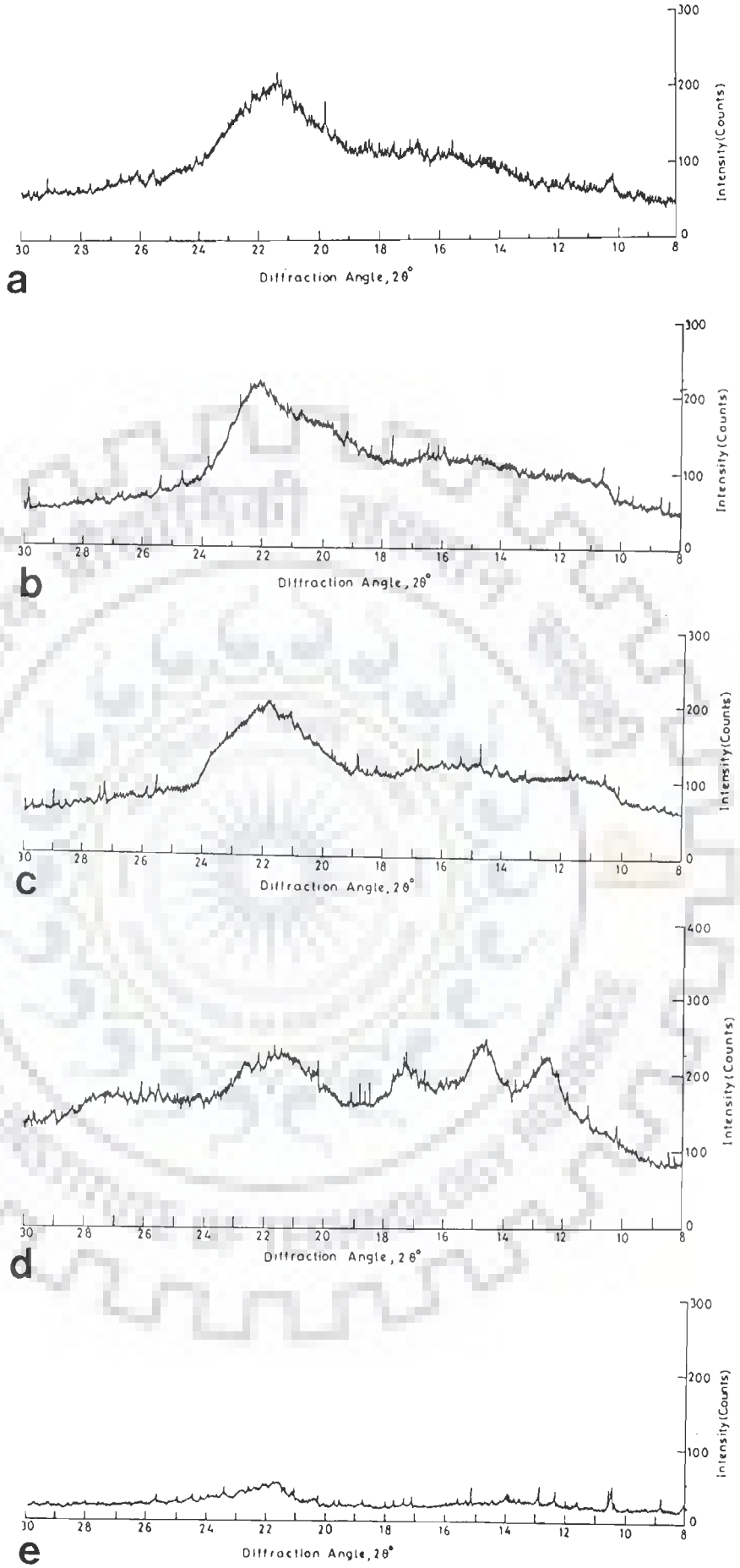

a
b
c
d
e
Intensity(Counts)
Diffraction Angle, 2θ°
300
200
100
0
400
30
28
26
24
22
20
18
16
14
12
10
8

Fig. 4

Higher intensity counts were observed for carboxymethylcellulose with decreased crystalline index as compared to the crystalline index of the other cellulosic substrates used. It therefore can be inferred that carboxymethylcellulose is less crystalline, more amorphous and therefore more susceptible to enzymatic attack on its surface as compared to other synthetic substrates. Interestingly, *Accacia* powder that appeared to be more crystalline. showed higher levels of CMCase and FPase as compared to the production of these enzymes with cellulose acetate apparently with lesser crystallinity. This has led to further investigation for the adsorption and saccharification pattern of the substrates. The observations indicated that adsorption of enzymatic proteins was less with cellulose acetate (30%) as compared with *Accacia* powder (43%) (Fig. 5a). Similarly the higher saccharification was observed with that of *Accacia* powder (28%) as compared with cellulose acetate (21%). The carboxymethylcellulose showing higher enzymatic levels also resulted into higher adsorption (52%) and saccharification (40%) as compared to the other substrates (Fig. 5b). These observations thus indicate that crystallinity to a greater extent accounts for the adsorption of enzymatic proteins on to cellulosic fibres. This subsequently promotes the substrate hydrolysis resulting into higher cellulolytic activities.

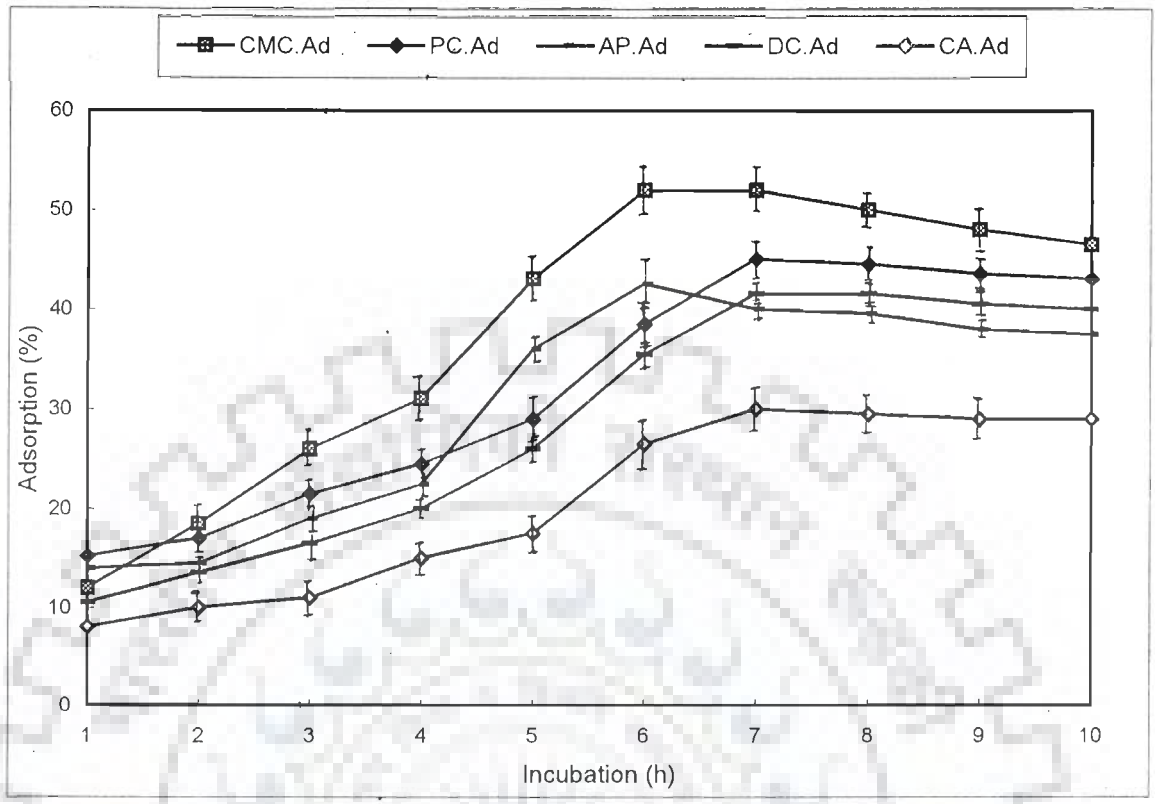
4.3 EVALUATION OF THE PRETREATMENT OF LIGNOCELLULOSIC SUBSTRATES

Cellulase derived bioconversion of the cheap and easily available cellulosic waste materials into fermentable sugars can be of utmost significance for industrial applications. The development of such process is a major objective of research at

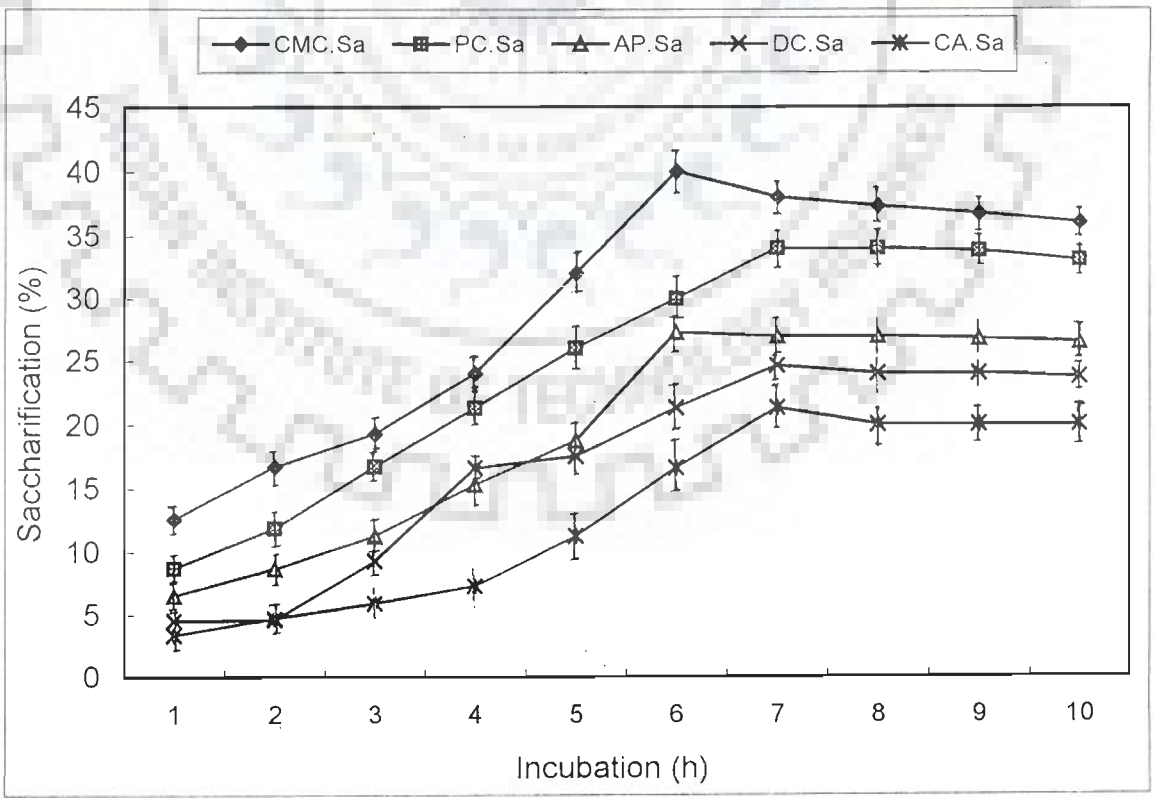
Fig. 5 **Adsorption behavior of cellulase proteins and relative saccharification of the synthetic cellulosic substrates at 50°C.**

a, Adsorption (Ad) with: Carboxymethylcellulose (CMC); PC, Phosphocellulose (PC); *Accaica* powder (AP); Cellulose acetate (CA); DEAE-Cellulose (DC).

b, CMC, Saccharification (Sa) with: Carboxymethylcellulose (CMC); Phosphocellulose (PC); *Accaica* powder (AP); Cellulose acetate (CA); DEAE-Cellulose (DC).


CMC.Ad
PC.Ad
AP.Ad
DC.Ad
CA.Ad
Adsorption (%)
Incubation (h)
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10

a


CMC.Sa
PC.Sa
AP.Sa
DC.Sa
CA.Sa
Saccharification (%)
Incubation (h)
0
5
10
15
20
25
30
35
40
45
1
2
3
4
5
6
7
8
9
10

b

Fig. 5

the present time. However, enzymatic hydrolysis of cellulose is restricted due to the crystalline structure of cellulose microfibrils that are aggregated and embedded within the lignified cell wall matrix. In order to increase the enzymatic susceptibility, the two lignocellulosic materials sugarcane bagasse and *Eicchornia crassipes* biomass selected for this study were subjected for pretreatment to expose and to separate the elementary cellulose microfibrils from the lignin counterpart of the lignocellulosic material.

4.3.1 High Pressure Steaming Followed By Alkali Pretreatment

The composition of cellulose, hemicellulose and lignin in the sugarcane bagasse, and water hyacinth *(Eicchornia crassipes)* biomass, obtained after high pressure steaming at 180-190°C for 30 min followed by alkali treatment was determined. Results obtained indicated that cellulose, hemicellulose and lignin contents are comparable in both lignocellulosic sources i.e. sugarcane bagasse and *Eicchornia crassipes* biomass (Table 7). The sugarcane is one of the major crops in the northern region (this region) of India and bagasse is thus generated as lignocellulosic waste material in abundance. This waste material has no further application and is subjected for burning. The water hyacinth (*Eicchornia crassipes*) with a significant amount of cellulose (48.3%) is found in the lakes, ponds and rivers and is prevalent in the many parts of India. The widespread occurrence of this biomass in aquatic resources not only affects the water quality but also the water content and current of the rivers and other resources.

Table 7 Composition of sugarcane bagasse and *Eicchornia crassipes* biomass lignocellulosic waste materials

Constituents	Composition (%)	
	Sugarcane bagasse	*Eicchornia crassipes*
Hemicellulose	16.0 ± 2.1	14.50 ± 2.0
Cellulose	54 ± 3.3	48.3 ± 3.4
Lignin	23 ± 2.8	18.60 ± 1.8
Material loss	7.0 ± 1.5	18.6 ± 1.2

4.3.1.1 X-ray diffraction analysis of lignocellulosic substrates

The crystallinity pattern for lignocellulosic substrates after pretreatment was studied using X-ray diffraction analysis. The peak at $2\theta = 23^0$ shows the degree of the crystallinity in the untreated lignocellulosic materials. A significant increase in the peak size was noticed after high pressure steaming followed by alkali pretreatment (Fig. 6a, b). The observations indicated that crystalline structure of these substrates was damaged due to lignin removal by alkali pretreatment as evident from the reduced crystalline index obtained after the treatment. The material that was therefore transformed into less crystalline or amorphous form thus was possibly susceptible to enzymatic attack for the bioconversion of the cellulosic fibres.

4.4 PRODUCTION OF CELLULASES IN SUBMERGED STATE FERMENTATION

In an attempt to fully exploit and to economize the process of cellulosic bioconversion, effort was made towards utilizing the pretreated sugarcane bagasse and water hyacinth (*Eicchornia crassipes*) biomass for the production of cellulases. The production was compared with the untreated substrates under submerged fermentation conditions using 1% of the cellulosic substrates. The production of extracellular enzymes was analyzed by using carboxymethylcellulose (for CMCase), filter paper (Whatman No.1) (for FPase) and para-nitrophenyl β-D-glucopyranoside (p-NPG) (for β-glucosidase) for assaying the enzymatic activities.

Fig. 6 **X-ray diffractographic analysis representing crystallinity of sugarcane bagasse (a), *Eicchornia crassipes* biomass (b).** Peak 1, represents the pattern of the untreated substrate. Peak 2, represent the pattern of the steam-alkali treated substrate.

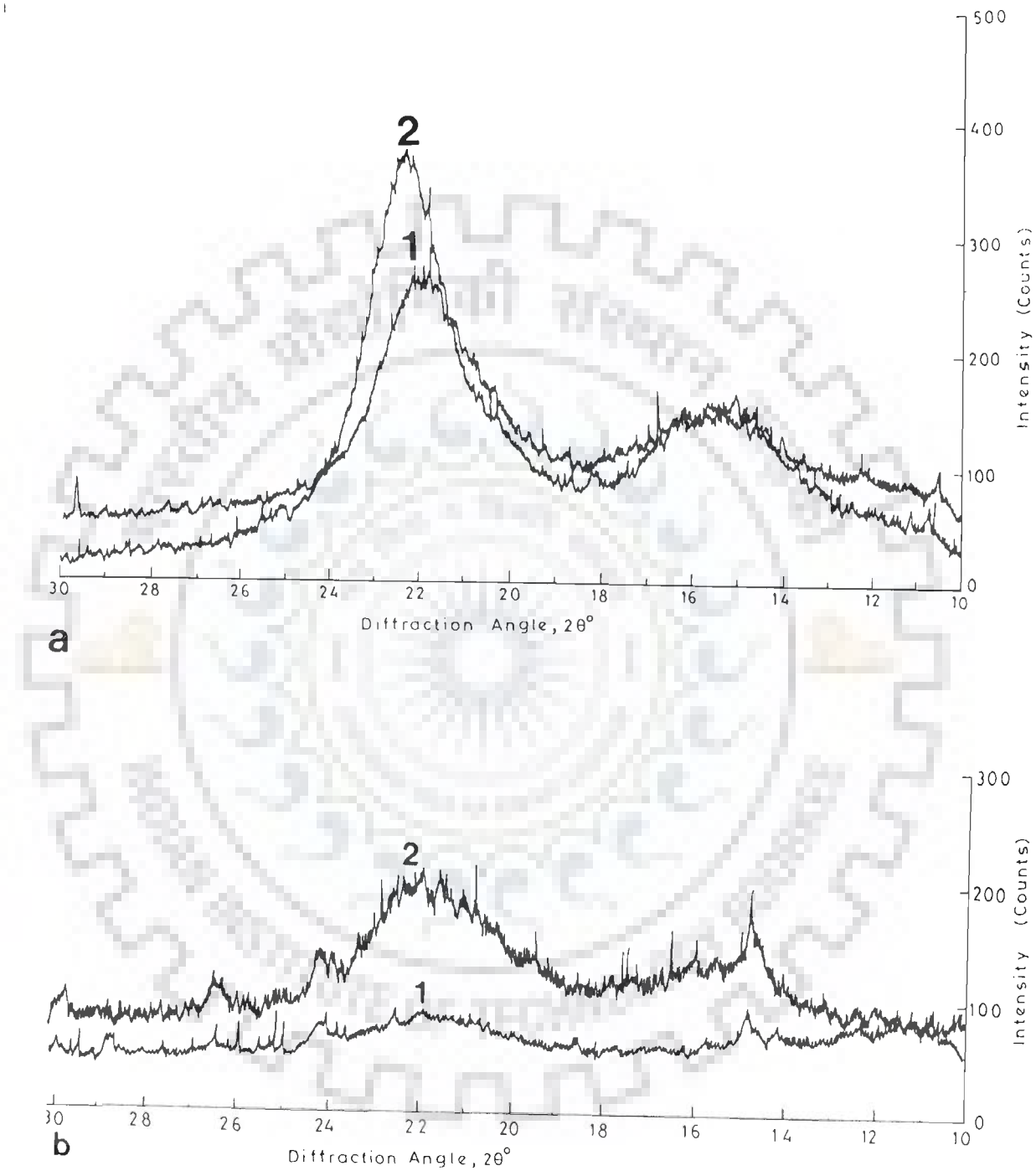

2
1
500
400
300
200
100
0
Intensity (Counts)
30
28
26
24
22
20
18
16
14
12
10
Diffraction Angle, 2θ°
a
2
1
300
200
100
0
Intensity (Counts)
30
28
26
24
22
20
18
16
14
12
10
Diffraction Angle, 2θ°
b

Fig. 6

4.4.1 Evaluation of Sugarcane Bagasse as Substrate

Cellulase production was studied with sugarcane bagasse as the substrate as it is predominantly present in this region and largely a waste. Maximum CMCase, FPase and β-glucosidase activities (2.08 IU, 1.64 IU, 0.425 IU) were obtained after 8 days of incubation with treated sugarcane bagasse (Fig. 7a). The activities were 48.5%, 49% and 41.1% higher compared to CMCase, FPase and β-glucosidase activities, obtained with the untreated substrate, (Fig. 7b). The production decreased on further incubation. Similarly higher level of protein (0.831 mg ml^{-1}) and cellulose degradation (45.1%) was observed with treated sugarcane bagasse which was 35.5% and 45.2% higher for protein production and cellulose degradation with respect to the untreated substrate respectively.

4.4.2 Evaluation of Water Hyacinth (*Eicchornia crassipes*) as Substrate

Water hyacinth is a widely prevalent biomass found in aquatic resources in the many parts of India. This biomass poses a serious threat to water quality and quantity of the lakes, ponds and the rivers. There is no significant use of this abundant biomass except its sporadic use as the animal feed. Attention therefore was focused on this biomass for its use as a potential cellulosic material (having 48% cellulose) for the production of cellulases leading to its bioconversion into fermentable sugars for industrial purposes. Analysis of the cellulase levels showed significant levels of CMCase (1.60 IU), FPase (1.68 IU) and β-glucosidase (0.216 IU) were produced when treated *Eicchornia crassipes* biomass (1%) was used as sole source of carbon in the fermentation medium (Fig. 8a). The activities

Fig. 7 **Production of cellulases and relative cellulose degradation by *Aspergillus niger* RK-3 with treated (a) and untreated (b) sugarcane bagasse.**

Mandels and Weber's modified medium containing 1% (w/v) of sugarcane bagasse as the substrate was inoculated with *Aspergillus niger* RK-3. Samples at indicated intervals were assayed for levels of enzymatic production and cellulose degradation.

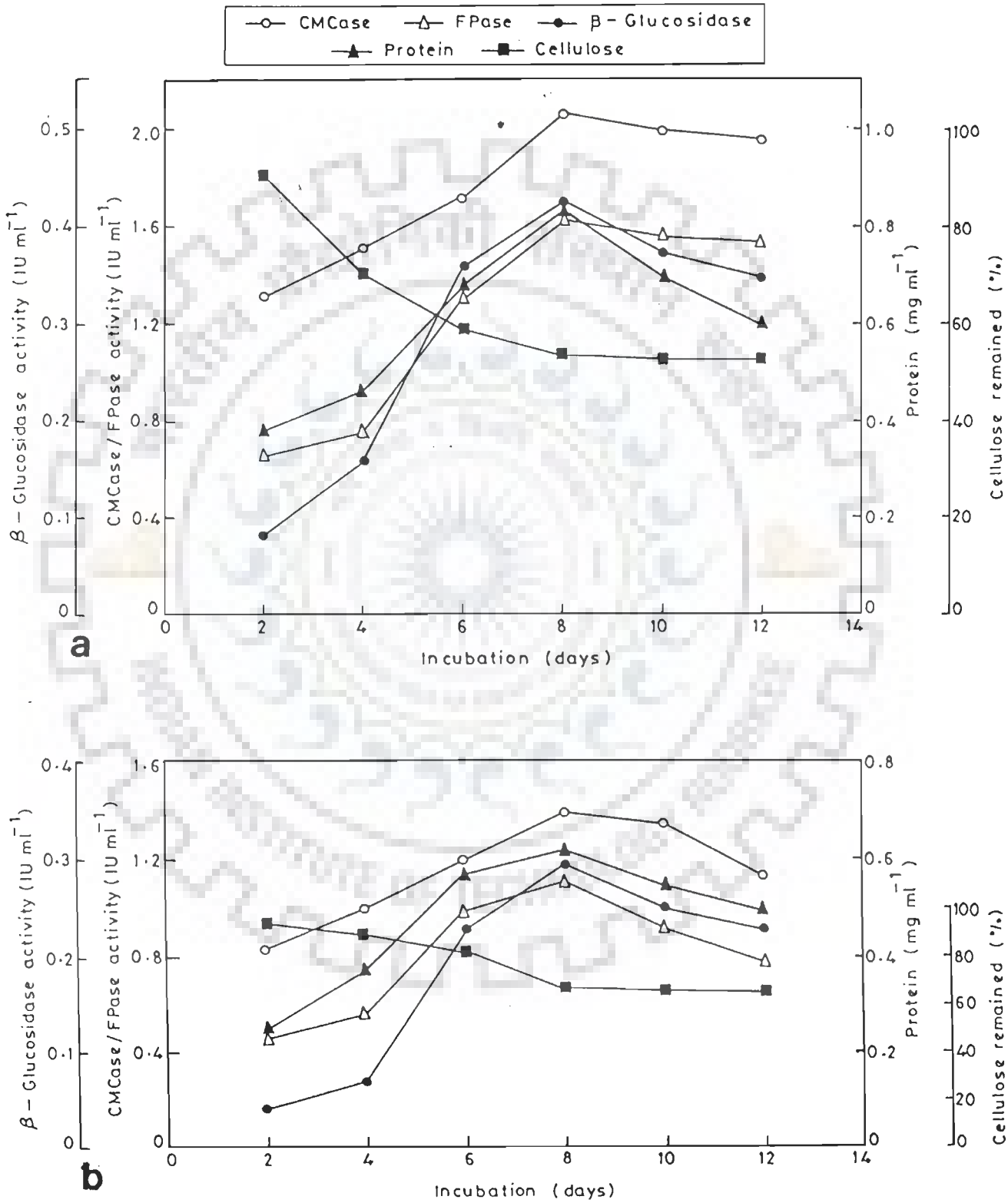

CMCase
FPase
β - Glucosidase
Protein
Cellulose
β - Glucosidase activity (IU ml⁻¹)
CMCase / FPase activity (IU ml⁻¹)
Protein (mg ml⁻¹)
Cellulose remained (%)
Incubation (days)
a
b

Fig. 7

Fig. 8 **Production of cellulases and relative cellulose degradation by *Aspergillus niger* RK-3 with treated (a) and untreated (b) *Eicchornia crassipes* biomass.**

Mandels and Weber's modified medium containing 1% (w/v) of *Eicchornia crassipes* biomass as the substrate was inoculated with *Aspergillus niger* RK-3. Samples at indicated intervals were assayed for levels of enzymatic production and cellulose degradation.

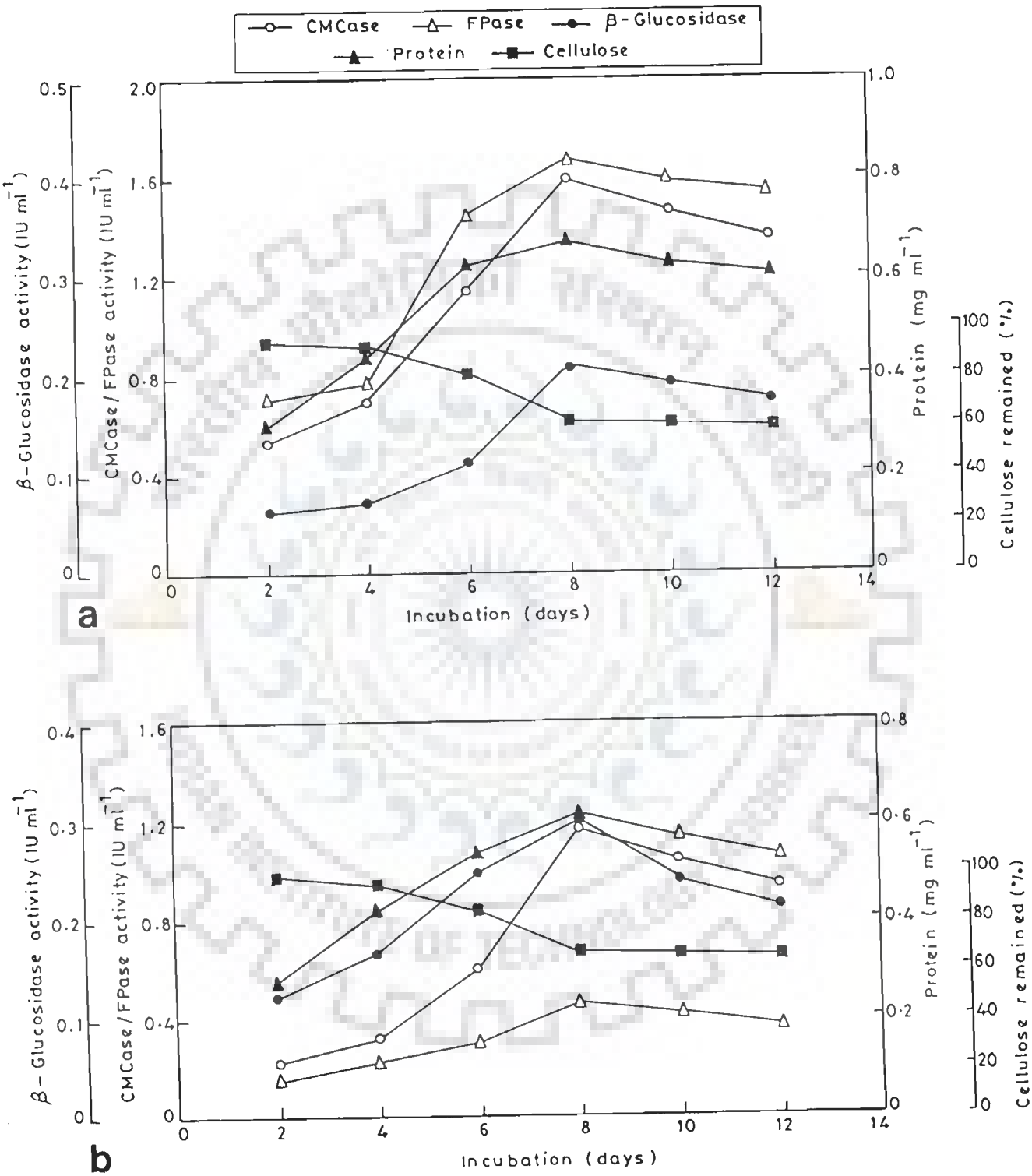

CMCase
FPase
β-Glucosidase
Protein
Cellulose
β-Glucosidase activity (IU ml⁻¹)
CMCase / FPase activity (IU ml⁻¹)
Protein (mg ml⁻¹)
Cellulose remained (%)
Incubation (days)
a
b

Fig. 8

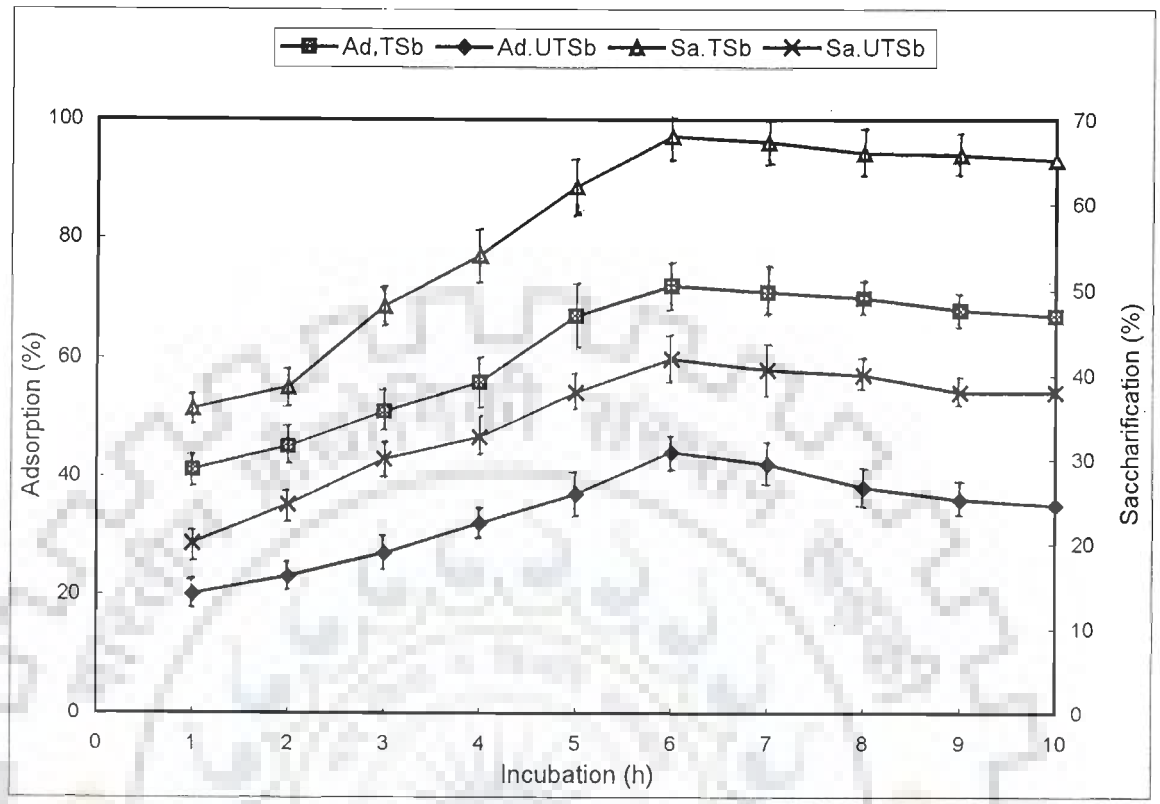
observed were 33.3%, 37.3% and 80% higher as compared to the activities observed with untreated substrate (Fig.8b). Concurrently higher level of protein (0.667 mg ml^{-1}) and cellulose degradation (37.9%) was observed with the treated *Eicchornia crassipes,* which was 10.9% and 26.0% higher, compared to protein production and cellulose degradation with untreated substrate, respectively.

These observations therefore suggested that steam-alkali pretreatment leads to the loss the ternary complex of the lignocellulosic materials due to lignin removal, thus reducing the crystallinity of the substrates. The substrate therefore becomes amorphous in nature (Fig 6a, b) and is highly susceptible to enzymatic attack, which results into its bioconversion to reducing sugars. Although both the substrates led to substantial levels of enzyme production, however sugarcane bagasse resulted into comparatively higher levels of production. Further, the adsorption studies were carried to analyze the differential levels of enzyme production from the treated and untreated lignocellulosic substrates used. The lignocellulosic substrates were taken and assayed for enzymatic adsorption and simultaneously for saccharification in the treated and untreated conditions. Extent of enzymatic adsorption was higher for both substrates i.e. sugarcane bagasse (71%), *Eicchornia crassipes* (65%) under treated conditions and in addition saccharification of sugarcane bagasse was relatively higher (66%) than the *Eicchornia crassipes* biomass (61%) under similar conditions (Fig 9a, b).

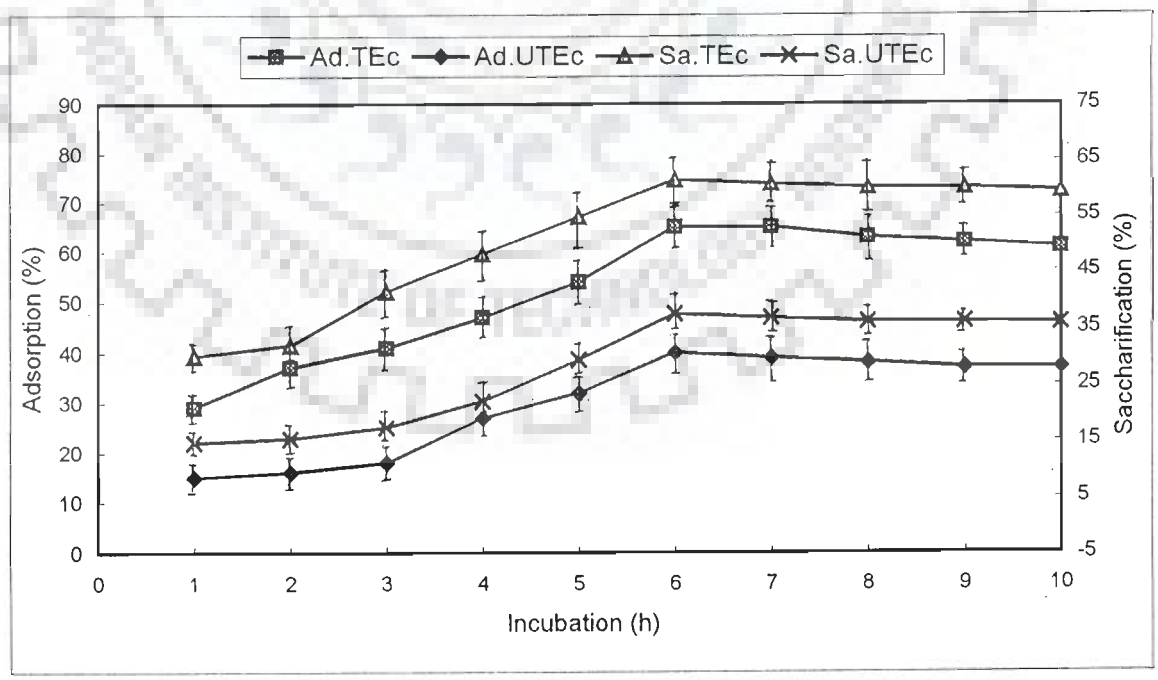
Fig. 9 **Adsorption behavior of cellulase proteins and the relative saccharification of sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b)**

Adsorption (Ad) with : treated sugarcane bagasse (TSb) and untreated sugarcane bagasse (UTSb); Saccharification (Sa) with: treated sugarcane bagasse (TSb) and untreated sugarcane bagasse (UTSb)

Adsorption (Ad) with : treated *Eicchornia crassipes* (TEc) and untreated *Eicchornia crassipes* (UTEc); Saccharification (Sa) with: treated *Eicchornia crassipes* (TEc) and untreated *Eicchornia crassipes* (UTEc).


Ad,TSb
Ad.UTSb
Sa.TSb
Sa.UTSb
Adsorption (%)
Saccharification (%)
Incubation (h)
0
20
40
60
80
100
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
9
10

a


Ad.TEc
Ad.UTEc
Sa.TEc
Sa.UTEc
Adsorption (%)
Saccharification (%)
Incubation (h)
0
10
20
30
40
50
60
70
80
90
-5
5
15
25
35
45
55
65
75
0
1
2
3
4
5
6
7
8
9
10

b

Fig. 9

4.2.3 Scanning Electron Microscopic Studies of Lignocellulosic Wastes

Scanning Electron Microscopic (SEM) analyses were performed to find out the morphological variations and the pattern of the fungal attack on the cellulosic substrates after the steam and alkali treatments. The SEM study revealed the sharp morphological variations after treatment of the lignocellulosic materials. The untreated substrates showed a rigid and compact structure with complex and hard epidermal layer known as cuticle (Fig.10a,c). On the other hand epidermal peeling was observed in treated substrates resulting into the removal of lignin and hemicellulose as compared to the untreated substrates (Fig.10b,d). The alkali treatment resulted into partial maceration of the compact tissues into simple fibres. Rapid growth of *Aspergillus niger* RK-3 mycelia was observed over the surface of the treated substrates (Fig.11b,d) compared to the slow growth over the surface of the untreated substrate (Fig.11a,c). The frequent appearance of small bodies attached with rib like structure with treated substrates (Fig.11b,d), indicated progressive consumption of organic matters by the fungus and possibly leaving behind the inorganic and other undigested elements.

4.5 ANALYTICAL EVALUATION OF FACTORS AFFECTING CELLULASE PRODUCTION BY *ASPERGILLUS NIGER* RK-3

4.5.1 Analysis of Physical and Biological Factors

The effect of physical (pH, temperature, incubation period, agitation speed) and biological factors (age and inoculum size) were studied. The production was estimated at the pH range 3.0-6.0. The results indicated the maximum enzymatic

Fig. 10 **Scanning electron micrographs of lignocellulosic substrates before and after the steam-alkali treatments. a, untreated sugarcane bagasse; b, treated sugarcane bagasse; c, untreated *Eicchornia crassipes* biomass; d, treated *Eicchornia crassipes* biomass.**

The morphological details of treated and untreated substrates were studied after washing, blotting and primary fixation followed by dehydration using alcohol gradients. The dried cellulosic fibers were examined under SEM (Leo-435 VP) using gold shadowing technique.

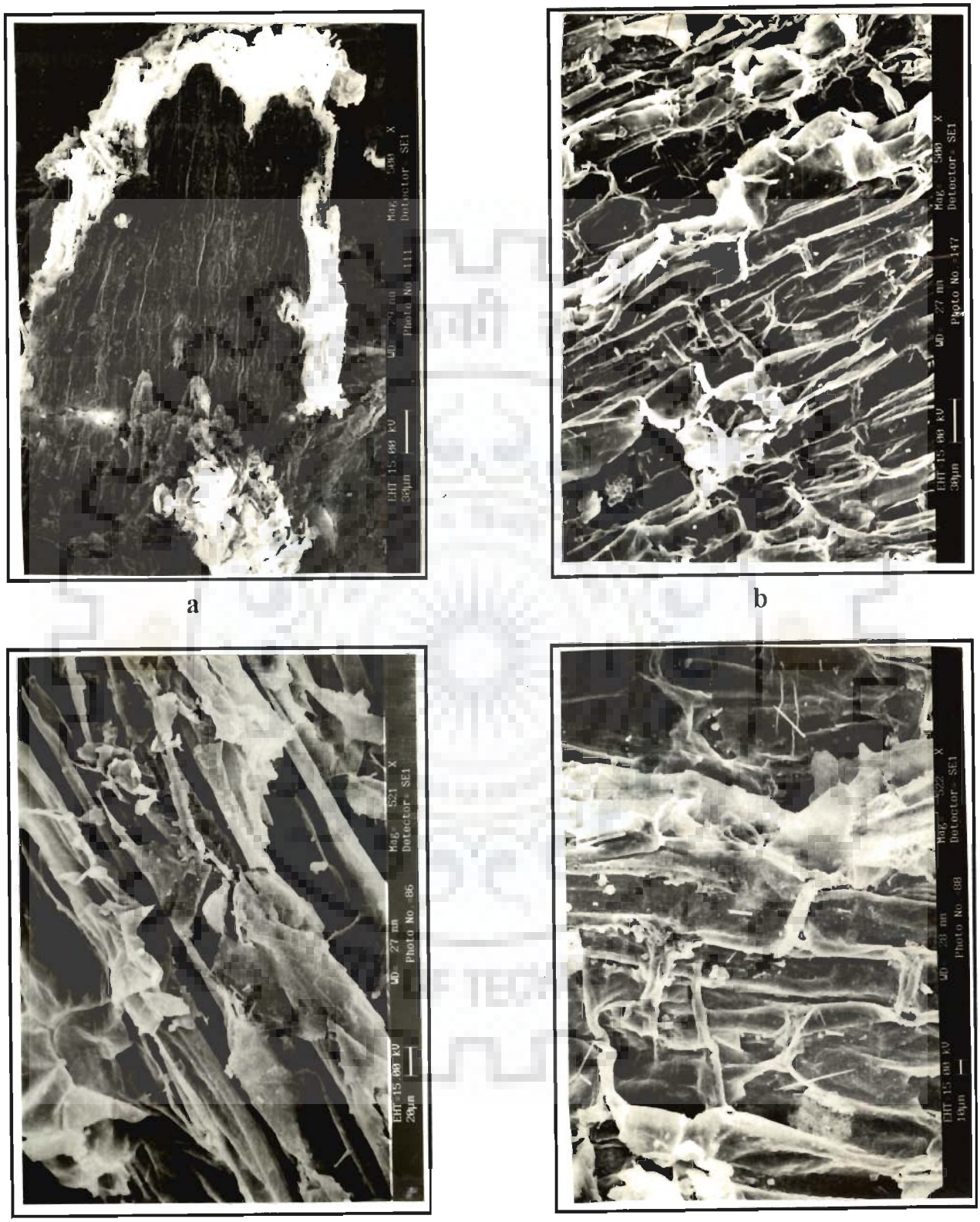


Fig. 10

Fig. 11 **Scanning electron micrographs representing colonization of fungal growth on treated and untreated lignocellulosic substrates.** a, growth of *Aspergillus niger* RK-3 on untreated sugarcane bagasse; b, growth of *Aspergillus niger* RK-3 on treated sugarcane bagasse; c, growth of *Aspergillus niger* RK-3 on untreated *Eicchornia crassipes* biomass; d, growth of *Aspergillus niger* RK-3 on treated *Eicchornia crassipes* biomass.

a

b

c

d

Fig. 11

activities at pH 4.8 (Fig. 12). The production levels of cellulases declined below and above this pH. Similarly a temperature range of 40-60°C was used for estimating the enzymatic activity that showed 50°C as the best suited temperature for activity measurements, however, a temperature of 30°C was found to be suitable for biomass accumulation (Fig.13). Variations in agitation speed also affected the biomass accumulation and an agitation speed of 130 rpm was favourable for higher biomass accumulation (Fig. 13). Inoculum levels is another major factor affecting cellulase production. An inoculum level of 5% led to increased production of cellulases (Fig 14), inoculum higher than 5% was inhibitory for cellulase production.

4.5.2 Analysis of Chemical Factors

The effect of various nutrients and chemical factors such a phenolics, organic acids and soluble sugars were analyzed for cellulase production. The physical and biological conditions derived earlier were utilized for these studies. The factor defined at every stage for maximum enzyme production was included as the medium constituent and medium thus designated severed as a control for next stage analysis of the factors affecting enzyme production on to subsequent steps.

4.5.2.1 Analysis of carbon sources

Production of cellulase in the presence of varying levels of cellulosic substrates i.e. sugarcane bagasse and *Eicchorinia crassipes* biomass was determined. The results denoted that the enzymatic activity increased with increasing

Fig. 12 Production of cellulases by *Aspergillus niger* RK-3 as a function of pH with treated cellulosic substrates. Sb, Sugarcane bagasse; Ec, *Eicchornia crassipes* biomass.

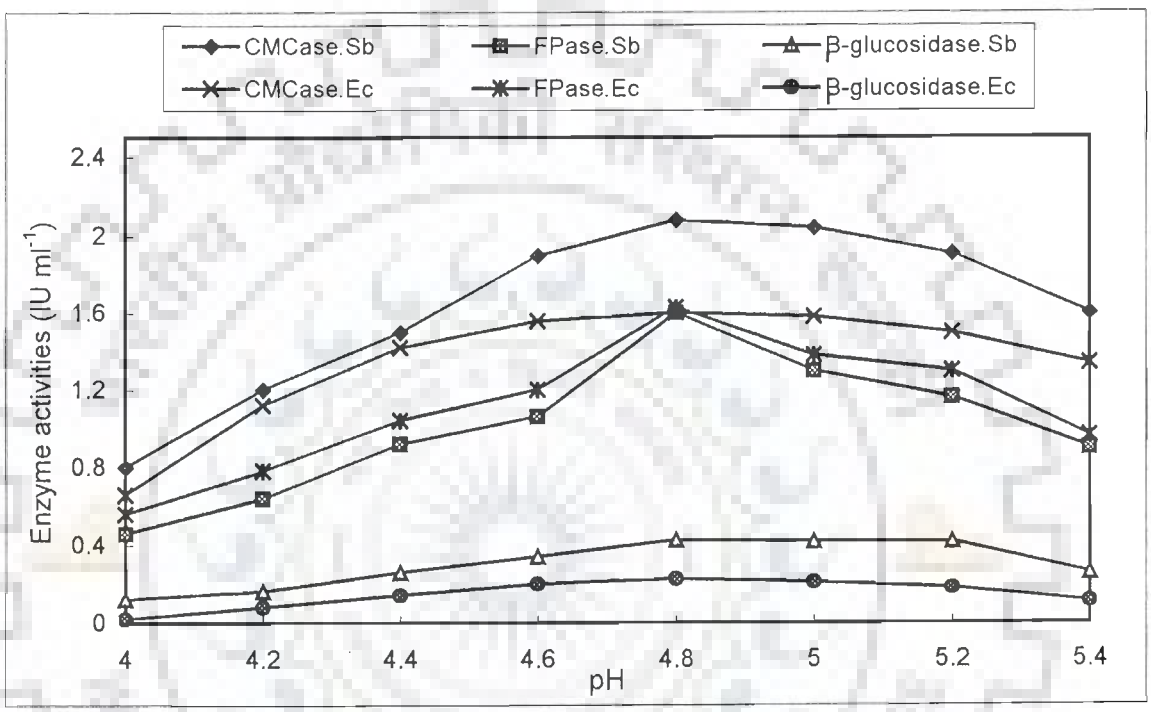

CMCase.Sb
FPase.Sb
β-glucosidase.Sb
CMCase.Ec
FPase.Ec
β-glucosidase.Ec
Enzyme activities (IU ml-1)
2.4
2
1.6
1.2
0.8
0.4
0
4
4.2
4.4
4.6
4.8
5
5.2
5.4
pH

Fig. 12

Fig. 13 Production of cellulases by *Aspergillus niger* RK-3 as a function of temperature and agitation with treated substrates. Sb, Sugarcane bagasse; Ec, *Eicchornia crassipes* biomass.

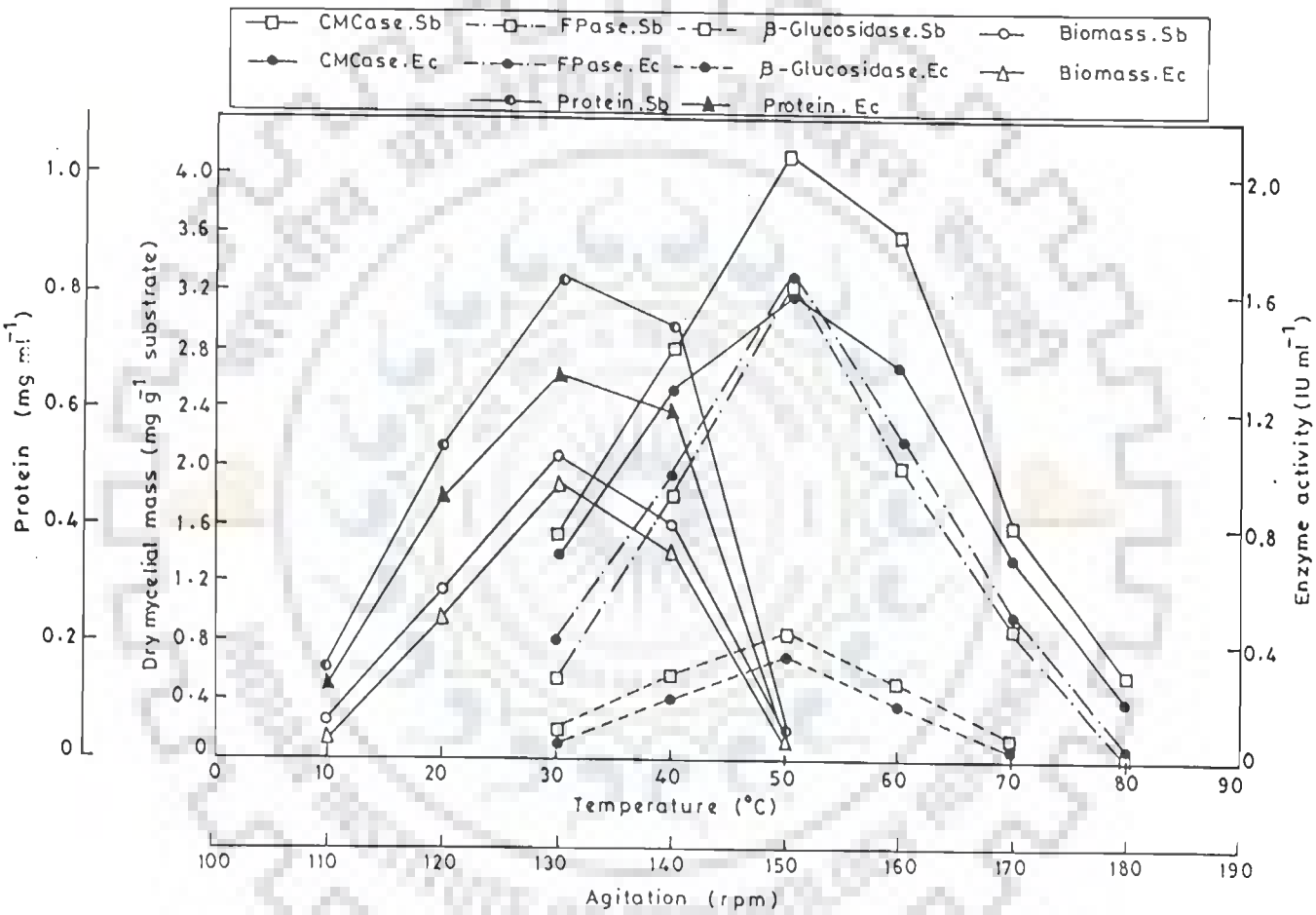

CMCase.Sb
FPase.Sb
β-Glucosidase.Sb
Biomass.Sb
CMCase.Ec
FPase.Ec
β-Glucosidase.Ec
Biomass.Ec
Protein.Sb
Protein.Ec
Protein (mg ml⁻¹)
Dry mycelial mass (mg g⁻¹ substrate)
Enzyme activity (IU ml⁻¹)
Temperature (°C)
Agitation (rpm)

Fig. 13

Fig. 14 **Production of cellulases by *Aspergillus niger* RK-3 as a function of inoculum level with treated cellulosic substrates. Sb, Sugarcane bagasse; Ec, *Eicchornia crassipes* biomass.**

Fig. 15 **Production of cellulases by *Aspergillus niger* RK-3 as a function of carbon source concentration with treated cellulosic substrates. Sb, Sugarcane bagasse; Ec, *Eicchornia crassipes* biomass.**

Mandels and Weber's medium (pH 4.8) supplemented with different amounts of carbon sources was inoculated with *Aspergillus niger* RK-3 and incubated under submerged conditions (130 rpm) for 8 days at 30°C. Samples following incubation were subjected for enzymatic estimation. The medium containing carbon concentration at 1% led to maximum enzyme production and hence designated as medium R_1m.

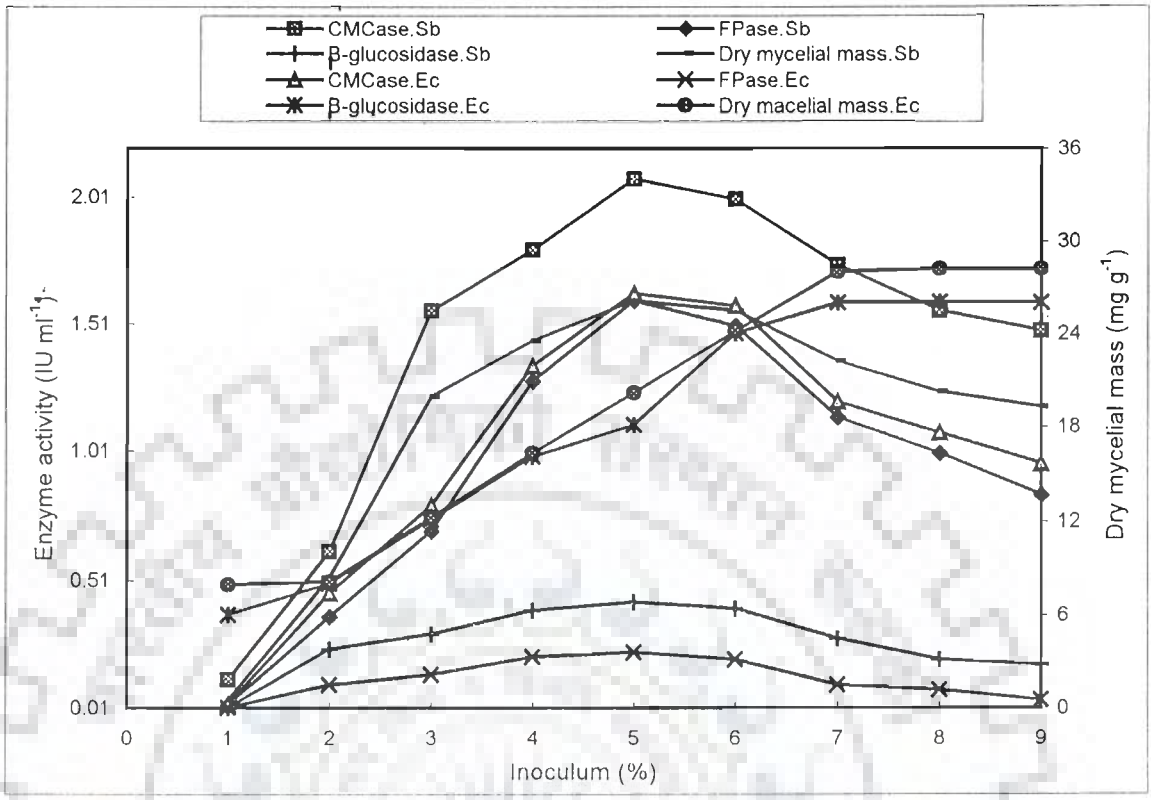

CMCase.Sb
FPase.Sb
β-glucosidase.Sb
Dry mycelial mass.Sb
CMCase.Ec
FPase.Ec
β-glucosidase.Ec
Dry macelial mass.Ec
Enzyme activity (IU ml-1)
Dry mycelial mass (mg g-1)
Inoculum (%)
2.01
1.51
1.01
0.51
0.01
36
30
24
18
12
6
0
0
1
2
3
4
5
6
7
8
9

Fig. 14

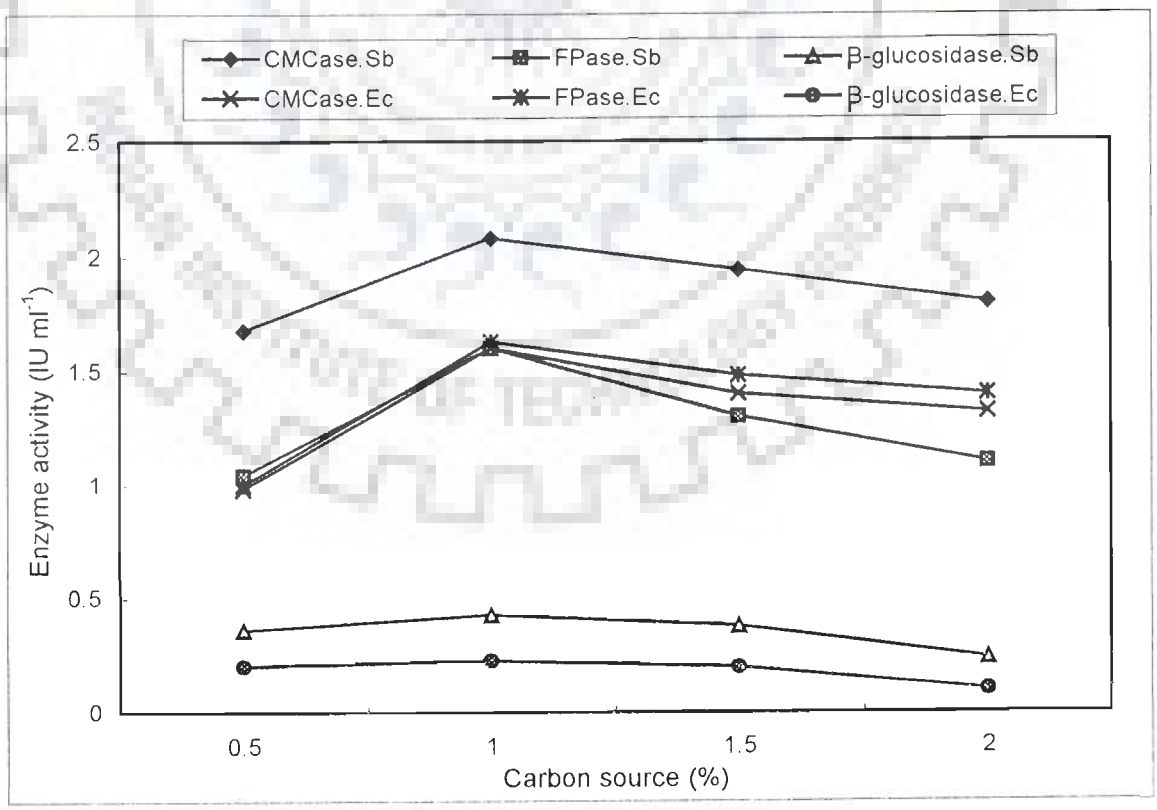

CMCase.Sb
FPase.Sb
β-glucosidase.Sb
CMCase.Ec
FPase.Ec
β-glucosidase.Ec
Enzyme activity (IU ml-1)
Carbon source (%)
2.5
2
1.5
1
0.5
0
0.5
1
1.5
2

Fig. 15

concentrations of substrate and yielding the maximum activity at 1% level of the substrate used. Activity gradually decreased thereafter (Fig. 15). The medium with defined components so far was designated as R_1 medium (R_1m).

4.5.2.2 Effect of nitrogen sources

The effect of different additional nitrogen sources (such as, ammonium chloride, ammonium ferrous sulfate, ammonium nitrate, sodium nitrate) added at different concentration (0.35-0.55 gl^{-1}) alongwith ammonium sulfate (2.1 gl^{-1}) as a major nitrogen source were analyzed. The presence of additional nitrogen sources did not influence the CMCase and FPase activity whereas a significant increase in β-glucosidase activity (51-105%) was observed with sugarcane bagasse and *Eicchornia crassipes* biomass in the presence of ammonium chloride added as an additional nitrogen for enzyme production (Fig.16a, b). Besides ammonium chloride, ammonium nitrate and sodium nitrate also showed a stimulatory effect on β-glucosidase activity. Ammonium ferrous sulfate on the other hand had inhibitory effect on β-glucosidase levels with both the substrates used. The medium containing the constituents as defined so far was designated as R_2 medium (R_2m).

4.5.2.3 Effect of phosphorus sources

The impact of adding some additional phosphorus sources (i.e. tetra-sodium pyrrophosphate, potassium dihydrogen phosphate, sodium β-glycero-phosphate and dipotassium hydrogen phosphate) at different concentrations (0.35-0.55 gl^{-1}) were analyzed. The phosphorus sources were added in addition to

Fig. 16 **Effect of nitrogen sources on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b). A, Ammonium chloride; B, Ammonium ferrous sulfate; C, Ammonium nitrate; D, Sodium nitrate**

R_1m medium supplemented with varying concentrations of nitrogen sources was inoculated with *Aspergillus niger* RK-3 under the conditions as described for Fig. 15. The medium composition yielding to maximum cellulase production was designated as medium R_2m.

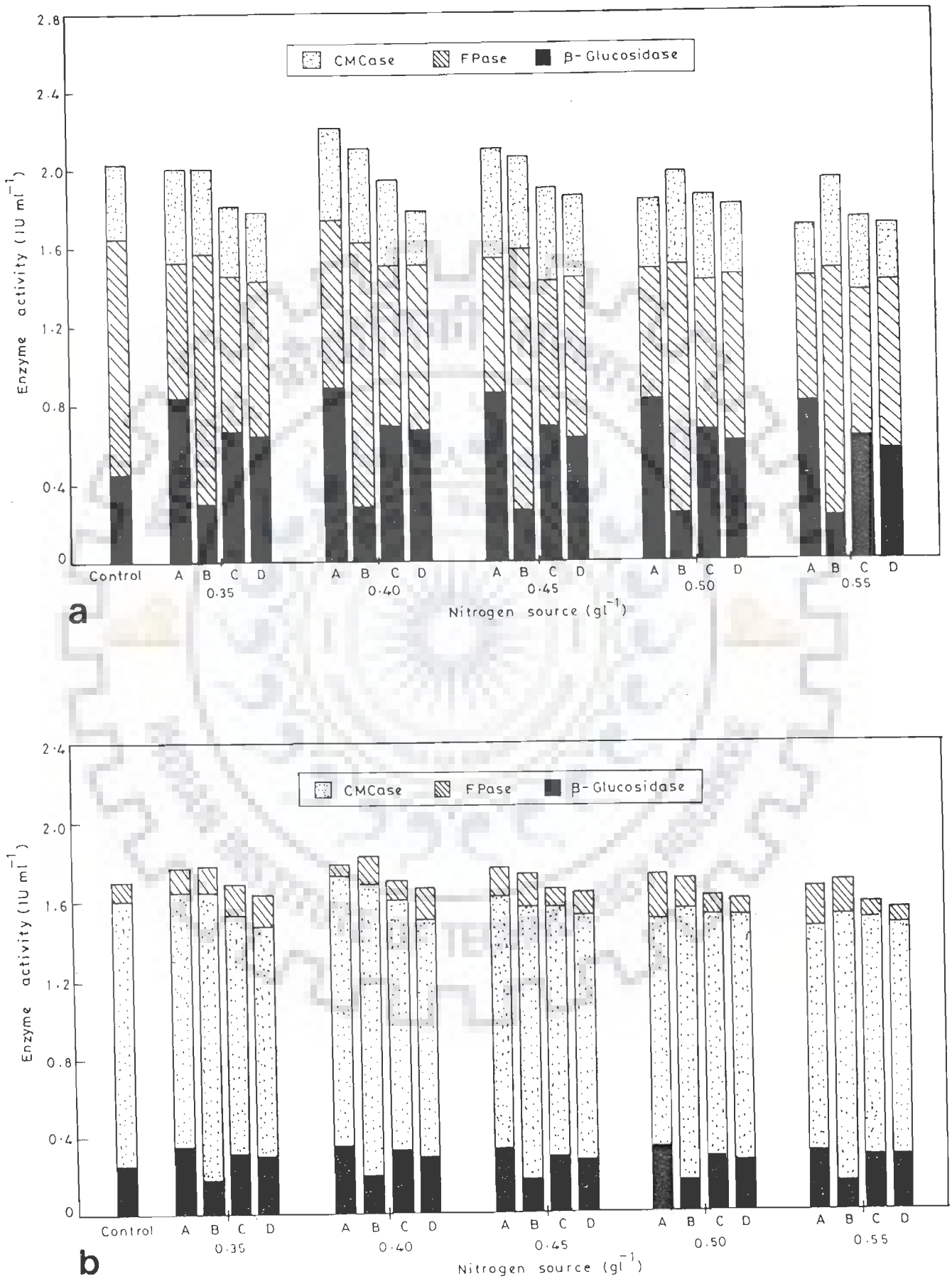

CMCase
FPase
β-Glucosidase
Enzyme activity (IU ml⁻¹)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
Control
A B C D
0.35
0.40
0.45
0.50
0.55
Nitrogen source (gl⁻¹)
a
b

Fig. 16

the potassium dihydrogen phosphate as major phosphorus sources (2.0 gl^{-1}) in the medium. Presence of additional phosphorus seemed to have an inhibitory effect on enzymatic activity except that a lower increase (20%) in β-glucosidase activity was observed by increasing the concentration of potassium dihydrogen phosphate upto 2.45 gl^{-1} with both the cellulosic substrates used (Fig. 17a, b). The medium added with the defined constituents so far was designated as R_3 medium (R_3m).

4.5.2.4 Effect of phenolic compounds

In addition to the above the effect of some phenolic compounds (0.05%) on cellulase levels was also investigated. Effect of phenolic compounds in particular vanillin was largely pronounced when *Eicchornia crassipes* biomass was used as the substrate. Addition of vanillin led to a 31% increase in the CMCase and 70% increase in the β-glucosidase activities with *Eicchornia crassipes* biomass as the substrate. (Fig.18). Whereas, FPase level remained unchanged. However, vanillin had no such influence on enzymatic activities with sugarcane bagasse as the substrate except that a lower level increase in CMCase activity was detected. Salicylic acid like vanillin had almost similar pattern of induction with both substrates used. On the other hand β-naphthol, tannic acid and guaicol showed strong inhibitory effects on cellulase levels. The medium including the constituents derived till the stage was denoted as R_4 medium (R_4m).

Fig. 17 **Effect of phosphorus sources on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b). A, Tetra sodium pyrrophosphate; B, Potassium dihydrogen phosphate; C, Sodium β-glycerophosphate; D, Dipotassium hydrogen phosphate.**

R_2m medium supplemented with varying concentrations of phosphorus sources was inoculated with *Aspergillus niger* RK-3 under the conditions as described for Fig. 15. The medium composition yielding to maximum cellulase production was designated as medium R_3m.

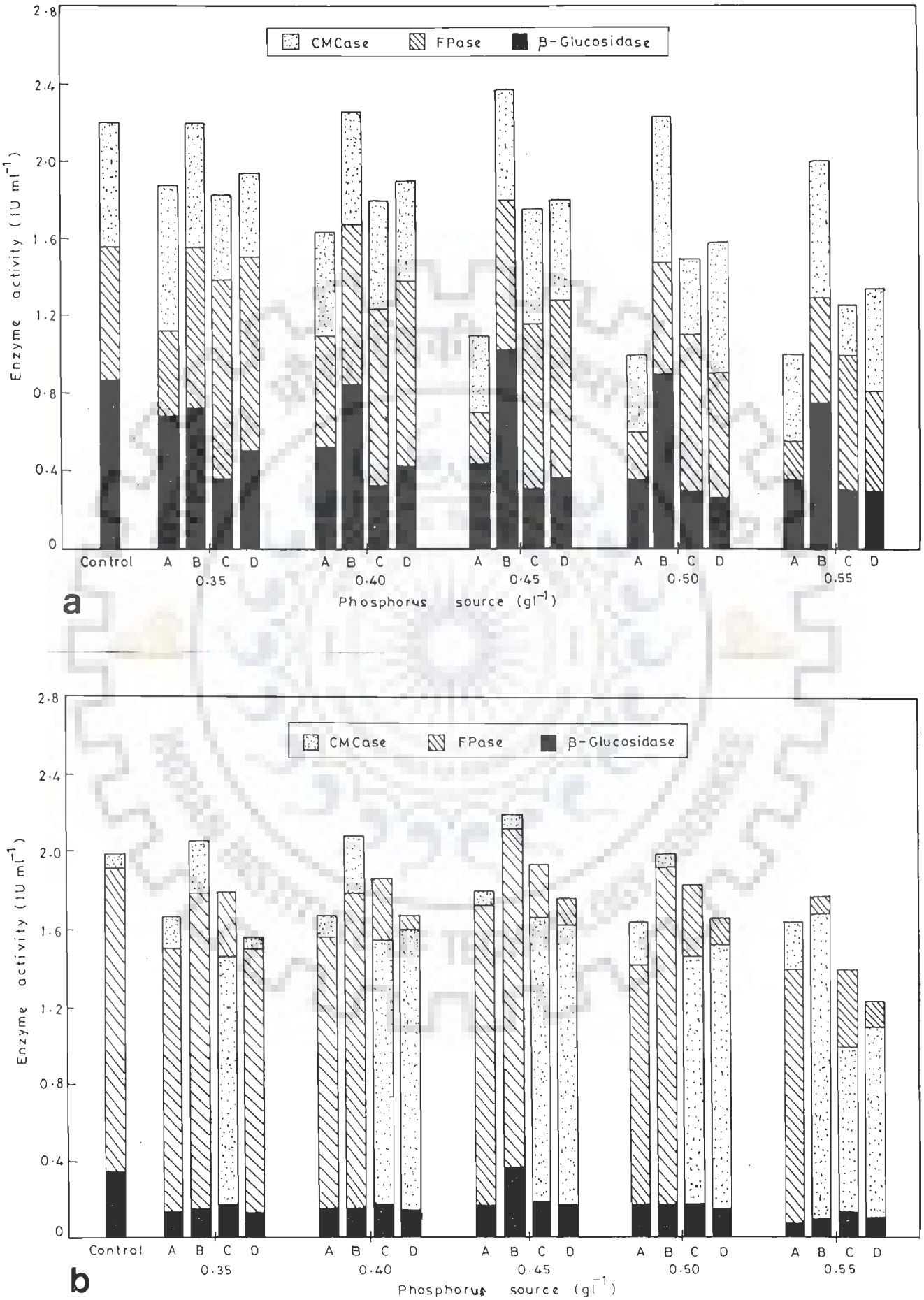

CMCase
FPase
β-Glucosidase
Enzyme activity (IU ml⁻¹)
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
Control
A B C D
0.35
0.40
0.45
0.50
0.55
Phosphorus source (gl⁻¹)
a
b

Fig. 17

Fig. 18 **Effect of phenolic compounds (0.05%) on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass as the substrates. A, Gallic acid; B, Tannic acid; C, Maleic acid; D, Salicylic acid; E, β-naphthol; F, Guaicol; G,Vanillinic acid; H,Vanillin.**

R_3m medium supplemented with different phenolic compounds was inoculated with *Aspergillus niger* RK-3 under the conditions as described for Fig. 15. The medium composition yielding to maximum cellulase production was designated as medium R_4m.

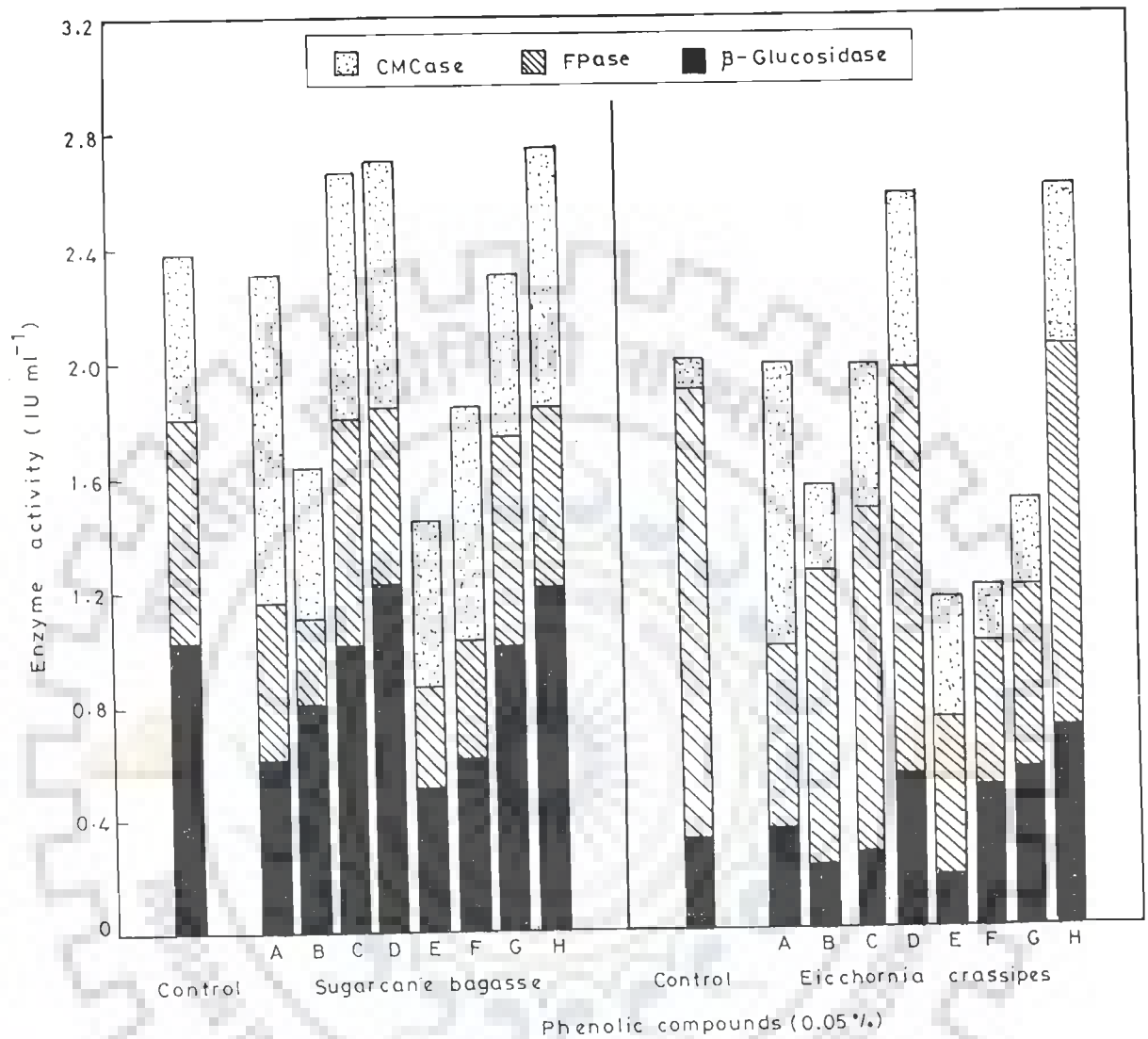

CMCase
FPase
β-Glucosidase
Enzyme activity (IU ml⁻¹)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
A B C D E F G H
Control
Sugarcane bagasse
Control
Eicchornia crassipes
Phenolic compounds (0.05%)

Fig. 18

4.5.2.5 Effect of organic acids

The Fig. 19 shows the effect of different organic acids (0.1%) i.e. acetic acid ascorbic acid, citric acid, oxoglutaric acid, propionic acid and succinic acid on the production of CMCase, FPase and β-glucosidase. Addition of organic acids to the fermentation medium had no effect on cellulase induction and in contrary a strong inhibition in CMCase, FPase and β-glucosidase levels was observed in presence of ascorbic, citric, oxoglutaric and propionic acid. A marginal induction in CMCase and β-glucosidase levels was noted with succinic acid in the medium. Succinic acid was included in the R_4m medium and the medium till this stage was designated as R_5m.

4.5.2.6 Effect of soluble sugars

The delignified cellulosic substrates (1% w/v) i.e. sugarcane bagasse or *Eicchornia crassipes* biomass were incubated in the production medium (R_5m) along with different sugars (0.2% w/v) mainly lactose, mannitol, fructose, sucrose, glucose, galactose, cellobiose and xylose and production of cellulases was measured after 8 days of incubation. Addition of sugars was generally stimulatory in nature. Among the sugars used cellobiose showed maximum induction and resulted into 30% and 39% increase respectively in CMCase and FPase levels with both the cellulosic substrates used. However, a varying level of induction in β-glucosidase levels i.e., 41% increase with *Eicchornia crassipes* biomass and a lower increase (18%) with sugarcane bagasse was observed (Fig. 20). Besides cellobiose, glucose also exhibited significant levels of induction.

Fig. 19 **Effect of organic acids (0.1%) on cellulase production by *Aspergillus niger* RK-3 when treated sugarcane bagasse and *Eicchornia crassipes* biomass was used as the substrates. A, Acetic acid; B, Ascorbic acid; C, Citric acid; D, Oxoglutaric acid; E, Propionic acid; E, Succinic acid.**

R_4m medium supplemented with different organic acids was inoculated with *Aspergillus niger* RK-3 under the conditions as described for Fig. 15. The medium composition yielding to maximum cellulase production was designated as medium R_5m.

Fig. 20. **Effect of sugars (0.2%) on cellulase production by *Aspergillus niger* RK-3 with treated sugarcane bagasse and *Eicchornia crassipes* biomass was used as the substrate. A, Lactose; B, Mannitol; C, Fructose; D, Sucrose; E, Glucose; F, Galactose; G, Cellobiose; H, Xylose.**

R_5m medium supplemented with different sugars was inoculated with *Aspergillus niger* RK-3 under the conditions as described for Fig. 15. The medium composition yielding to maximum cellulase production was designated as medium R_6m.

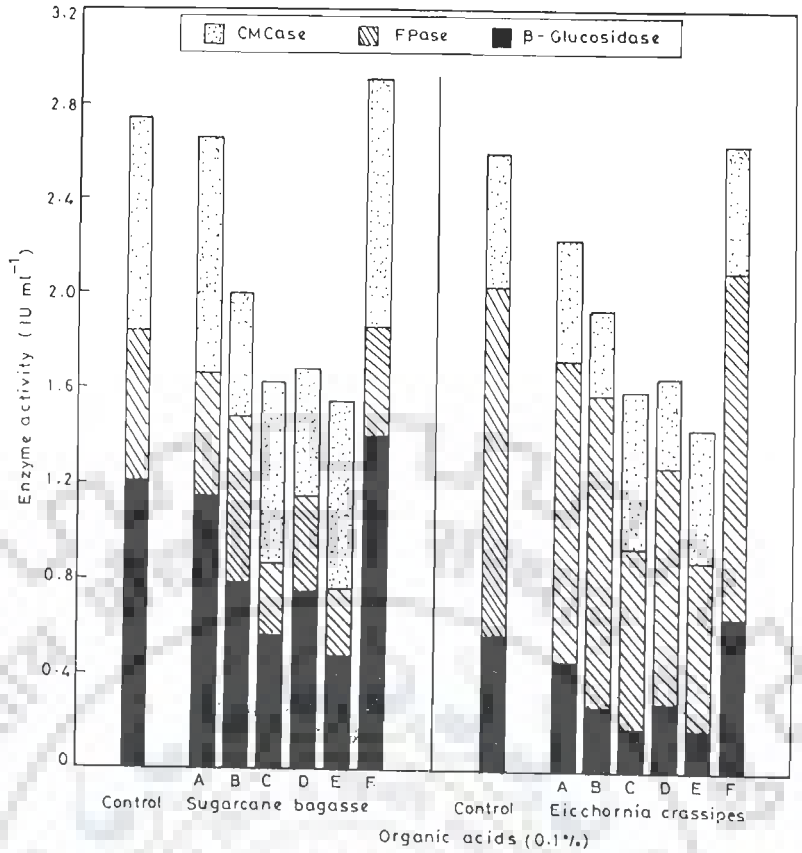

CMCase
FPase
β-Glucosidase
Enzyme activity (IU ml⁻¹)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
A B C D E F
Control
Sugarcane bagasse
Control
Eicchornia crassipes
Organic acids (0.1%)

Fig. 19

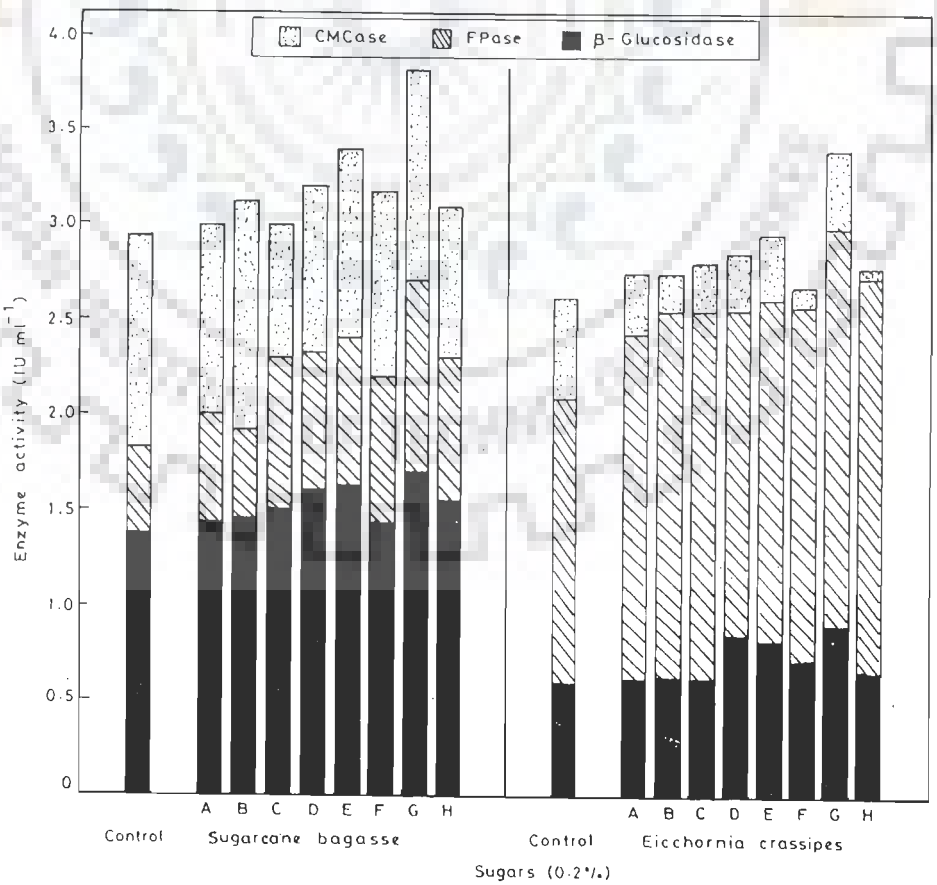

CMCase
FPase
β-Glucosidase
Enzyme activity (IU ml⁻¹)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
A B C D E F G H
Control
Sugarcane bagasse
Control
Eicchornia crassipes
Sugars (0.2%)

Fig. 20

Therefore, the results indicated that delignified cellulose in the earlier defined medium along with soluble sugar (particularly cellobiose) in a ratio of 5:1 could be used for increased level of cellulase production. This combination of constituents thus defined for the production medium was designated as R_6m.

4.6 PRODUCTION OF ENZYMES IN THE FINALLY DEFINED MEDIUM (R_6m)

The various factors as derived from the earlier studies (Table 8) were used for estimating cellulase production levels at different time periods. The defined constituents of the medium (R_6m) and the factors led to substantial increase in cellulase production over the enzymatic activities obtained initially with Mandels and Weber's medium. The maximum CMCase (3.80 IU ml^{-1}), FPase (2.6 IU ml^{-1}) and β-glucosidase (1.65 I U ml^{-1}) activities were observed with sugarcane bagasse after 8 days of incubation (Fig. 21a). These activities respectively were 83%, 59% and 288% higher as compared to the CMCase, FPase and β-glucosidase activities observed with sugarcane bagasse using the Mandels and Weber's medium. Similar results were also observed when *Eicchornia crassipes* biomass was used as the substrate. The activities of CMCase, FPase and β-glucosidase observed with *Eicchornia crassipes* biomass (Fig 21b) were 112%, 79% and 316% higher as compared to the activities observed with Mandels and Weber's medium. Further, higher rates of cellulose degradation (60-65%) and protein production (1.9-2.6 mg ml^{-1}) were observed with defined medium (R_6m), that was 18-26% and 180-210% higher as compared to the cellulose degradation and protein production with

Table 8 Defined set of conditions and factors for the medium designated as R_6m

Parameters	Conditions / Factors derived
(A) Physical parameters	
(i) pH	4.8
(ii) Temperature	30-32^{0}C (growth)
	50^{0}C (enzyme activity)
(iii) Agitation speed	130 rpm
(B) Biological parameters	
(i) Age of inoculum	24 h
(ii) Concentration of inoculum	5%
(C) Chemical parameters	
(i) Carbon source	1% (any one of the treated substrate*)
(ii) Nitrogen source	(Ammonium sulfate, 2.1 gl^{-1} alongwith ammonium chloride, 0.4 gl^{-1})
(iii) Phosphorous source	Potassium dihydrogen phosphate (2.45 gl^{-1})
(iv) Phenolic compounds	Vanillin (0.05%)
(v) Organic acid	Succinic acid (0.1%)
(vi) Soluble sugars	Cellobiose (0.2%)

* Delignified sugarcane bagasse or *Eicchornia crassipes* biomass

Fig. 21 **Production of cellulases and relative cellulose degradation by wild type *Aspergillus niger* RK-3 at different time intervals using R_6m defined medium containing treated sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b) as the substrates.**

Medium R_6m was inoculated with *Aspergillus niger* RK-3 for measuring enzymatic production under the conditions as described for Fig. 15.

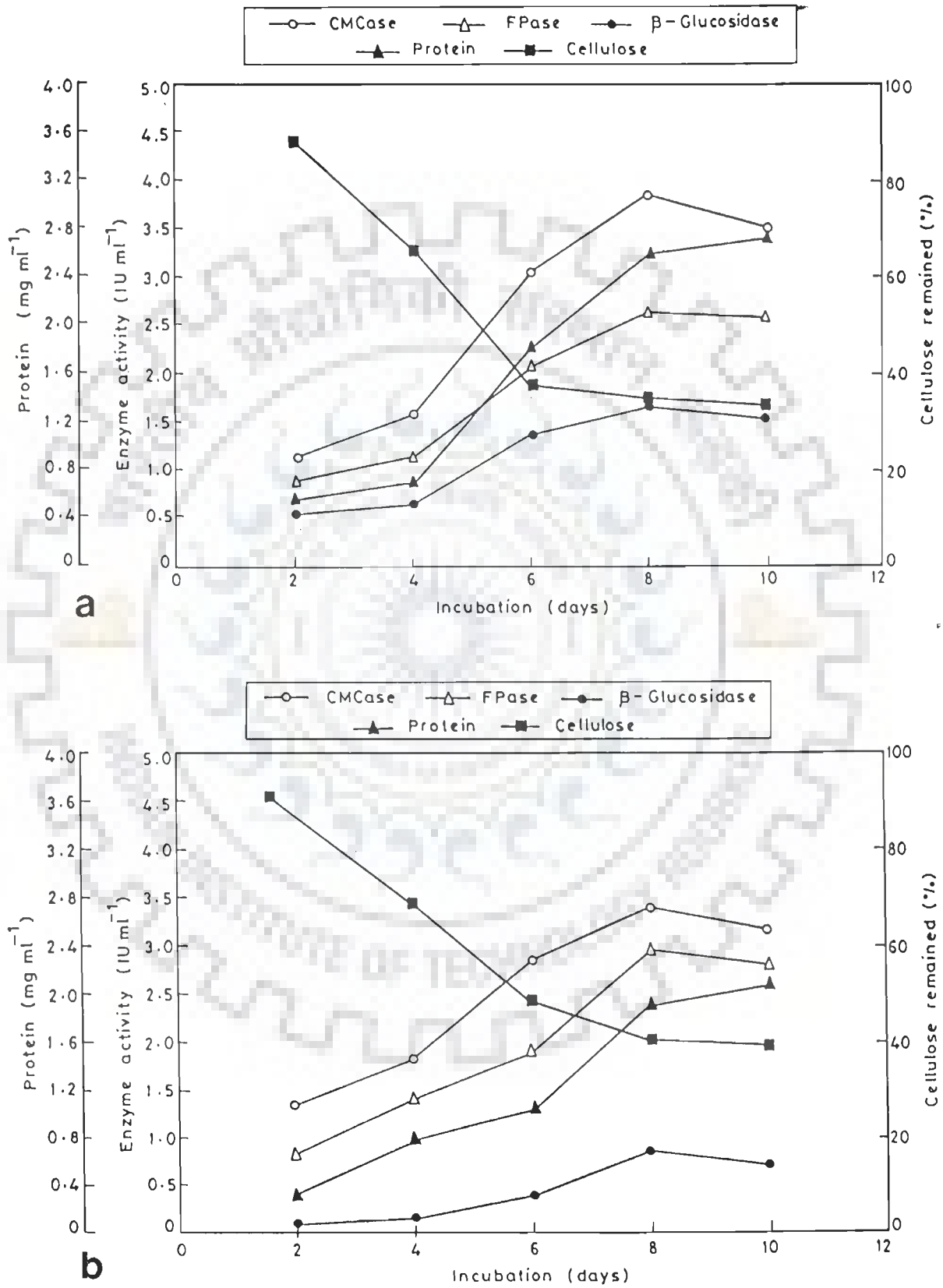

CMCase
FPase
β-Glucosidase
Protein
Cellulose
Protein (mg ml⁻¹)
Enzyme activity (IU ml⁻¹)
Cellulose remained (%)
Incubation (days)
a
b

Fig. 21

Table 9 Analysis of variance for cellulase, protein production and relative cellulose degradation before and after optimization of the production medium

Source of variation	Degree Of Freedom	Mean Square (Ms)									
		Sugarcane bagasse					*Eicchornia crassipes* biomass				
		CMCase	FPase	β–gluco-sidase	Soluble Protein	Cellulose Degradation	CMCase	FPase	β-gluco-sidase	Soluble Protein	Cellulose Degradation
Variation in enzymes, protein production and relative cellulose degradation between before and after optimization of production medium	1	4.44*	1.38*	2.25*	4.35*	1.57#	4.72*	2.66#	0.70*	2.17	238.52#
Variation in enzymes, protein production and relative cellulose degradation within before and after optimization of production medium	2	0.07	0.27	0.009	0.04	0.011	0.077	0.001	0.001	0.02	0.11
Error	3	0.02	0.009	0.0089	0.03	2.00	0.01	0.03	0.0008	0.0002	7.86

* Significant at 1% level; # Siginificant at 5% level

both the substrates used with Mandels and Weber's medium after 8 days of incubation. Variations in the enzymatic levels and relative cellulose degradation were significant before and after the derivation of suitable conditions and the factors (Table 9).

4.7 MUTAGENESIS OF *ASPERGILLUS NIGER* RK-3 FOR IMPROVED CELLULASE PRODUCTION

The experiments conducted with the wild type isolate *Aspergillus niger* RK-3 showed that this strain has the following advantages: (i) it can utilize a wide variety of synthetic and natural substrates for cellulase production and (ii) it has notable levels of the desired enzymatic components for cellulosic bioconversion.

Natural isolates usually produce the desired enzymatic components in limited concentrations. Attempts therefore have to be made to induce the cellulase production level of the chosen organism. An increase in the cellulase production levels was achieved with the wild type isolate *Aspergillus niger* RK-3 by defining the physico-chemical and biological conditions or by adding the regulators. This approach was limited due to the strain's maximum ability to synthesize the cellulases under defined conditions. Further increase in the production of cellulases therefore was attempted by mutagenesis of the strain. The production of cellulases is potentially controlled by the genome, attempts therefore were made to mutagenize the wild type strain for improving the cellulase production level of the organism. The strain improvement was done with continual genetic modification of the culture followed by the analysis of resulting mutants for induced levels of enzymatic production.

Aspergillus niger RK-3 was subjected to physical (ultraviolet radiation), chemical (N-methyl-N′-nitro-N-nitrosoguanidine, sodium azide, colchicine) and mixed physical and chemical mutagenic treatments. The level of enzyme production by the mutant strains was analyzed by determining the zone diameters on congo-red CMC-agar plate and the subsequently assaying the cellulases by culturing the strains in the R_6m defined production medium. Both of these media were also enriched with 1% glucose as additional constituent so as to specifically select the mutants with resistance to end product inhibition.

4.7.1 Single Step Mutagenesis

The wild type isolates from natural resources usually have limited ability to produce the material of commercial significance. Enhancement in the levels of production can be achieved either by mutagenic treatment or by molecular cloning of the essential genetic components. In the present study, attempts were made to increase the cellulases production levels of the *Aspergillus niger* RK-3 by subjecting the strain to physical, chemical and mixed mutagenic treatments

4.7.1.1 Ultraviolet mutagenesis

Exposure with ultraviolet radiation (12-168 Jm^{-2} min^{-1}) of *Aspergillus niger* RK-3 spores resulted into various mutants. The mutants RKU-40 and RKU-45 exhibited maximum zone diameter (4.0 mm) on congo-red CMC-agar plate. The rate of survivability of the spores showed the percentage of the positive mutants was lower (12-15%) compared to corresponding or negative mutants (Table 10).

4.7.1.2 Chemical mutagenesis

Wild type RK-3 strain was treated with N-methyl-N′-nitro-N-nitrosoguanidine (NTG, 100 μg ml^{-1}) for different time intervals. Mutants RKN-400 and RKN-450 obtained after 40 and 45 minutes of treatments showed greater zone diameter of 3.0 mm (Table 11). Similarly strain RK-3 was also treated separately with sodium azide (1-4 μg ml^{-1}) and colchicine (0.01-2.0 μg ml^{-1}) for a period of 24 h. Treatment with sodium azide resulted into mutants RKS-20 and RKS-30 having the zone diameter of 2.6 and 2.5 respectively (Table 12), whereas colchicine treatment led to the generation of mutants RKC-50 and RKC-60 yielding the zone diameters of 2.6 and 2.7 mm respectively (Table 13). In all the chemical treatments the rate of survivability of positive mutants was considerably lower (10% or less) than the negative or corresponding mutants. The rate of survivability of positive mutants obtained with chemical mutagenesis was generally lower than the ultraviolet mutagenesis.

The mutants thus obtained and showing higher zone diameters after ultraviolet and chemical mutagenesis were further analyzed for cellulase production with R_6m medium under submerged conditions.

4.7.1.2.1 Production of cellulases with mutants obtained after single mutagenesis

Production of cellulases was measured with carboxymethylcellulose (CMC) as substrate under submerged conditions. A notable increase in the enzyme activities was observed in the mutants obtained after ultraviolet radiation and the

Table 10 Single step mutagenesis of *Aspergillus niger* RK-3 by ultraviolet radiation

Dose of UV radiation (Jm^{-2} min^{-1})	Mutants	Zone diameter (mm)	Rate of survivability (%)		
			Corresponding mutants	Negative mutants	Positive mutants
12	RKU-5	2.0	90	10	ND
24	RKU-10	2.0	82	18	ND
36	RKU-15	2.0	80	20	ND
48	RKU-20	2.2	80	15	5
60	RKU-25	2.2	80	42	8
72	RKU-30	2.4	50	55	10
84	RKU-35	3.0	35	40	18
96	RKU-40	4.0	42	45	15
108	RKU-45	4.0	40	55	12
120	RKU-50	3.0	33	58	8
132	RKU-55	2.8	34	60	6
144	RKU-60	2.5	35	60	5
168	RKU-70	2.1	30	68	2

Zone diameter : *Aspergillus niger* RK-3 wild type / corresponding mutants, 2.0 mm; positive mutants > 2.0 mm; negative mutants < 2.0 mm

ND : not detected

Table 11 Single step mutagenesis of *Aspergillus niger* RK-3 by N-methyl-N′-nitro-N-Nitrosoguanidine (NTG) treatment

NTG (100 μ g ml^{-1}) (min)	Mutants	Zone diameter (mm)	Rate of survivability (%)		
			Corresponding mutants	Negative mutants	Positive mutants
30	RKN-300	1.8	75	25	ND
35	RKN-350	1.5	70	30	ND
40	RKN-400	3.0	68	25	7
45	RKN-450	3.0	60	35	5
50	RKN-500	2.2	72	26	2
55	RKN-550	2.0	68	30	2
60	RKN-600	2.0	70	23	2

Zone diameter : *Aspergillus niger* RK-3 wild type / corresponding mutants, 2.0 mm; positive mutants > 2.0 mm; negative mutants < 2.0 mm

ND : not detected

Table 12 Single step mutagenesis of *Aspergillus niger* RK-3 by sodium azide treatment

Sodium azide ($\mu g\ ml^{-1}$)	Mutants	Zone diameter (mm)	Rate of survivability (%)		
			Corresponding mutants	Negative mutants	Positive mutants
1.0	RKS-10	2.2	65	28	7
2.0	RKS-20	2.6	60	30	10
3.0	RKS-30	2.5	63	27	10
4.0	RKS-40	2.4	65	27	8

Zone diameter : *Aspergillus niger* RK-3 wild type / corresponding mutants, 2.0 mm; positive mutants > 2.0 mm; negative mutants < 2.0 mm

Table 13 Single step mutagenesis of *Aspergillus niger* RK-3 by colchicine treatment

Colchicine ($\mu g\ ml^{-1}$)	Mutants	Zone diameter (mm)	Rate of survivability (%)		
			Corresponding mutants	Negative mutants	Positive mutants
0.01	RKC-10	1.5	95	5	ND
0.02	RKC-20	1.7	90	10	ND
0.1	RKC-30	2.1	95	5	ND
0.2	RKC-40	2.4	85	7	8
0.5	RKC-50	2.6	75	15	10
1.0	RKC-60	2.7	70	19	11
2.0	RKC-70	2.3	68	24	8

Zone diameter : *Aspergillus niger* RK-3 wild type / corresponding mutants, 2.0 mm; positive mutants > 2.0 mm; negative mutants < 2.0 mm

ND : not detected

maximum CMCase, FPase and β-glucosidase activities (1.96 IU ml^{-1}, 1.62 IU ml^{-1}, 0.679 IU ml^{-1}) were determined with mutant RKU-40 among all the mutants obtained after single step mutagenesis (Fig. 22). The activities were 21%, 20% and 33% higher compared to the CMCase, FPase and β-glucosidase activities obtained with the wild type *Aspergillus niger* RK-3 strain using the defined medium (R_6m) alongwith 1% of the glucose. The mutant RKU-45 also showed an almost similar increase in the activities. A lesser degree of increase or no changes in enzymatic activities were observed with remaining mutants obtained after chemical mutagenesis (Fig 22). Mutants RKU-40 and RKU-45 showing maximum cellulases activities among the mutants obtained were therefore selected and subjected for mixed chemical mutagenesis.

4.7.2 Mixed Chemical Mutagenesis

For mixed chemical mutagenesis, the mutants RKU-40 and RKU-45 obtained after single treatment and having maximum ability of cellulase production were treated with NTG (100 μg ml^{-1}) for 40 and 45 minutes. Among the mutants obtained the mutant UN-440 and UN-445 had maximum zone diameters (4.6 and 4.4 mm respectively) on congo-red CMC-agar plate, were selected (Table 14) for further mutagenesis. Strains UN-440 and UN-445 were subjected to sodium azide treatment (1-4 μg ml^{-1}) and in the resulting mutant strains UNS-442 and UNS-443 with respective zone diameters of 5.3, 5.0 mm (Table 15) on congo-red CMC-agar plate and were selected for next stage mutagenesis. Interestingly the survivability of the positive and negative mutants increased with respect to the survivability of the

Fig. 22 **Production of cellulases by mutants derived by the physical or chemical mutagenesis of the wild type *Aspergillus niger* RK-3.**

Mutant RKU-40 and RKU-45 were obtained after UV mutagenesis; RKN-400 and RKN-450 after NTG treatment; RKS-20 and RKS-30 after sodium azide treatment; RKC-50 and RKC-60 after colchicine treatment. The medium R_6m containing carboxymethylcellulose (1% w/v) as the carbon source was inoculated with *Aspergillus niger* RK-3 or the mutants and the production of cellulases were measured under the conditions as described for Fig. 20.

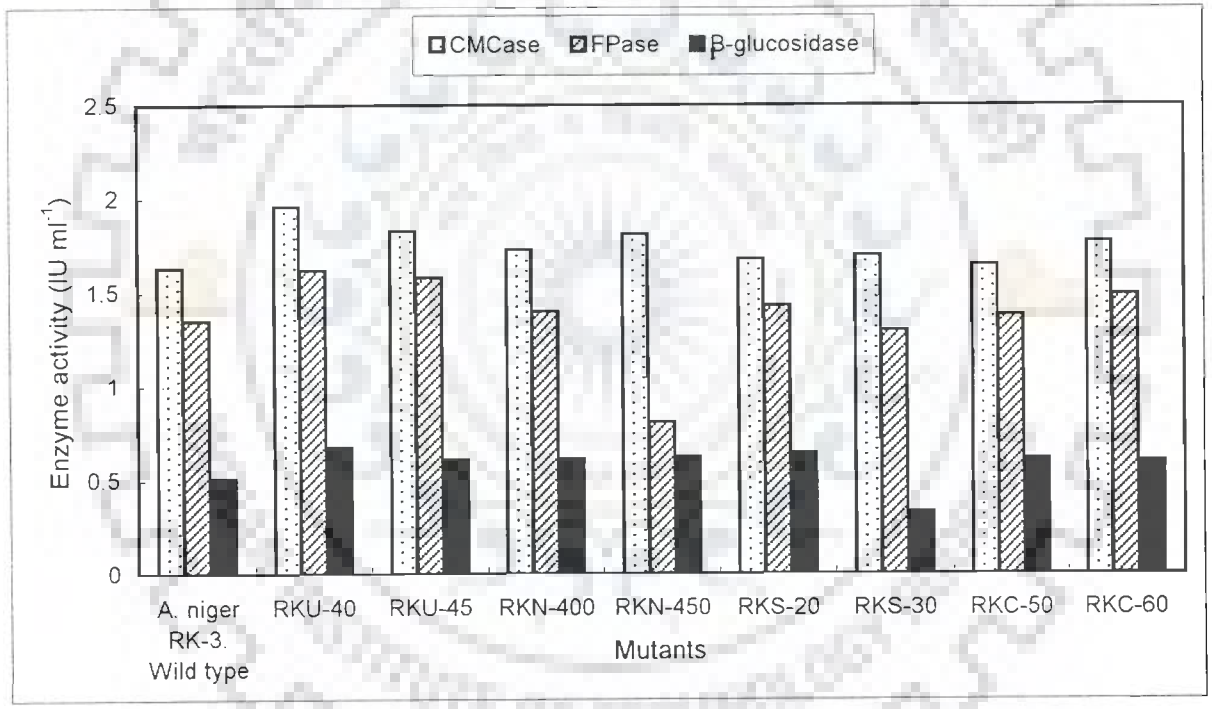

CMCase
FPase
β-glucosidase
Enzyme activity (IU ml-1)
2.5
2
1.5
1
0.5
0
A. niger RK-3. Wild type
RKU-40
RKU-45
RKN-400
RKN-450
RKS-20
RKS-30
RKC-50
RKC-60
Mutants

Fig. 22

Fig-1

Table 14 NTG mutagenesis of RKU-40 and RKU-45 mutant (UV-treated) strains

Mutants	NTG (100µg ml^{-1}) (min)	Mutants	Zone diameter (mm)	Rate of survivability (%)		
				Corresponding mutants	Negative mutants	Positive mutants
RKU-40	40	UN-440	4.6	82	10	8
RKU-40	45	UN-445	4.4	78	12	10
RKU-45	40	UN-540	3.3	80	15	5
RKU-45	45	UN-550	3.0	74	14	12

Zone diameter : RKU-40 / RKU-45, corresponding mutant, 4.0 mm; positive mutants > 4.0 mm; negative mutants < 4.0 mm

Table 15 Sodium azide mutagenesis of UN-440 and UN-445 mutant (UV-NTG treated) strains

Mutants	Sodium azide (µg ml^{-1})	Mutants	Zone diameter (mm)	Rate of survivability (%)		
				Corresponding mutants	Negative mutants	Positive mutants
UN-440	1	UNS-441	3.4	67	20	13
	2	UNS-442	5.3	63	22	15
	3	UNS-443	5.0	57	25	18
	4	UNS-444	4.2	62	18	20
UN-445	1	UNS-451	3.0	62	15	23
	2	UNS -452	2.8	65	15	20
	3	UNS -453	2.2	71	12	17
	4	UNS-454	2.3	70	13	17

Zone diameter : For UN-440 / corresponding mutant, 4.6 mm; positive mutants > 4.6 mm; negative mutants < 4.6 mm;
For UN-445 / corresponding mutant, 4.4 mm; positive mutants > 4.4 mm; negative mutants < 4.4 mm

corresponding mutants after NTG treatment. The mutant strains UNS-442, UNS-443 as obtained after UNS treatment (UV-NTG-sodium azide) were further subjected to colchicine treatment. The mutants obtained after this stage of mixed treatment UV-NTG-sodium azide-colchicine (UNSC) are described in the table 16. The mutant strains UNSC-442 and UNSC-443 showed highest zone diameters (8.3 and 8.0 mm respectively) on congo-red CMC-agar plate (Fig. 23) and were finally selected as the potential mutant strains. The rate of survivability of the positive mutants was comparable with the mutants as obtained for the previous stage of mutagenesis.

4.7.2.1 Production of cellulases with mutants obtained after mixed mutagenesis

Mutants showing higher zone diameters as detected at various stages of mixed mutagenesis were analyzed for cellulase production. CMCase, FPase and β-glucosidase activities were estimated in submerged state fermentation conditions with carboxymethylcellulose using R_6m defined medium. Increased levels of all three components of the cellulase complex were observed with the mutant strains selected at different stages of mixed mutagenesis with CMC as the substrate. The highest enzymatic activities i.e. CMCase (2.87 IU ml^{-1}), FPase (1.96 IU ml^{-1}) and β-glucosidase (0.88 IU ml^{-1}) were observed with mutant UNSC-442 (Fig. 24). These activities were 76%, 45% and 72% higher as compared to CMCase, FPase and β-glucosidase activities obtained with the wild type strain using the carboxy-methylcellulose as the substrate in the R_6m defined medium.

Table 16 Colchicine mutagenesis of UNS-442 and UNS-443 mutant (UV-NTG-Sodium azide treated) strains

Mutants	Colchicine ($\mu g\ ml^{-1}$)	Mutants	Zone diameter (mm)	Rate of survivability (%)		
				Corresponding mutants	Negative mutants	Positive Mutants
UNS-442	0.01	UNSC - 440	2.3	77	15	8
	0.02	UNSC - 441	2.8	72	18	10
	0.1	UNSC - 411.1	2.0	59	20	11
	0.2	UNSC - 441.2	2.9	49	28	23
	0.5	UNSC - 441.5	4.3	44	31	25
	1.0	UNSC - 442	8.3	39	40	21
	2.0	UNSC - 443	8.0	37	45	18
UNS-443	0.01	UNSC - 42.01	4.0	50	35	15
	0.02	UNSC - 42.02	4.2	50	38	12
	0.1	UNSC - 442.1	3.8	51	35	14
	0.2	UNSC - 442.2	3.6	65	25	10
	0.5	UNSC - 442.5	4.1	71	20	9
	1.0	UNSC - 443.1	3.8	71	13	16
	2.0	UNSC - 443.2	2.8	68	20	12

Zone diameter : For UNS-442 / corresponding mutant, 5.3 mm; positive mutants > 5.3 mm; negative mutants < 5.3 mm.

Zone diameter : For UNS-443 / corresponding mutant, 5.0 mm; positive mutants > 5.0 mm; negative mutants < 5.0 mm.

Fig. 23 **Screening of the end product resistant mutants**
1 *Aspergillus niger* RK-3 wild type strain;
2,3 Mutant RKU-40 strains;
4,5,6 Mutant UNSC-442.

Strains were growing in R_6m medium containing 1% glucose were analyzed for white zone formation on congo-red CMC–agar as described in methods.

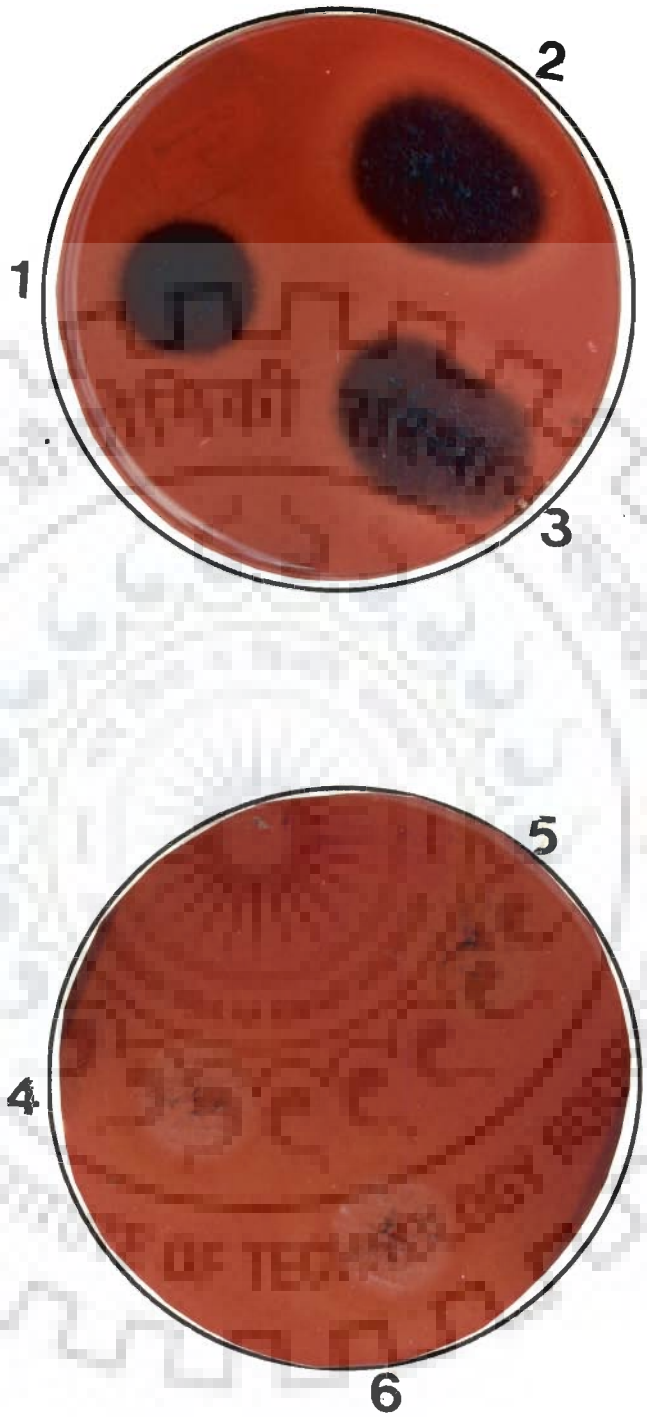


Fig. 23

Fig. 24 **Production of cellulases by mutant strains derived after mixed mutagenesis of RKU-40 mutant which after NTG treatment resulted into mutants UN-440, 445 which after sodium azide treatment, resulted into mutants UNS-442, 443, which after colchicine treatment resulted into mutants UNSC-442, 443. Mutants selection plan was as described in methods.**

The medium R_6m containing carboxymethylcellulose (1% w/v) as the carbon source was inoculated with *Aspergillus niger* RK-3 or the mutants and the production of cellulases were measured under the conditions as described for Fig. 22.

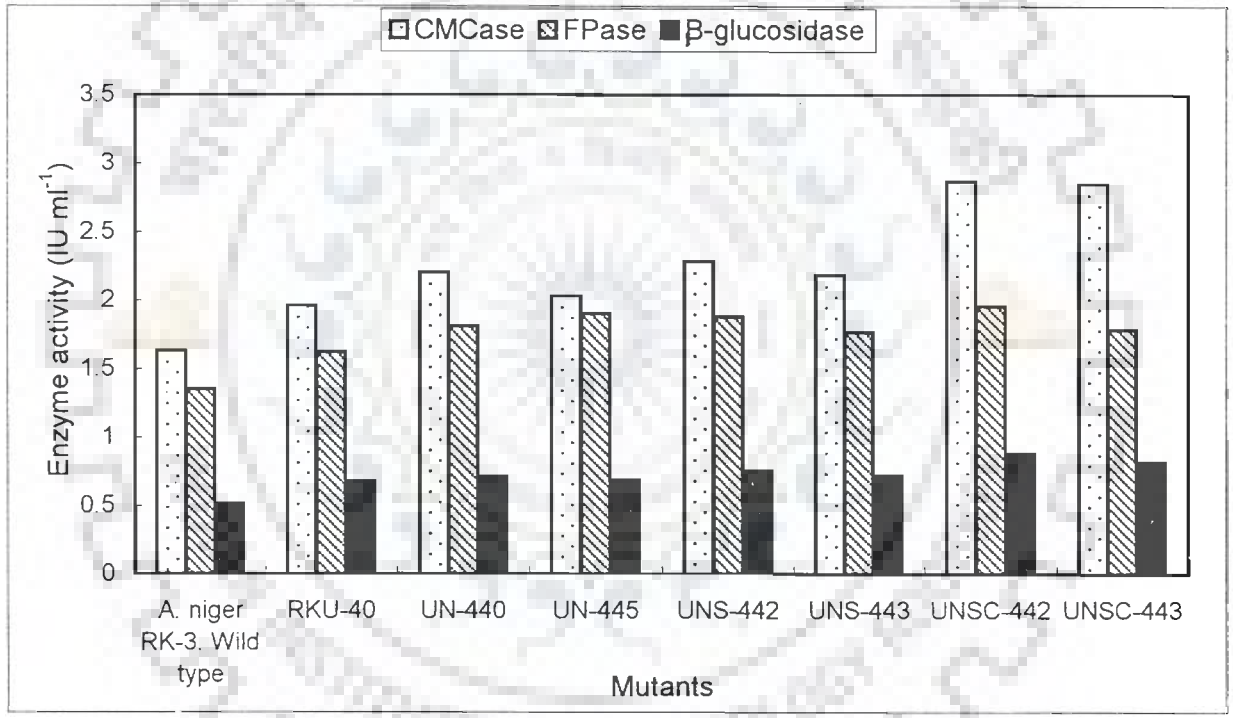

CMCase
FPase
β-glucosidase
Enzyme activity (IU ml-1)
3.5
3
2.5
2
1.5
1
0.5
0
A. niger RK-3. Wild type
RKU-40
UN-440
UN-445
UNS-442
UNS-443
UNSC-442
UNSC-443
Mutants

Fig. 24

These observations therefore denoted strain UNSC-442 as the potential strain and hence selected for analysing its ability to degrade the delignified sugarcane bagasse and *Eicchornia crassipes* biomass cellulosic waste materials.

4.7.2.2 Evaluation of cellulase production by mutant UNSC-442 with treated cellulosic waste materials

Treated sugarcane bagasse and *Eicchornia crassipes* biomass were used for cellulase production with mutant UNSC-442. Significant levels of cellulases were generated with both the substrates used. The maximum CMCase (7.18 IU ml^{-1}) and β-glucosidase (2.55 IU ml^{-1}) were observed with sugarcane bagasse when modified medium R_6m was used in submerged state fermentation after 8 days of incubation (Fig 25a). These activities were 88.9% and 54.5% higher compared to the CMCase and β-glucosidase activities obtained with wild type strain using similar substrate and the conditions. Increase in the FPase activity was still higher (130%). Similar levels of increase in the enzymatic activity were observed with *Eicchornia crassipes* biomass. Levels of activity achieved with mutant UNSC-442 were 108% higher for CMCase, 145% for FPase and 53.1% for β-glucosidase (Fig. 25b) with respect to the enzymatic levels as obtained from the wild type strain. Similar patterns for increasing level of protein and cellulose degradation (61.5-67%) was observed with both the substrates. Variations in the enzymatic levels and relative cellulose degradation were significant in the wild type *Aspergillus niger* RK-3 and its mutant UNSC-442 (Table 17).

Table 17 Analysis of variance for cellulase, protein production and relative cellulose degradation by wild type *Aspergillus niger* RK-3 and its mutant UNSC-442

Source of variation	Degree Of Freedom	Mean Square (Ms)									
		Sugarcane bagasse					*Eicchornia crassipes* biomass				
		CMCase	FPase	β-gluco-sidase	Soluble Protein	Cellulose Degradation	CMCase	FPase	β-gluco-sidase	Soluble Protein	Cellulose Degradation
Variation in enzymes, protein production and relative cellulose degradation between wild type *Aspergillus niger* RK-3 and its mutant UNSC-442	1	17.17*	17.54*	1.21*	1.33*	1747.6*	20.31*	28.25*	1.59*	1.49*	1572.05*
Variation in enzymes, protein production and relative cellulose degradation within wild type *Aspergillus niger* RK-3 and its mutant UNSC-442	2	0.092	0.016	0.097	0.39	1.48	0.015	0.000016	0.013	0.076	8.29
Error	3	0.014	0.076	0.0008	0.0072	13.31	0.025	0.072	0.0012	0.0001	0.153

* Significant at 1% level

Fig. 25 Production of cellulases and relative cellulose degradation by mutant UNSC-442 with treated sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b) using the defined R_6m. medium. Conditions for cellulase production were as defined for Fig. 21.

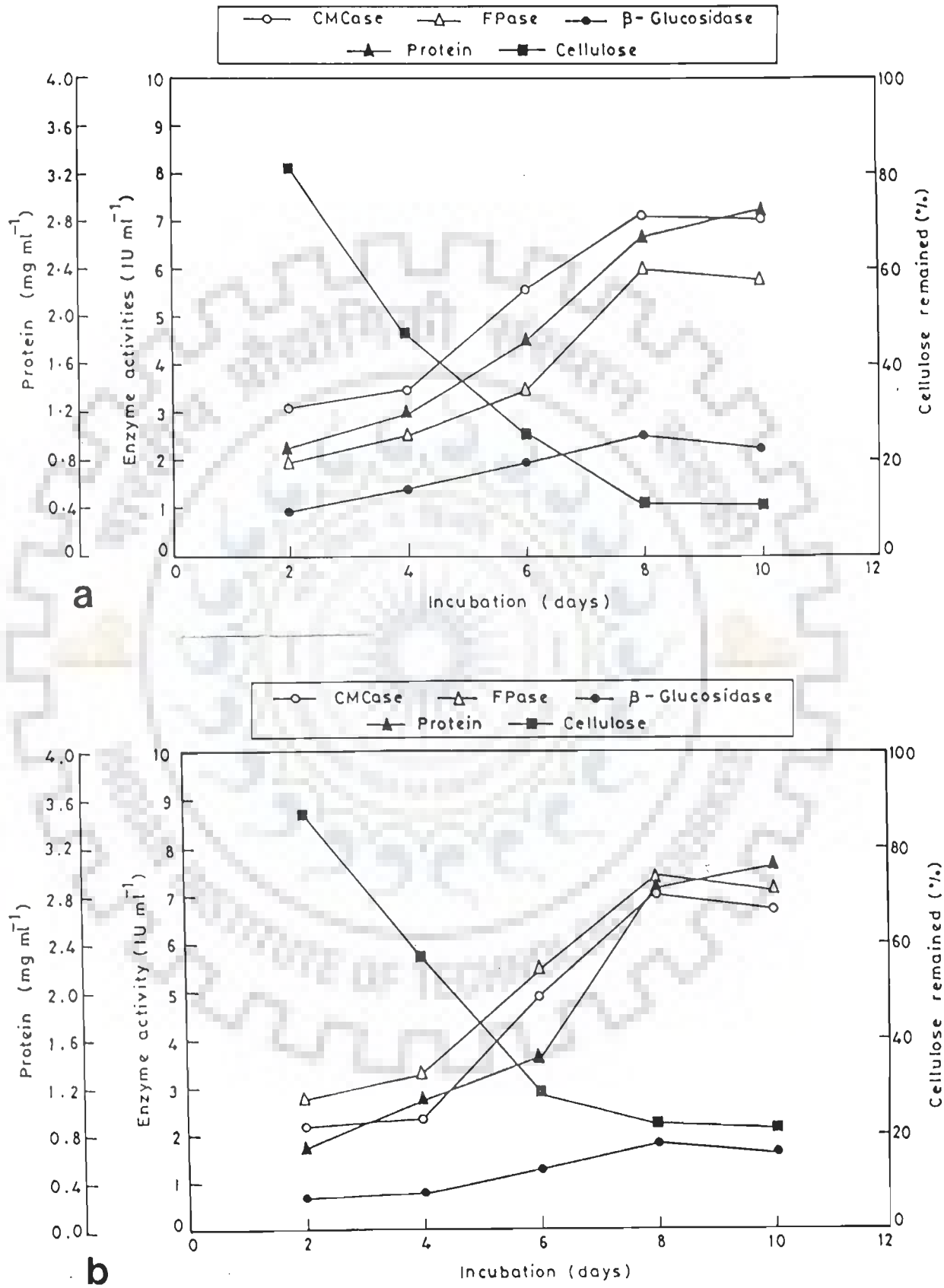

CMCase
FPase
β-Glucosidase
Protein
Cellulose
Protein (mg ml⁻¹)
Enzyme activities (IU ml⁻¹)
Cellulose remained (%)
Incubation (days)
a
CMCase
FPase
β-Glucosidase
Protein
Cellulose
Protein (mg ml⁻¹)
Enzyme activity (IU ml⁻¹)
Cellulose remained (%)
Incubation (days)
b

Fig. 25

The protein profiles of the fermentation broth was analyzed using mutant UNSC- 442 and compared with that of wild type RK-3 and UV- derived mutant RKU-40. The SDS-polyacrylamide gel analysis had shown increased levels of 95.5 kD, 68 kD and 52.4 kD bands (Fig. 26) from mutant UNSC-442 as compared to the similar bands from the wild type RK-3 or mutant RKU-40 strains. Fermentation broth was subjected for ammonium sulphate precipitation followed by dialysis and than fractionation through Sephadex columns as described in methods. The zymogram analysis of cellulase components had shown higher levels of CMCase, FPase and β-glucosidase activities from the partially purified fractions collected from mutant UNSC-442 derived cellulase production with respect to the enzymatic production from RK-3 or RKU-40 strains. Molecular weight analysis of the respective bands indicated CMCase, FPase and β-glucosidase to be of 91.2 kD, 68 kD, 52.4 kD bands respectively (Fig. 27 i-iii).

4.7.3 Analysis of Stability and Morphological Features of Mutant UNSC-442

It may be inferred therefore that sugarcane bagasse and *Eicchornia crassipes* biomass are the useful lignocellulosic waste materials for the production of fermentable sugars. Stability of the mutant UNSC-442 with respect to the enzymatic production was evaluated. Mutant UNSC-442 was subcultured monthly in the defined R_6m medium for a total period of 10 months followed by enzymatic assay using carboxymethylcellulose as the substrate at every stage of subculturing. Enzymatic levels were found to be significantly stable with minor variations at

Fig. 26 **SDS-PAGE analysis of proteins produced by wild type *Aspergillus niger* RK-3 and mutants RKU-40 and UNSC-442 after 18 days of incubation.**

Lane 1, marker proteins.
Lane 2-5, protein profile of wild type *Aspergillus niger* RK-3 strain.
Lane 6-8, protein profile of mutant RKU-40 strain.
Lane 9-11, protein profile of mutant UNSC-442 strain.

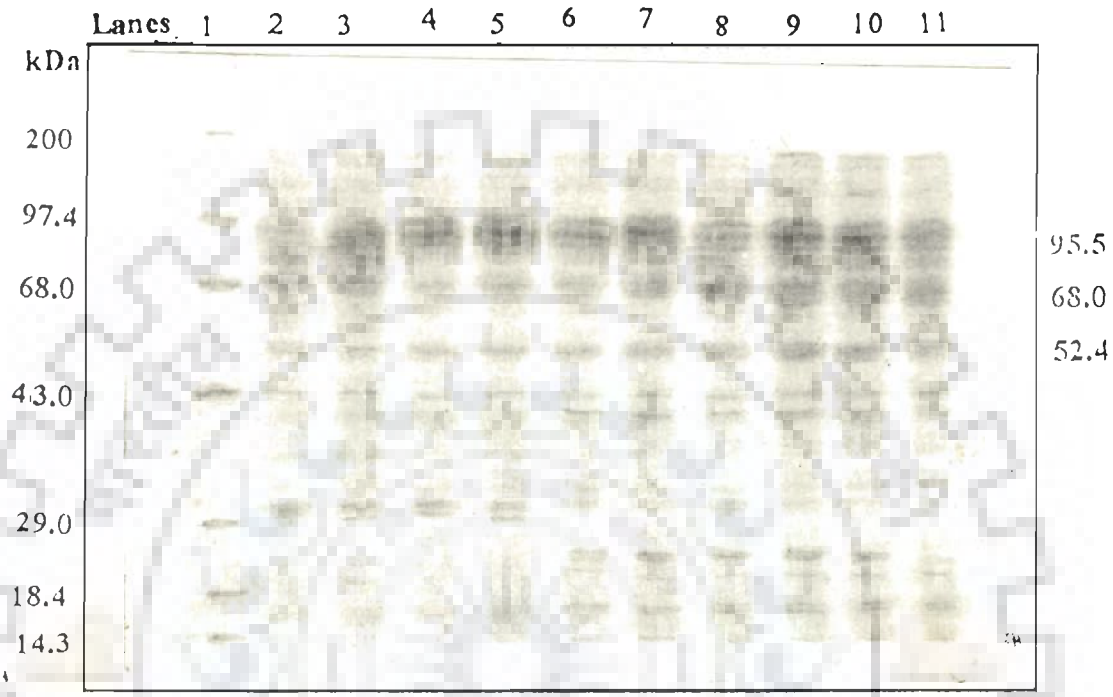

Lanes 1 2 3 4 5 6 7 8 9 10 11
kDa
200
97.4
68.0
43.0
29.0
18.4
14.3
95.5
68.0
52.4

Fig. 26

Fig.27 Zymogram analysis of cellulase components using the native polyacrylamide gel

(i) Analysis of CMCase component by incorporating carboxymethyl cellulose (0.1%) as the substrate. The bands were visualized after 30 min incubation at 50°C followed by congo-red staining and washing with 5% NaCl.

Lane 1, CMCase from *Aspergillus niger* RK-3 wild type strain.
Lane 2, CMCase from mutant RKU-40 strain.
Lane 3,4, CMCase from mutant UNSC-442 strain.

(ii) Analysis of FPase component by incorporating 4-methylumbelliferyl β-D-cellobioside (1 mM) as the substrate. The bands were visualized under ultraviolet illuminator in the dark.

Lane 1, FPase from *Aspergillus niger* RK-3 wild type strain.
Lane 2,3, FPase from mutant RKU-40 strain.
Lane 4, FPase from mutant UNSC-442 strain.

(iii) Analysis of β-glucosidase component by incorporating 4-methylumbelliferyl β-D-glucoside (1 mM) as the substrate. The bands were visualized under ultraviolet illuminator in the dark.

Lane 1, β-glucosidase from *Aspergillus niger* RK-3 wild type strain.
Lane 2,3, β-glucosidase from mutant RKU-40 strain.
Lane 4, β-glucosidase from mutant UNSC-442 strain.

FPase and β-glucosidase bands were visualized after 30 min incubation at 50°C under ultraviolet light in the dark.

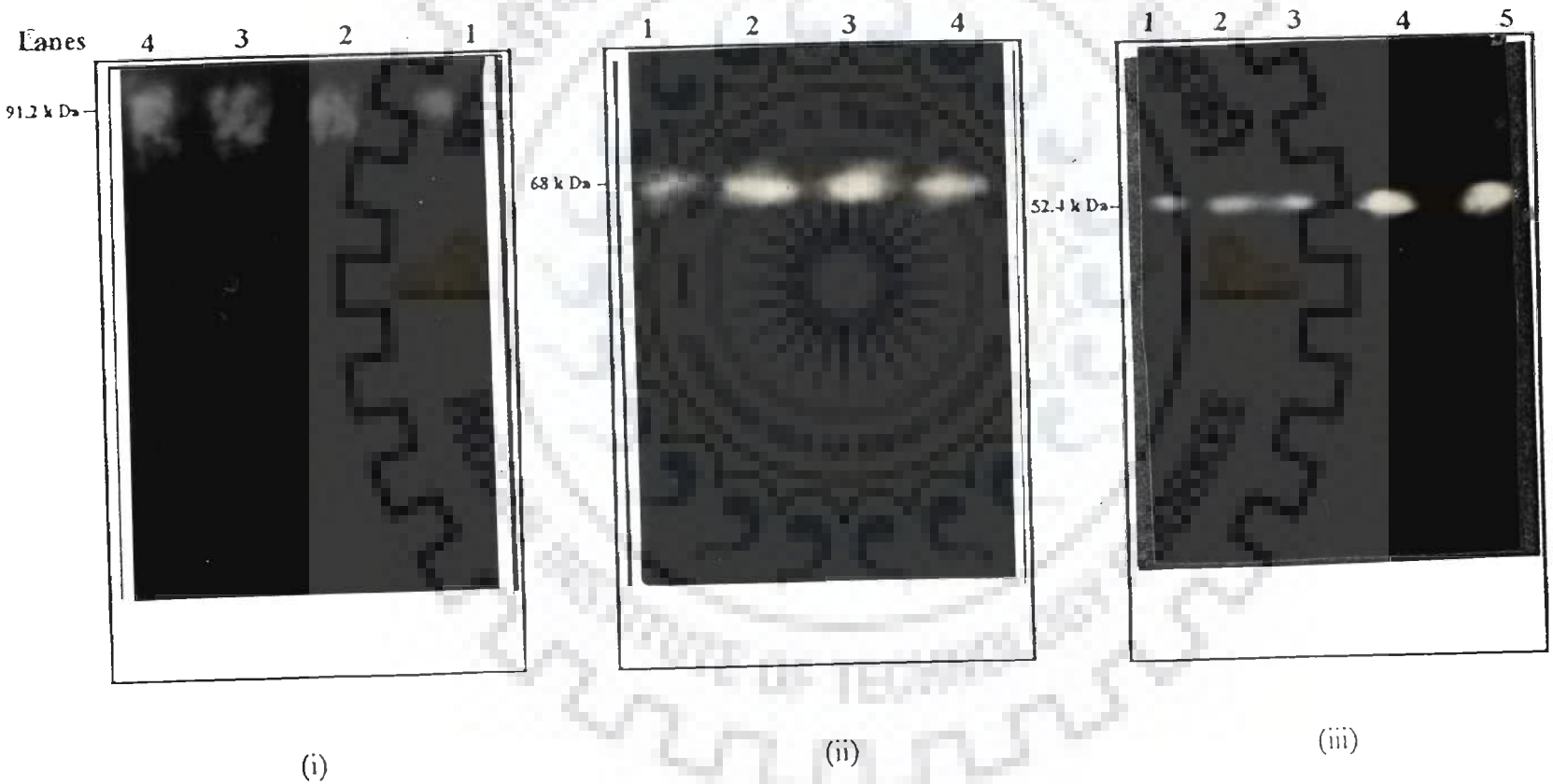


Fig. 27

every stage of subculturing (Fig. 28). The mutant UNSC-442 therefore appears to be a highly stable strain with remarkable ability for producing cellulase components.

Morphological changes in the mutant strain were analyzed by the Scanning Electron Microscopic (SEM) studies compared with that of the wild type strain. The growth of wild type strain was slow and the mycelia observed were thin, long and sporadically branched, whereas the mutant UNSC-442 was comparatively fast growing having the extended mycelia that were highly branched and swollen leading to the increased biomass production (Fig. 29a, b). Extent of sporulation in the mutant strain was comparatively lesser and spore morphology as observed was less globular and irregular (Fig 29c, d).

4.8 EVALUATION OF CELLULASE PRODUCTION UNDER CO-CULTIVATION CONDITIONS WITH STRAINS *ASPERGILLUS NIGER* RK-3, MUTANT UNSC-442 AND *TRICHODERMA REESEI* MTCC-164

Bioconversion of cellulosic material requires the synergistic activity of atleast three enzymatic components i.e. endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Among the various cellulolytic organisms known *Trichoderma reesei* has optimal levels of the first two enzymatic components, whereas its ability to produce β-glucosidase is low. Attempts have been made either to increase the β-glucosidase activity or by supplementing the

Fig. 28 Stability analysis of the mutant UNSC-442 for enzymatic production.

The cultures were transferred every month for a total period of ten months. The levels of enzymatic production were measured using the defined medium R_6m containing carboxymethylcellulose (1% w/v) as the carbon source.

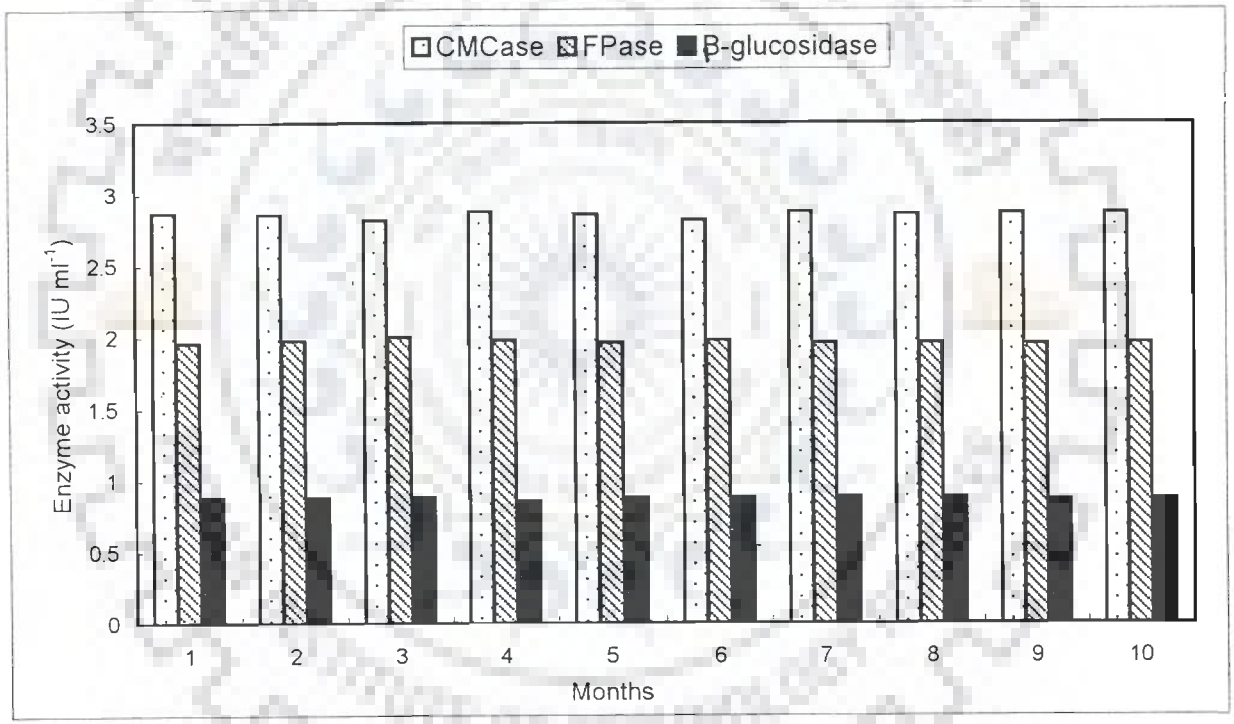
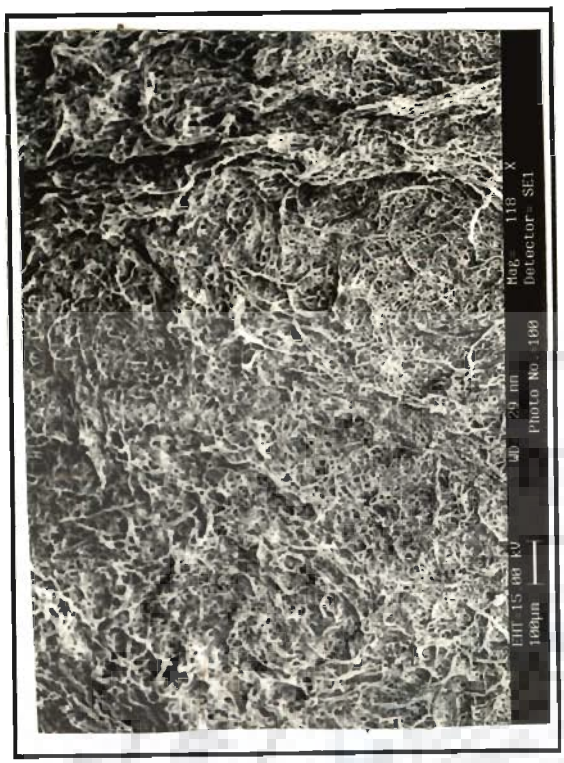
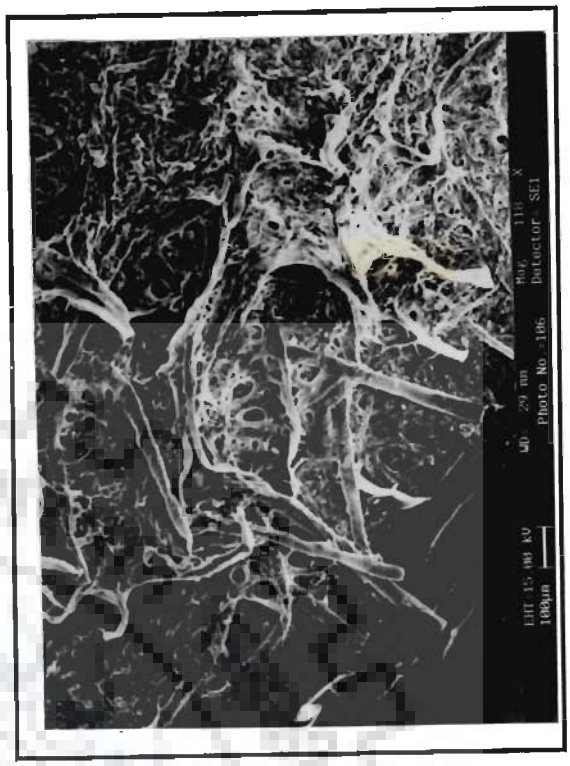

CMCase
FPase
β-glucosidase
Enzyme activity (IU ml-1)
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
Months

Fig. 28

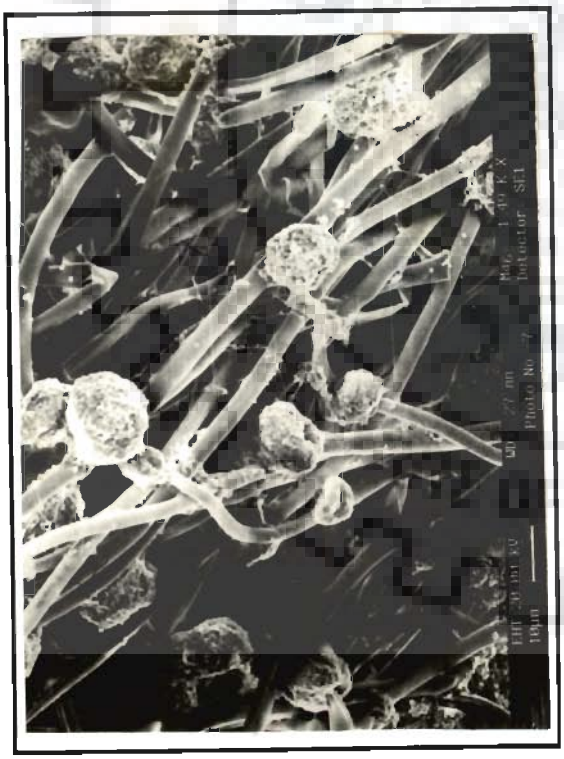
Fig. 29 Scanning electron micrographs of 72 h old mycelia of the wild type *Aspergillus niger* RK-3 (a) and the mutant UNSC-442 (b) and the pattern of sporulation from the wild type *Aspergillus niger* RK-3 (c) and the mutant UNSC-442 strain (d).


Mag 118 X
Detector SE1
WD 29 mm
Photo No. 180
EHT 15.00 kV
100µm

a


Mag 118 X
Detector SE1
WD 29 mm
Photo No. 186
EHT 15.00 kV
100µm

b



c

d

Fig. 29

β-glucosidase for achieving higher cellulolytic ability of the *Trichoderma reesei* strain. The selection of the compatible organisms with appropriate β-glucosidase level and its subsequent co-cultivation with *Trichoderma reesei* strain may lead to improved level of bioconversion

In present investigation, co-cultivation of *Aspergillus niger* RK-3, its mutant UNSC-442 alongwith *Trichoderma reesei* MTCC-164 using sugarcane bagasse and *Eicchornia crassipes* biomass under semi-solid state fermentation have been attempted for achieving increased levels of lignocellulosic bioconverson. The semi-solid state fermentation was performed using the defined medium R_6m except for the substrate concentration that was derived as below.

4.8.1 Analysis of Substrate Concentration for Cellulase Production

The varying concentrations (6-12% w/v) of the treated cellulosic substrates i.e., sugarcane bagasse and *Eicchornia crassipes* biomass using R_6m medium were utilized under semi-solid state fermentation. The production of cellulases from the wild type *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 was estimated. The results obtained, indicated that higher production of cellulases was observed after 18 days of incubation with 10% concentration of either sugarcane bagasse or the *Eicchornia crassipes* biomass used as the substrate. The production decreased with an increase in substrate concentration. Maximum CMCase (27.99 IU g^{-1} , 28.81 IU g^{-1}), FPase (14.21IU g^{-1}, 15.18 IU g^{-1}) and β-glucosidase (10.72 IU g^{-1}, 6.78 IU g^{-1}) activities were detected respectively with *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 at 10% sugarcane bagasse when used as the

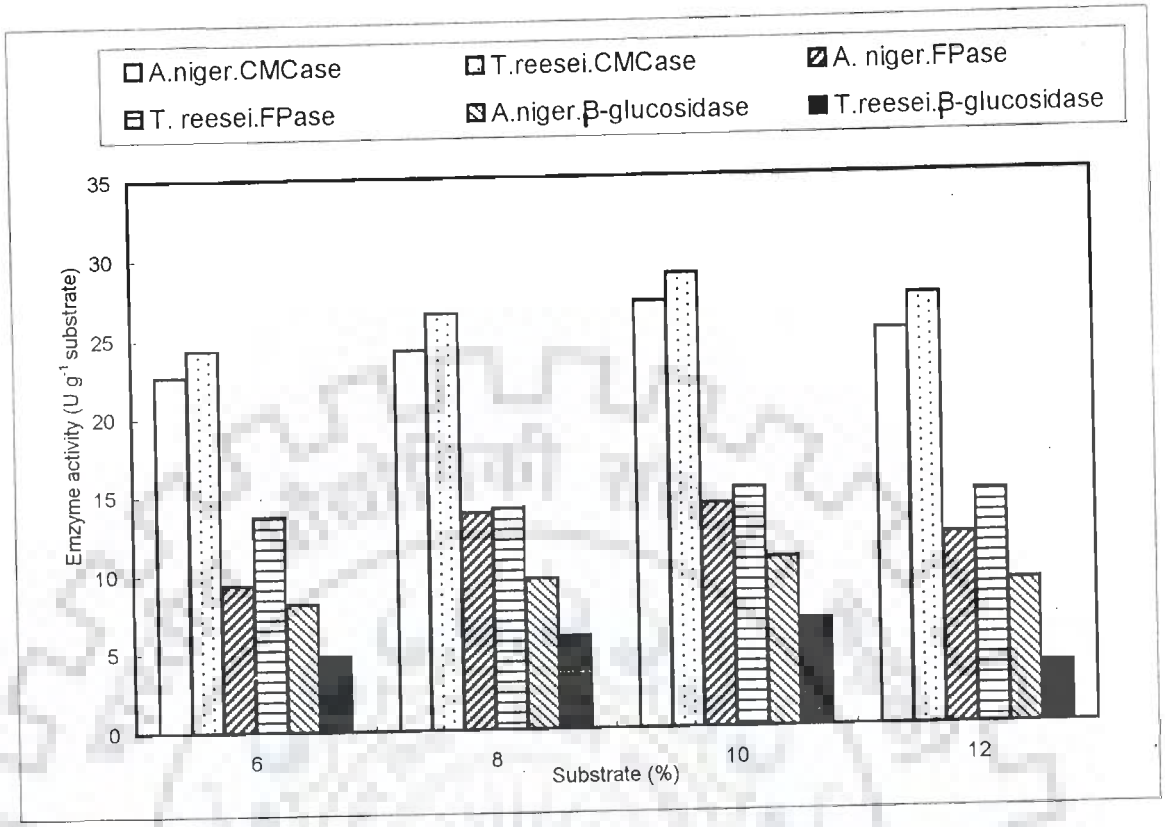
cellulosic substrate (Fig. 30a). Similarly *Eicchornia crassipes* biomass at 10% concentration resulted into maximum enzymatic activities i.e., CMCase (25.06 IU g^{-1}, 27.99 IU g^{-1}), FPase (16.33 IU g^{-1}, 18.48 IU g^{-1} and β-glucosidase (9.23 IU g^{-1}, 5.95 IU g^{-1}) as observed by *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 strains after 18 days of incubation (Fig. 30b). The total soluble proteins and the dry mycelial mass also had the similar patterns (Fig. 31a, b). Thus 10% concentration of the cellulosic substrates was used for co-cultivation studies in semi-solid state fermentation.

4.8.1.1 Evaluation of Sugarcane Bagasse

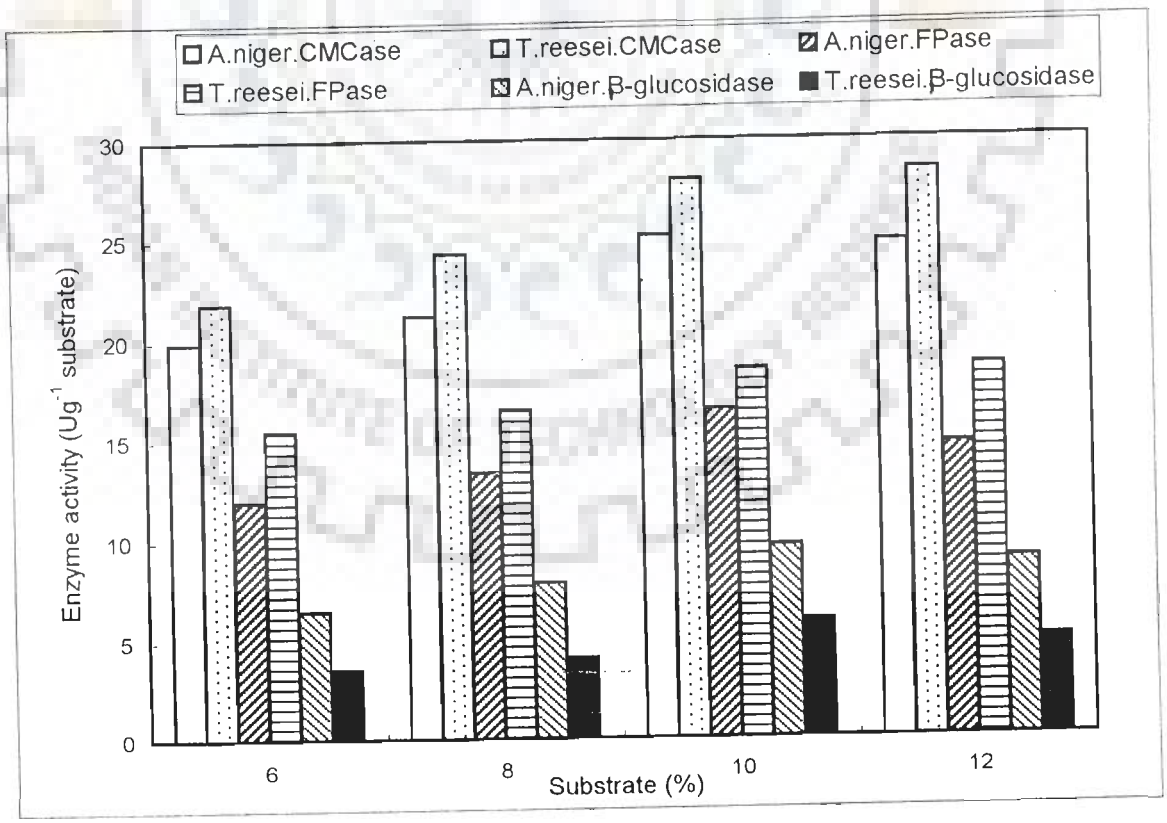
The co-cultivation of strain *Aspergillus niger* RK-3, mutant UNSC-442 along with *Trichoderma reesei* MTCC-164 (3:1) were used for estimating cellulase production. Incubation of *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 resulted into 23.3% and 32.8% increase in CMCase and FPase activities over the respective maximum activities observed under single culture conditions. However, the increase in β-glucosidase was only 13.5% as compared to the maximum β-glucosidase activity observed with *Aspergillus niger* RK-3 when used under single culture conditions (Fig. 32a). The co-cultivation of mutant UNSC-442 along with *Trichoderma reesei* MTCC-164 showed a marginal increase (8.5%) in CMCase activity over the maximum activity under single culture condition. However, comparatively higher increase was observed for FPase (22.7%) and β-glucosidase (24.1%) levels by co-cultivation of mutant UNSC-442 together with *Trichoderma reesei* MTCC-164 over the maximum activities observed under single

Fig. 30 **Effect of substrate concentration on cellulases production in Semi-solid state fermentation using treated sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b) as the substrate after 18 days of incubation at 30°C.**

Production of cellulases were estimated using medium R_6m added with indicate concentration of substrates as mentioned above.


A.niger.CMCase
T.reesei.CMCase
A. niger.FPase
T. reesei.FPase
A.niger.β-glucosidase
T.reesei.β-glucosidase
Emzyme activity (U g-1 substrate)
35
30
25
20
15
10
5
0
6
8
10
12
Substrate (%)

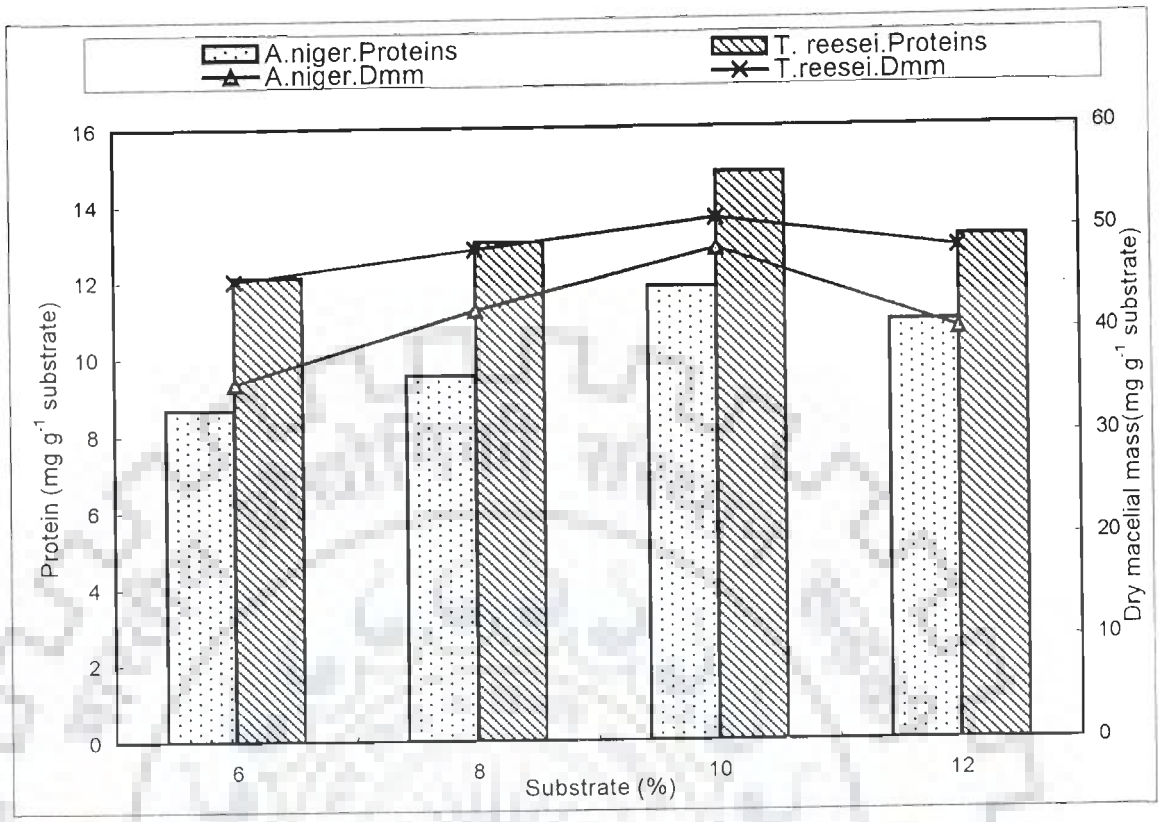
a


A.niger.CMCase
T.reesei.CMCase
A.niger.FPase
T.reesei.FPase
A.niger.β-glucosidase
T.reesei.β-glucosidase
Enzyme activity (Ug-1 substrate)
30
25
20
15
10
5
0
6
8
10
12
Substrate (%)

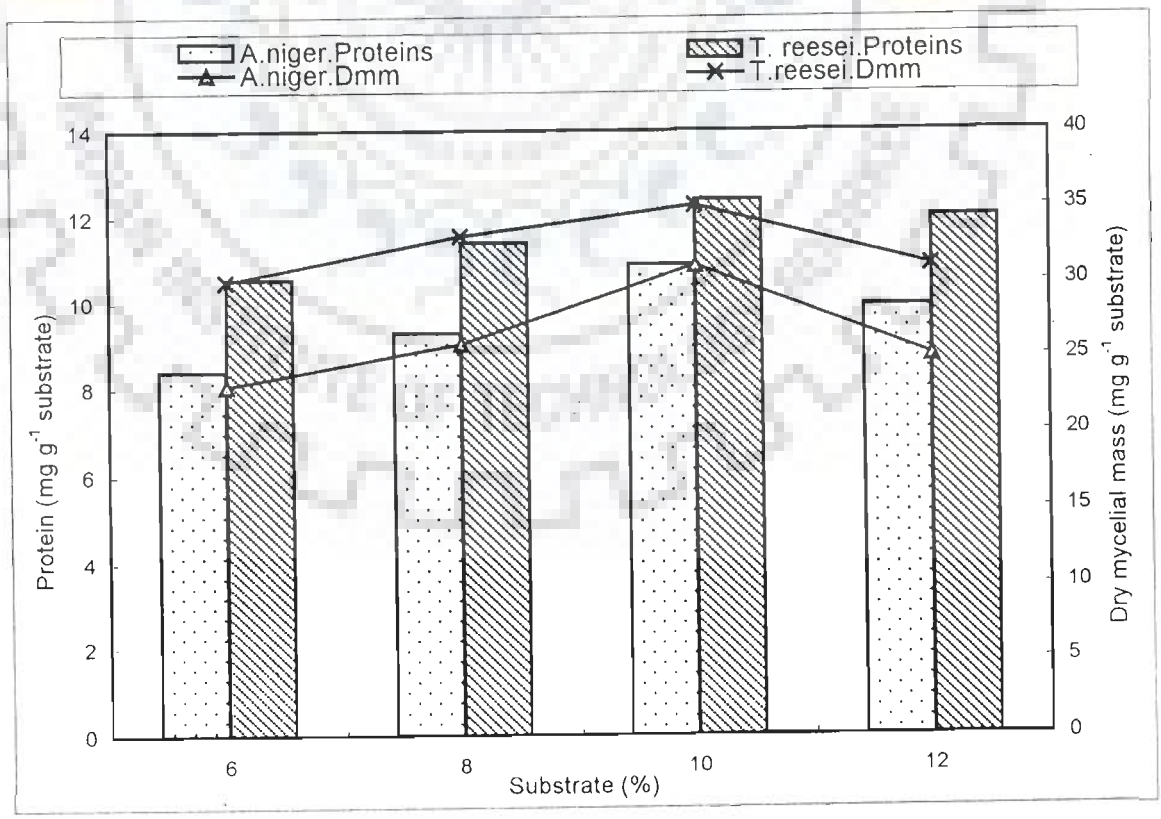
b

Fig. 30

Fig. 31 Effect of substrate concentration on total soluble proteins and dry mycelial mass (Dmm) in semi-solid state fermentation using sugarcane bagasse (a) and *Eicchornia crassipes* biomass (b) as the cellulosic substrate after 18 days of incubation at 30°C.


A.niger.Proteins
A.niger.Dmm
T. reesei.Proteins
T.reesei.Dmm
Protein (mg g-1 substrate)
Dry macelial mass(mg g-1 substrate)
Substrate (%)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
6
8
10
12

a


A.niger.Proteins
A.niger.Dmm
T. reesei.Proteins
T.reesei.Dmm
Protein (mg g-1 substrate)
Dry mycelial mass (mg g-1 substrate)
Substrate (%)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
35
40
6
8
10
12

b

Fig. 31

culture condition (Fig. 32a). The high enzymatic activities were related to increased soluble protein and total dry mycelial mass under co-cultivation conditions (Fig. 32b) as compared to under single culture condition.

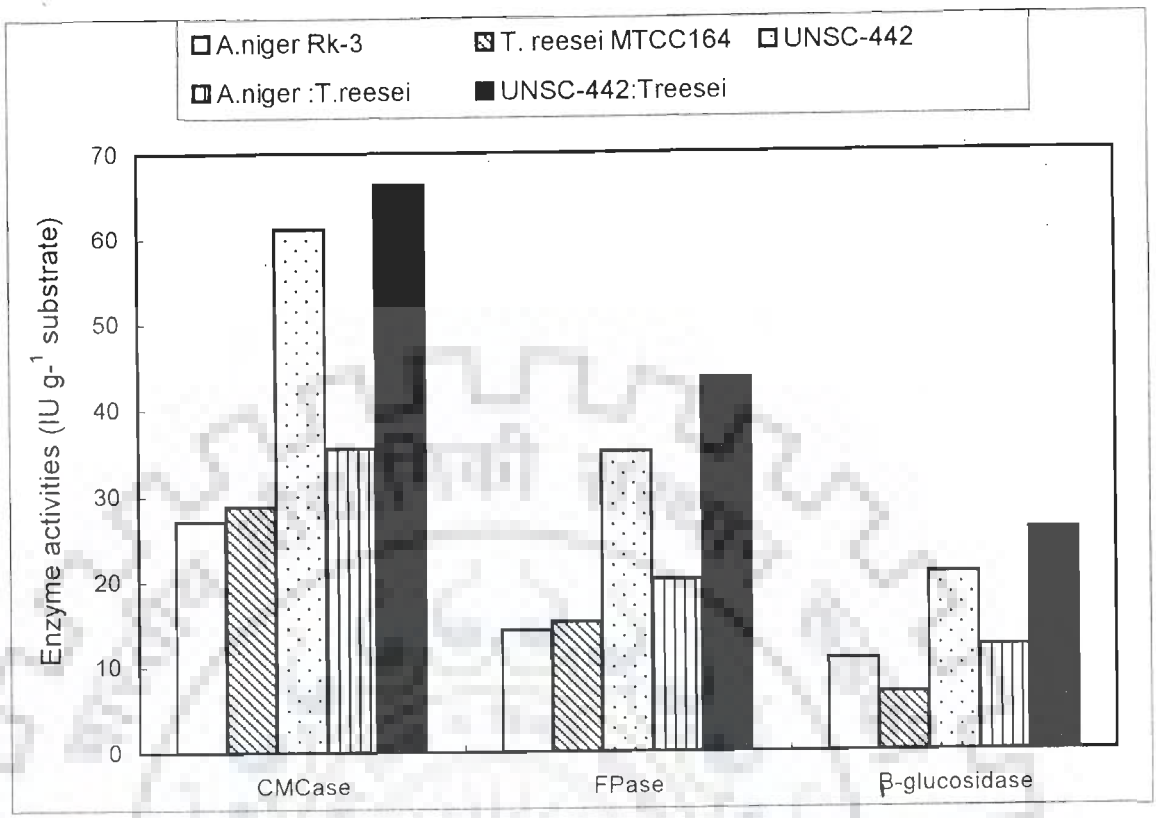
4.8.1.2 Evaluation of *Eicchornia crassipes* Biomass

The effect of co-cultivation of the wild type and the mutant strain as described for sugarcane bagasse was also studied for *Eicchornia crassipes* biomass as the substrate for cellulase production. Incubation of wild type strain *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 under co-cultured conditions with *Eicchornia crassipes* biomass resulted 20-24% increase in CMCase and FPase activities (Fig. 33a) over the respective maximum activities observed under single culture conditions. However, the increase in β-glucosidase activity was 13% higher compared to the maximum β-glucosidase activity observed when *Aspergillus niger* RK-3 was used as single culture for incubation under similar conditions. The co-cultivation of mutant UNSC-442 together with *Trichoderma reesei* MTCC-164 also showed moderate increase in CMCase (65.21 IU g^{-1}), FPase (43.21 IU g^{-1}) activities that were 10-11% higher as compared to the maximum enzymatic activities obtained under single culture conditions. However, the β-glucosidase activity observed was 23.47 IU g^{-1} under similar co-cultivation conditions and the increase in the activity was 29.5% higher as compared to the maximum β-glucosidase activity obtained under single culture condition. The high enzymatic activities were related to increased soluble protein and total dry mycelial mass under co-cultivation condition (Fig. 33b) as compared to under single culture condition.

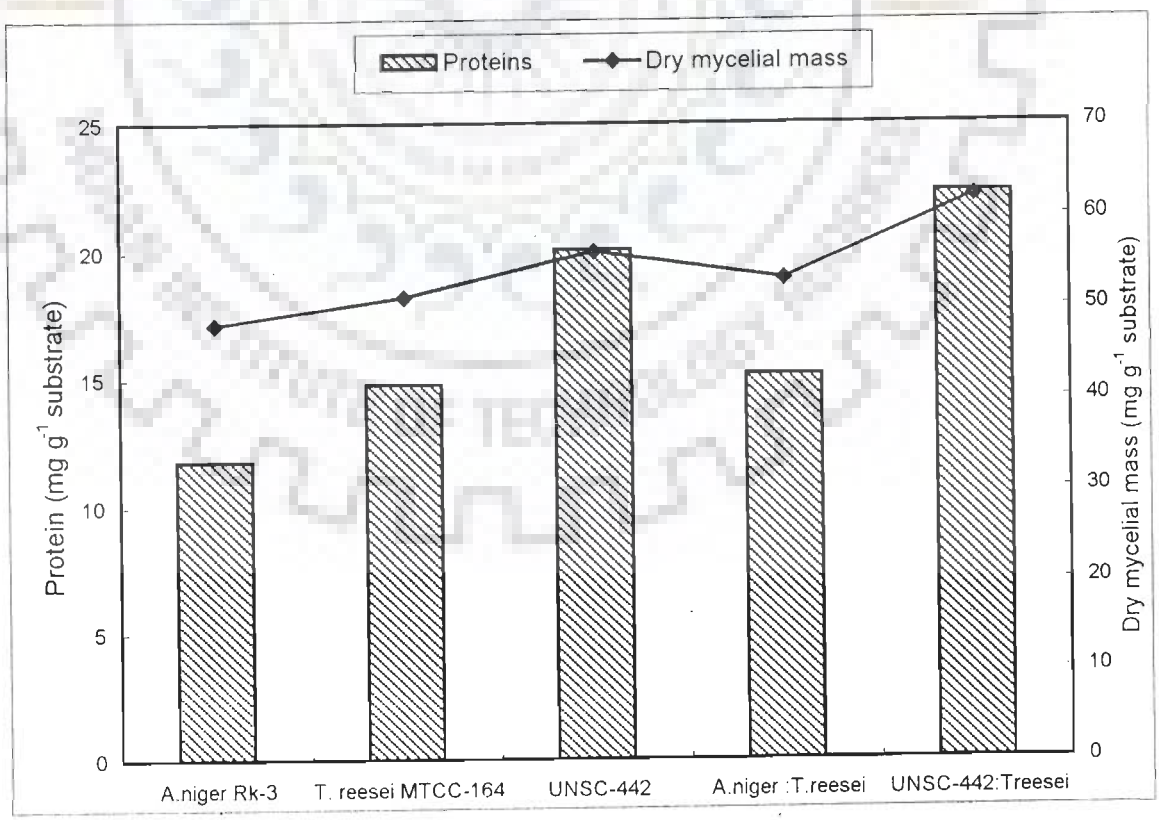
Fig. 32a. **Production of CMCase, FPase and β-glucosidase by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi-solid state fermentation using treated sugarcane bagasse as the substrate.**

The medium R_6m containing 10% of cellulosic substrate was used for the production of cellulases under the conditions as described for Fig. 30.

Fig. 32b. **Production of soluble proteins and dry mycelial mass by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi-solid state fermentation using treated sugarcane bagasse.**


A.niger Rk-3
T. reesei MTCC164
UNSC-442
A.niger :T.reesei
UNSC-442:Treesei
Enzyme activities (IU g-1 substrate)
70
60
50
40
30
20
10
0
CMCase
FPase
β-glucosidase

a


Proteins
Dry mycelial mass
Protein (mg g-1 substrate)
Dry mycelial mass (mg g-1 substrate)
25
20
15
10
5
0
70
60
50
40
30
20
10
0
A.niger Rk-3
T. reesei MTCC-164
UNSC-442
A.niger :T.reesei
UNSC-442:Treesei

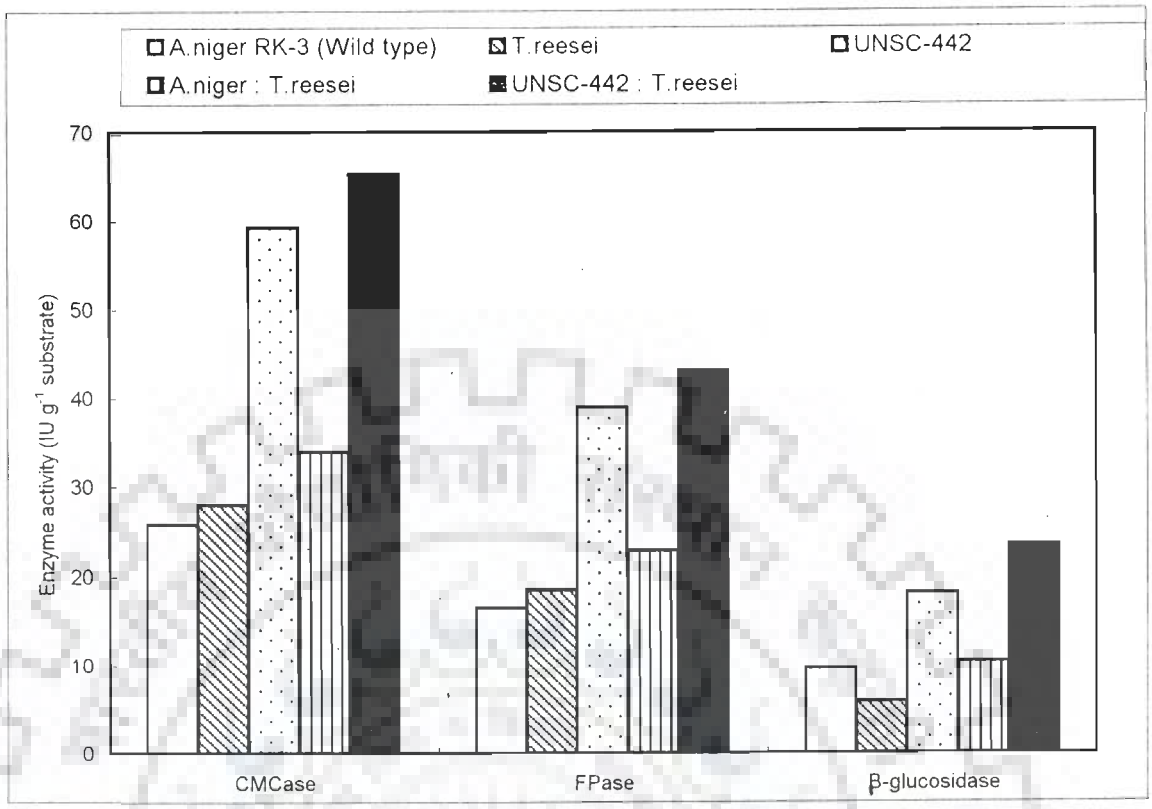
b

Fig. 32

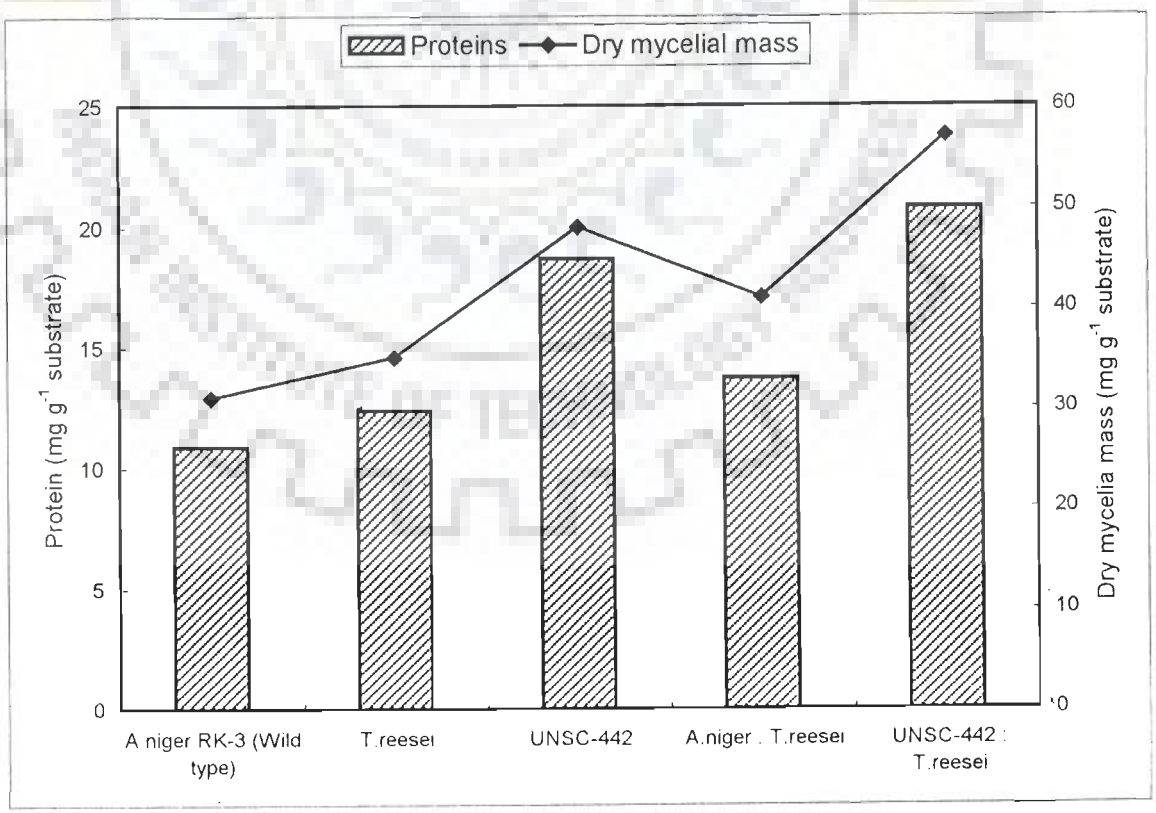
Fig. 33a **Production of CMCase, FPase and β-glucosidase by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi-solid state fermentation using treated *Eicchornia crassipes* biomass as the substrate.**

The medium R_6m containing 10% of cellulosic substrate was used for the production of cellulases under the conditions as described for Fig. 30.

Fig. 33b **Production of soluble proteins and dry mycelial mass by single and co-cultivation of *Aspergillus niger* RK-3, mutant UNSC-442 and *Trichoderma reesei* MTCC-164 in semi-solid state fermentation using treated *Eicchornia crassipes* biomass as the substrate.**


A.niger RK-3 (Wild type)
T.reesei
UNSC-442
A.niger : T.reesei
UNSC-442 : T.reesei
Enzyme activity (IU g-1 substrate)
70
60
50
40
30
20
10
0
CMCase
FPase
β-glucosidase

a


Proteins
Dry mycelial mass
Protein (mg g-1 substrate)
Dry mycelia mass (mg g-1 substrate)
25
20
15
10
5
0
60
50
40
30
20
10
0
A niger RK-3 (Wild type)
T.reesei
UNSC-442
A.niger . T.reesei
UNSC-442 : T.reesei

b

Fig. 33

4.9 APPLICATIONS OF CELLULOSE BIOCONVERSION TECHNOLOGY

4.9.1 Use of Cellulose Hydrolyzates for the Production of Baker's Yeast

The enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass were used as a carbon source for cultivating the yeast cells in carbon free medium (Czapex dox medium) supplemented with 0.1% peptone as described in the methods. The final concentration of reducing sugars, measured as glucose, in the hydrolyzate was adjusted to 2%. Similar medium containing 2% D-glucose was also used as control. A 10% inoculum of the yeast was added in the growth medium on a rotatory shaker (150 rpm) for upto 72 h at 30°C.

Yeast cells at indicated periods were harvested, washed thrice with double distilled water and the freeze dried over night. The results of triplicate experiments are presented in Table 18. The maximum production of yeast biomass was observed after 72 h of incubation with enzymatic hydrolyzates of either sugarcane bagasse (178 mg ml^{-1}) or *Eicchornia crassipes* (163 mg ml^{-1}) biomass. The biomass production under these conditions is significantly comparable with the biomass generated with 2% D-glucose (188 mg ml^{-1}) used as the carbon source. Thus use of enzymatic hydrolyzates of either sugarcane bagasse or *Eicchornia crassipes* biomass appears to be promising for yeast growth and the process can be suitably extended for pilot scale production.

Table 18 Cultivation[a] of *Saccharomyces cerevisiae* (baker's yeast) on enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass

Carbon source	Sugar concentration (%)	Incubation (h)	Dry weight of cells (mg ml^{-1})
Enzymatic hydrolyzate of Sugarcane bagasse.	2	12	18
		24	109
		48	167
		72	178
Enzymatic hydrolyzate of *Eicchornia crassipes* biomass.	2	12	16
		24	98
		48	144
		72	163
D-glucose	2	12	21
		24	128
		48	171
		72	188

[a], after 10 days of fermentation.

4.9.2 Use of Cellulose Hydrolyzates for Gluconic Acid Production

The enzymatic hydrolyzates prepared as described in methods were used as the substrate (carbon source) for the production of gluconic acid by *Aspergillus niger* ORS-4 (isolated in this laboratory). Hydrolyzates containing sugar equivalent to 4 to 14% glucose were added in the fermentation broth (as describe in the methods). The fermentation was initiated by adding 5% inoculum of *Aspergillus niger* ORS-4 spores (2×10^6 spores ml^{-1}) produced after incubating the *Aspergillus niger* ORS-4 on potato dextrose agar plates for 5 days. After 10 days of fermentation period, gluconic acid formed into the medium was estimated which showed 64.40 mg ml^{-1} and 45.18 mg ml^{-1} of the gluconic acid with the enzymatic hydrolyzates (sugar content 10%) of sugarcane bagasse and *Eicchornia crassipes* biomass respectively. These levels of gluconic acid production were comparable with the production levels obtained (68.2 mg ml^{-1}) with similar amount of the D-glucose (Table 19). These results clearly showed that the sugars produced by the enzymatic hydrolyzates of either sugarcane bagasse or *Eicchornia crassipes* may be used as a cheap substrate for gluconic acid fermentation and may require no pretreatment as usually required in the case of molasses.

4.9.3 Use of Cellulose Hydrolyzates for Citric Acid Production

The enzymatic hydrolyzates prepared was used as a substrate (carbon source) for citric acid production by *Aspergillus niger* KCU-5. Hydrolyzates containing sugar equivalent to 4 to 14% glucose were added in to the fermentation broth (as described in the methods). A 5% inoculum of *Aspergillus niger* KCU-5

Table 19 Production[a] of gluconic acid by enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass by *Aspergillus niger* ORS-4

Carbon source	Sugar concentration (%)	Gluconic acid (mg ml^{-1})
Enzymatic hydrolyzate of Sugarcane bagasse	4	21.67
	6	25.73
	8	51.35
	10	64.40
	12	58.43
	14	52.88
Enzymatic hydrolyzate of *Eicchorina crassipes* biomass	4	16.88
	6	20.67
	8	32.81
	10	45.18
	12	44.00
	14	40.91
D- glucose	4	24.33
	6	32.18
	8	55.15
	10	68.20
	12	62.11
	14	60.73

[a], after 10 days of fermentation.

Table 20 Production[a] of citric acid using enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass by *Aspergillus niger* KCU-5

Carbon source	Sugar concentration (%)	Citric acid (mg ml^{-1})
Enzymatic hydrolyzate of Sugarcane bagasse	4	22.55
	6	35.11
	8	50.31
	10	54.80
	12	52.10
	14	51.30
Enzymatic hydrolyzate of *Eicchorina crassipes* biomass	4	20.0
	6	33.51
	8	45.38
	10	48.33
	12	44.30
	14	40.10
D-glucose	4	25.32
	6	40.14
	8	54.50
	10	60.31
	12	58.83
	14	55.38

[a], after 10 days of fermentation.

spores ($2x10^6$ spores ml^{-1}) was added and fermentation continued for 10 days. The concentration of citric acid was measured. A maximum production of i.e. 54.80 mg ml^{-1} and 48.33 mg ml^{-1} of citric acid was obtained from the enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass respectively having 10% sugar concentration (Table 20). In addition the level of citric acid production (60.31 mg ml^{-1}) with D-glucose as a substrate was found to be comparable. The results therefore clearly indicated that the sugars produced by the enzymatic hydrolyzates of either sugarcane bagasse or *Eicchornia crassipes* biomass may be used as a cheap substrate for citric acid fermentation. Further studies are under way for scale up the process for improving the economics of the products of the commercial significance.

Chapter 5

DISCUSSION

DISCUSSION

Lignocelluloses as agricultural, industrial and forest residues account for majority of the total biomass present in the world and around 10 to $50x10^9$ tons of this potential biomass is generated as wastes per annum. The majority of this waste material remains unutilized thus causing loss of this potentially valuable resource material (Singh et al. 1988b). The bioconversion of these lignocellulosic waste material is an attractive proposition for the production of less expensive fermentable sugar (Dale, 1987) such as glucose which is the precursor for organic acids, alcohols, single cell proteins, and many other products of industrial significance. The major emphasis of this study therefore was (i) to identify a high cellulase producing microorganism (ii), to define precise conditions for maximum bioconversion and (iii) to utilize the high cellulose containing and abundantly available agricultural and natural lignocellulosic waste materials for bioconversion into fermentable sugars.

In the present investigation two major lignocellulosic waste materials particularly sugarcane bagasse and water hyacinth (*Eicchornia crassipes)* biomass were evaluated for bioconversion. Sugarcane bagasse is a major waste material generated from the sugarcane industries that are prevalent in and around this region. It remains largely unutilized and is subjected for burning, causing environmental pollution. On the other hand *Eicchornia crassipes* biomass is another major lignocellulosic waste which is present in plenty in the ponds, lakes and rivers and is a major concern for water quality and quantity (Basu et al. 2000). The biomass in some instances is used as animal feed and otherwise is of no particular value.

The bioconversion of these major lignocellulosic materials was attempted by isolating microbial strains from decomposing bagasse and the paper waste materials. Eleven fungal and eleven bacterial strains were isolated and analyzed for cellulase producing ability on congo-red CMC-agar plates as described (Schwarz et al. 1989). Among these, the RK-3 strain was found to have the maximum level of enzymatic activities on plate assay as well as in submerged state fermentation. This strain was identified as *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi, India and designated as *Aspergillus niger* RK-3. The present work embodies a detailed investigation toward the ability of the isolated strain *Aspergillus niger* RK-3 for bioconversion of sugarcane bagasse and *Eicchornia crassipes* biomass lignocellulosic waste materials. In 1950 Dr. Elwyn Reese and his co-workers at the Natick Laboratories USA, isolated and identified *Trichoderma* strain with ability to produce an active cellulase complex (Ryu and Mandels, 1980). Similarly many thermophilic fungi isolated from wood chips and baled sugarcane bagasse were studied for degradation of plant cell wall (Chang, 1967). Later on Tansey (1971) and Sugden and Bhat (1994) reported strains of *Chaetomium thermophile*, *Sporotrichum thermophile* and *Thermoascus aurantiacus* having higher cellulolytic activities than *Trichoderma viride* a potent cellulolytic fungus as reported (Mandels and Weber, 1969). Subsequently El-Refai et al. (1984) surveyed about fourteen fungal, three bacterial and two yeast strains for cellulolytic activities with beet bagasse and found *Penicillium funiculosum* to be the best strain. Further, *Aspergillus niger* AS 101, *Aspergillus terreus*, *Aspergillus wentii* have been isolated and identified to be cellulase producing strains (Singh et al. 1988a;

Srivastava et al. 1987; Yadav and Neelkantan, 1988). At the same time nine white rot basidiomycetes strains were isolated from fruiting bodies from the decaying wood in forest of Sourthern Germany and observed only the *Trametes versicolor* as a phenol resistant and the cellulase producing strain (Muller et al. 1988). Similarly *Chaetomium thermophile* var. *coprophile*, *Pencillium purpurogenum*, and *Humicola fuscoatra* were also isolated from different substrates (Gunju and Murthy, 1990; Steiner et al. 1994; Rajenderan et al. 1994).

Cellulase production ability of *Aspergillus niger* RK-3 was assessed initially with the synthetic cellulosic substrates. Among these substrates, carboxymethyl cellulose, followed by phosphocellulose and other substrates used resulted into significant levels of enzyme production. The variation in the enzyme production pattern may be due to the crystallinity of the substrates. The nature and relative distribution of the substituent groups e.g. methyl, ethyl, carboxymethyl, hydroxymethyl etc. may account for the solubility and crystallinity of the cellulosic materials (Cowling, 1975; Fan et al. 1980; Fan et al. 1982; Bisaria and Mishra, 1989). Besides crystallinity, the adsorption profile of the cellulases onto these materials may equally affect the degree of hydrolysis of the substrates. As observed the carboxymethylcellulose had a lower crystalline index and had the maximum adsorption as compared to other substrates and possibly therefore resulted into higher enzymatic production. A positive correlation between the adsorption of cellulases and enzymatic hydrolysis had been observed by many groups (Ghose and Bisaria, 1979; Castanon and Wilke, 1980; Klyasov et al. 1986). Adsorption of cellulases is related to the adsorption sites and the surface area of the

cellulose fibers (Fan et al. 1980; Kim et al. 1988;). Besides these, hydrogen bonds, Vanderwall's forces and hydrophobic interaction of cellulases may also contribute towards adsorption and subsequently to hydrolysis of the substrate as described (Reinkainen et al. 1995; Tomme et al. 1995; Duff and Murray, 1996).

5.1 EVALUATION OF NATURAL LIGNOCELLULOSIC MATERIALS AS THE SUBSTRATES

The prime emphasis of the present study was to utilize the agricultural and natural lignocellulosic waste materials i.e. sugarcane bagasse and *Eicchornia crassipes* biomass for bioconversion into simple sugars. The cellulase production ability of *Aspergillus niger* RK-3 with synthetic substrates was further analyzed using agro (sugarcane bagasse) and natural (*Eicchornia crassipes* biomass) waste materials. As described earlier, the sugarcane bagasse is a major waste from sugarcane industry and generally remains unutilized whereas the water hyacinth (*Eicchornia crassipes*) is a natural waste present in abundance in the ponds, lakes and rivers and is a serious threat to the precious aquatic resources as well as is an environmental menace (Basu et al. 2000). The high cellulosic content of these materials indicate these as the promising substrates for their further utilization. One promising way to utilize these plentiful biomass materials is to convert them into fermentable sugars for production of the industrially significant products.

The lignocellulosic substrates i.e. sugarcane bagasse and *Eicchornia crassipes* biomass like any other lignocellulosic materials are characterized by a ternary complex composed of hemicellulose, lignin and cellulose. The presence of

lignin component of the lignocellulosic materials makes them resistant to enzymatic hydrolysis. Therefore in order to make the cellulose susceptible to hydrolysis, the lignocellulosic substrates were subjected to pretreatment for breaking the ternary complex that would reduce the crystallinity and increase the surface area which therefore would enhance the rate of saccharification to a greater extent (Tanahashi et al. 1982; Puri and Pearce, 1986). The different approaches had been employed for the pretreatment (Oliver and Toit, 1986; Singh et al. 1988c; Singh et al. 1990; Weil, 1994). The steam treatment followed in the present investigation was a modified version of Jurasek (1978) and Lipinsky (1979) approach where material was heated rapidly to 243 – 250^0 C by high pressure steam for 1 minute and then exploded by a quick release of the pressure. In the present investigation the material was steam heated at a lower temperature (180 – 190^{0}C) for 30–40 minutes. Steam treatment as reported results an increase in the surface area, pore size and thus effecting the partial decrystallization of the cellulose, in addition, removal of acetyl groups from hemicellulose and it's depolymerization have also been reported (Fan et al. 1980; Doppelbauer et al. 1987; Hartree et al. 1987). The ternary complex (hemicellulose-lignin-cellulose) under these conditions is broken thus damaging the crystallinity of the substrates and making these susceptible to enzymatic degradation (Tanahashi et al. 1982). The high pressure steam treatment was followed by extraction of lignocellulosic material with aqueous and alkaline (0.25 N NaOH) solutions for further disintegration and removal of hemicellulose and lignin components of the substrates. Steam and alkali treatment have widely been attempted by several investigators (Doppelbauer et al. 1987; Singh et al. 1990;

Maheshwari et al. 1992; Weil et al. 1994; Gupte and Madamwar, 1997) and has been found to be highly effective for optimal cellulose hydrolysis.

5.1.1 Cellulase Production with Treated Cellulosic Materials

The results obtained had indicated that production of cellulases significantly increased with steam-alkali treated substrates. Lower level of production with untreated substrates was possibly due to the lignin content of the cellulosic substrates, which may not only inhibit the cellulases chemically but also act as a barrier, rendering the cellulases unsusceptible to the enzymatic action (Singh et al. 1991; Singh et al. 1990). The digestibility of the treated cellulosic material depends on the adsorption of enzymatic proteins on cellulosic surface that in turn depends on the cellulose binding domains (CBDs) of the enzymes, surface area and the adsorption sites present on the surface of the cellulosic fibers (Kim et al. 1988). Moreover the interaction of cellulose binding domains (CBDs) of the cellulase macromolecule with the cellulose surface has been shown to affect the decrystallization of the cellulosic substrates (Himmel et al. 1999). Of the two substrates used for the present study treated sugarcane bagasse had comparatively higher adsorption than the *Eicchornia crassipes* biomass. This variation may be related to the crystalline structure of the substrates; and as observed, the sugarcane bagasse was less crystalline than the *Eicchornia crassipes* biomass and hence more suitable for the enzymatic adsorption. These observations had substantiated our findings that comparatively higher enzymatic production was obtained with sugarcane bagasse than the *Eicchornia crassipes* biomass. Our observations had

also indicated that the crystalline structure of the substrates damaged because of lignin removal due to steam-alkali treatment, resulting into reduced crystalline index and thus effecting an increase in the amorphosity of cellulose (Blackwell, 1982; Weil et al. 1994). Higher amorphosity of the substrate was suitable for enzymatic adsorption and then hydrolysis of cellulosic substrates (Kim et al. 1988).

Scanning electron microscopy had shown that untreated substrates had compact and rigid structure compared to the treated substrates, which are loose and fibrous. Further, the removal of external cuticle leads to the epidermal peeling followed by the removal of lignin and hemicellulose components of the lignocellulosic materials. The slow growth of fungus was noted on the surface of untreated substrate as compared to the treated substrates, where rapid growth took place on the outer surface accompanied with the penetration of mycelia in the intracellular locations. Similar observations were reported by Kuhad and Johri (1992) who had demonstrated the removal of surface cuticle and disintegration of fibrous structure are the major factors affecting microbial attack on cellulosic substrates. Koeings (1974) has proposed that in the brown rot fungi Fenton's reaction producing hydroxyl radical in combination with the enzyme is mainly active in depolymerizing the cellulose. However, the Fenton's reaction mechanism has not been proven to be the primary mode of cellulose degradation in wood, mainly due to the unknown life span of extracellular H_2O_2 in the environment and its subsequent diffusion into the wood cell components (Veness and Evans, 1989). However, H_2O_2 is necessary for the action of the lignolytic enzymes as reported by Kersten (1990). Further, Evans et al., (1994) have presented a hypothesis

concerning to the role of some low-molecular-mass (LMM) mediators such as hydrogen peroxide, veratryl alcohol, oxalate and manganese contributing toward fungal attack to lignocellulosic materials. As proposed, the LMM mediators diffuse into the wood cell wall structure to initiate the decay, thus, opening up the pore size in the wood and allowing enzyme penetration for the degradative process. Veratryl alcohol and oxalate are produced as a result of fungal metabolism and their secretion enabled fungi to colonize and degrade the wood cell wall more effectively than the other organisms. Moreover, the fermented cellulosic substrates showed strikingly prominent nodular bodies attached to rib like structure known as silica bodies (Kuhad and Johri, 1992; Chahal et al. 1995). The frequent appearance of silica bodies indicated progressive consumption of organic matter by the fungus and leaving behind the undigested inorganic elements as reported (Kuhad and Johri, 1992).

5.2 ANALYSIS OF FACTORS AFFECTING CELLULASE PRODUCTION

Among the factors analyzed the pH of the medium is correlated to the enzymatic adsorption thus significantly affecting the saccharification process (Doppelbauer et al. 1987; Kim et al. 1988; Rajenderan et al. 1994; Reinikainen et al. 1995). Analysis of pH indicated 4.8 to be the optimal for cellulase production by *Aspergillus niger* RK-3 with both the substrates and is in agreement with earlier groups (Mandel et al. 1975; Eriksson, 1976; Toyama and Ogowa, 1976; Ismail et al. 1985). However, a pH of 5.5 was found suitable for β-glucosidase production from *Penicellium purpurogenum* (Bronnenmeier and Staudenbauer, 1988). Similarly

Menon et al., (1994) suggested a pH range of 5.5-6.0 yielded maximum β-glucosidase and pH below 3-4 and above 7.0 showed inhibitory effects on enzymatic production (Steiner et al. 1994).

Temperature like pH influences the enzyme production. A temperature of 30-32° C was suited for the growth of the isolated *Aspergillus niger* RK-3 strain, whereas higher i.e. 50°C was optimal for reaction and was similarly reported by other investigators (Bronnenmeier and Staudenbauer, 1988; Steiner et al. 1994 and Vipan et al. 1994). The temperature besides affecting growth and enzyme production also affects adsorption of the cellulases for hydrolytic reaction (Van-Wyk, 1997). The optimum pH and temperature for production of cellulases may vary to some extent depending onto the physiology of the cellulolytic organisms (McCarthy et al. 1987; Tuncer et al. 1999).

Besides the above, 5% of the 24 h old inoculum was best suited for cellulase production, higher level of inoculum decreased the enzyme activity due to clumping of the cells, that could reduce the sugar and oxygen uptake and also the enzyme release (Srivastava et al. 1987). A 4-6% level of inoculum works the best as reported (Srivastava et al. 1987; Singh et al. 1989a and Gunju et al. 1990).

The cellulose concentration appeared critical for cellulase production as cellulose greater than 1% (w/v) led to a decline in the cellulase levels. Cellulose at 3% (w/v) nearly inhibited the enzyme production (Lakhani, 1990; Menon et al. 1994; Steiner et al. 1994) *Penicillium rubrum* O stoll and *Penicellium purpurogenum* also had maximum enzyme production with 1% (w/v) cellulose as the substrate. Similarly 1% (w/v) of the carboxymethylcellulose, cereal straw and avicel were observed

suitable for cellulolytic activities of the *Chaetomium globosum* and *Sporotrichum thermophile* (Aikane and Patil, 1989; Sudgen and Bhat, 1994). The decrease in cellulase production at increasing cellulose concentration may be attributed either to the physical adsorption of the enzyme on cellulose or the repression of cellulase synthesis due to accumulation of the hydrolytic products in the medium (Singh et al. 1990; Rajendran et al. 1994). On the other hand cellulose at 10% (w/v) led to maximum cellulase production in semi-solid state fermentation. These observations are similar to that of Singh et al., (1989a), who showed 12% cellulose, for maximum enzyme production in semi-solid state fermentation. Higher substrate concentrations decreased the enzymes production and may possibly be due to depletion of oxygen, moisture contents and because of the exhaustion of nutrients (Phillips, 1966; Garg and Neelkantan, 1982; Singh et al 1991; Rajenderan et al. 1994).

Of the various nitrogen sources used, except ammonium ferrous sulphate, the others showed an inductive effect on β-glucosidase activity and maximum induction observed was due to ammonium chloride and may be attributed due to chloride ions having as shown an inductive effect for CMCase and β-glucosidase activity (Singh et al. 1991). However, a lower level increase in CMCase activity was observed with ammonium chloride in the present investigation. A decreased β-glucosidase activity with presence of ammonium ferrous sulphate may be due to ferrous ions as reported (Garg and Neelkantan, 1982; Yeoh et al. 1986; Singh et al. 1991). Among the phosphorus sources potassium dihydrogen phosphate is the most favourable phosphorus source for increased enzymatic production. Garg and Neelkantan (1982) and Singh et al., (1991) had also demonstrated potassium

dihydrogen phosphate to have an inductive effect on cellulase activity. Addition of other phosphorus sources was inhibitory.

Besides the studying the effect of nutrients as discussed the effect of some other regulatory compounds i.e. phenolics, organic acids and sugars were analyzed. In the phenolic compounds, vanillin has resulted into maximum stimulation to the cellulase levels as similarly reported by Muller et al., (1988). The stimulation has been shown to be due to induce the laccase production, which stimulates the cellobiose-quinone oxidoreductase (CQOase) (Arora and Sandhu, 1986). The CQOase enzyme complex converts cellobiose into cellobionic acid thus cellobiose (a cellulase inhibitor) formed during cellulose hydrolysis is continuously utilized by oxidation. In another observation, Muller et al., (1988) proposed that a compound cellobiono-lactone was formed by the enzymatic interactions of laccase, phenol, cellobiose and CQOase. This lactone enhanced the cellulase production significantly.

Addition of organic acids in general were inhibitory except for succinic acid that marginally induced the cellulase levels. Singh et al., (1988b) has however observed the increased production of CMCase, FPase and β-glucosidase with acetic acid. The pH of the system during cellulolytic activity increases slightly during incubation as observed by Doppelbauer et al., (1987) and may be regulated by addition of the organic acids.

Sugars, particularly the glucose or cellobiose added in the medium in 1:5 ratio with cellulose (1% w/v), showed a notable enhancement in cellulase activity. Presence of glucose/cellobiose or any other soluble sugar along with cellulose may

initially lead to cell growth to yield necessary cell mass which was then induced by cellulose for enzyme production (Singh et al, 1989b). The results obtained in the present study was also supported by the Paul and Varma (1990), who had studied the effects of glucose and cellobiose on xylanase biosynthesis. Similarly, Waki et al., (1976) proposed a mechanism of cellulase biosynthesis and stated that cellulose is degraded into soluble oligosaccharide and cellobiose. Cellobiose then enters in the cell wall and degraded into glucose due to cellobiase (β-glucosidase). The glucose or its catabolite generated acts as catabolite repressor and thus cellobiose acts as inducer. Effects of various soluble sugars such as cellobiose, sophorose, lactose and cellobionolactone as the inducers of the cellulase synthesis have been described (Sternberg and Mandels, 1979; Bisaria and Mishra, 1989). It was presumed that cytosolic sucrose phosphorylase may play a role in initiation of cellulase synthesis. Maltose an α-1, 4- linked disaccharide containing two glucose moieties is analogous to β-1, 4- linked cellobiose, seems acting like glucose, as a repressor delaying cellulase synthesis and still stimulating the enzyme production in *Aspergillus cellulolyticus* as reported (Shiang, 1991). However, inhibitory effect of the soluble sugars at higher concentration was reported by Yeoh et al., (1986). Shiang et al., (1991) proposed the possible regulatory mechanism of cellulase biosynthesis. According to this, sugar analogues i.e. cellobiose, glucose, sucrose, sorbose, xylose etc. at a particular concentration induce cellulase activator molecule (CAM), a cellulase regulatory protein. CAM may or may not be the same component as suggested by Stutzenberger (1985) who described the regulation of cellulase synthesis using a modification of the arabinose operon (Shiang, 1991).

The formation rate and yield of CAM are reportedly dependant on substrates, substrate concentration and some unknown factors imparted by moderators.

5.3 STRAIN IMPROVEMENT BY MUTAGENESIS

The widespread occurrence of lignocellulosic materials and its potential for generation of fermentable sugars for production of fuels and other value added products of commercial significance has resulted into extensive work towards it bioconversion. The materials with lower cellulosic contents and restriction in availability of suitable strains appear to be a major barrier for achieving higher rates of bioconversion. There appears to be a little immediate scope for using gene transfer technique due to the complexity and diversity of the bioconversion process. The major success for increasing the bioconversion level is basically due to the extensive application of mutation and selection. Hence this approach was applied to *Aspergillus niger* RK-3.

The strain *Aspergillus niger* RK-3 as detected had significant levels of cellulase production with lignocellulosic materials under defined conditions containing nutrients and regulators. The strain RK-3 was further subjected for physical and chemical mutagenesis for achieving higher levels of cellulase production for further economizing the process of bioconversion. UV mutagenesis of strain RK-3 resulted into mutants RKU-40, RKU-45 with higher zone diameters and cellulase activities with respect to the other mutants obtained either by UV or chemical mutagenesis. The mutants RKU-40 and RKU-45 were further subjected for mixed chemical mutagenesis. During the treatments, mutants showing

maximum zone diameters were selected and subjected for next step of chemical mutagenesis. After the final step, mutant UNSC-442 was found to have the maximum zone diameter and subsequently had the higher cellulase activity. The mutant UNSC-442 thus obtained respectively had 89% and 130% higher CMCase and FPase activities with sugarcane bagasse and about similar increase in CMCase (108%) and FPase (145%) activities as also observed with the *Eicchornia crassipes* biomass as the substrate. However, increase in β-glucosidase activity was about similar with both the substrates. Mutant strains using physical and chemical mutagenesis of *Trichoderma reesei* (Bisaria and Ghosh, 1981), strain AANTG-43 from *Aspergillus awomori* (Smith and Wood, 1991) and strain DSM-841 and NTG-19 from *Fusarium oxysporium* (Kuhad et al. 1994) have been isolated. Bailey and Nevalainen (1981) and Montencourt (1983) developed the mutants RL-P 37 and VTT-D-79125 and suggested that the improved cellulolytic activities of the mutants were not due to the synthesis of cellulase enzyme with improved catalytic activity, but rather due to the increased synthesis of cellulase proteins at the expense of other non cellulase proteins. In addition Whelan and Prembroke (1989) had also constructed a protease negative mutant of *Cellulomonas* with improved cellulase activities. Similarly Reyes and Noyola (1998) constructed several mutants (M4, M9, M9-80, M9-82, M11 and M12) from *Cellulomonas flavigena* using NTG and mixed NTG-EMS treatments. Strain UNSC-442 as derived from mixed mutagenesis appeared a promising strain since subculturing and simultaneously measuring the enzymatic activities for several generations demonstrated almost stable enzymatic levels with minor variations. Scanning electron microscopic

studies of the mutant UNSC-442 mycelia indicated these to be highly branched, extended and swollen with respect to wild type mycelia. The molecular basis for morphological variation leading to alter enzymatic production is not well defined. The mutant UNSC-442 was observed to be catabolically depressive strain and was able to produce higher cellulase levels in presence of glucose (1% w/v) as similarly reported by Stewart and Leatherwood (1976). Allen and Roche (1989) reported that elimination of glucose repression in native *Trichoderma reesei* QM6a strain led to even higher activity than that of mutants and concluded that the mutations may not affect the cellulase genes directly but probably affected the mechanisms regulating the synthesis and secretion of cellulase components. Similarly Szabo et al. (1996) had successfully derived the conditions to alleviate the catabolite repression for cellulase production by *Phenarochaete chrysosporium*.

5.3.1 SDS- PAGE Analysis of Cellulase Components

The extracellular protein profile produced by the wild type *Aspergillus niger* RK-3 and its mutants RKU-40 and UNSC-442, using *Eicchornia crassipes* biomass as the substrate were analyzed by electrophoresis of samples on SDS-polyacrylamide gel. A wide range of proteins from 10-190 kD was observed. Mason et al., (1988) had also demonstrated the proteins from 10-100 kD from *Thermonospora mesophila* DSM-43048, *Streptomyces cyaneus* MT-813 and *Actinomadura* sp MT-809 with ball-milled straw as the cellulosic substrate. Molecular mass of the cellulase components was determined by zymogram analysis with native gel using CMC (for endoglucanase), 4-methylumbelliferyl-β-D-

cellobioside (for exoglucanase or MUC activity) and 4-methylumbelliferyl-β-D-glucoside (for β-glucosidase or MUG activity). Many workers (Schwarz et al. 1989; Hamamoto et al. 1990; Sharma et al. 1991) had used similar approach. Bands of molecular masses equivalent to 91.2 kD, 68 kD and 52.4 kD were detected in the native gel, corresponding to the CMCase, FPase (MUC) and β-glucosidase (MUG) activities respectively. Cellulase complex components with a broad range of molecular mass i.e. CMCase (12.5 – 145 kD), FPase (5.6-76 kD) and β-glucosidase (47-400 kD) have earlier been reported (Bisaria and Ghosh, 1981; Singh et al. 1990; Hamamoto et al. 1990; Petit et al. 1990; Zurbriggen et al. 1990; Teunissen et al. 1992). The higher degree of variation among cellulase components has been due to the variations in the conserved regions and glycosylation (Sprey and Bochem, 1991; Meldgaard and Svendsen, 1994).

5.4 ANALYSIS OF CO-CULTIVATION CONDITIONS FOR CELLULASE PRODUCTION

Lignocellulosic waste materials are available in abundance and is considered a major source for the production of fermentation sugars for generation of fuel, solvents, chemical feed stocks and other products of commercial significance. A major emphasis has been towards the enzymatic hydrolysis of cellulosic substrates that mainly depends on the source of cellulosic substrates, pretreatment strategy and the organism chosen for the bioconversion process. The *Trichoderma reesei* has been observed as a highly cellulolytic strain and has significant levels of endo and exoglucanases but production level of β-glucosidase is low (Ryu and Mandel,

1980). On the other hand another potential cellulolytic organism *Aspergillus niger* has comparatively lower levels of endo/exoglucanase but has higher β-glucosidase levels (Madamwar and Patel, 1992). Efforts have therefore been made to derive a system supplemented with limiting enzymatic components for improving the hydrolytic process. Co-cultivation of the compatible strains with significant levels of the desired enzymatic components has been increasingly useful towards improving the economics of cellulose bioconversion technology.

The co-cultivation using the isolated *Aspergillus niger* RK-3 strain, its mutants along with the *Trichoderma reesei* MTCC-164 was attempted for evaluation of the cellulase levels under semi-solid state fermentation (SSSF) conditions. The solid state fermentation has advantages over submerged fermentation such as the mimicking nature, lower capital, smaller operational expenses and control, simpler cultivational requirements, production of concentrated enzyme, improved recovery and smaller waste water output (Madamwar and Patel 1992). Most of the work with regard to cellulase production during the last two decades has been done in submerged fermentation (Stahlberg et al. 1991; Jackson 1996, Kaya et al. 1996) and little attention has been focused on to solid state fermentation (Muniswarn and Charyulu, 1994).

The substrate concentration is a major factor for semi-solid state fermentation that can significantly influence the moisture content and the optimal microbial growth for bioconversion. Maximum levels of cellulases were observed at 10% substrate concentration (sugarcane bagasse or *Eicchornia crassipes* biomass) and similar finding have been reported by Singh et al. (1989) with

corncobs and Muniswaran and Charyulu (1994) with coconut coirpith. However, higher concentrations (20%) of agricultural wastes e.g. paddy straw, wheat straw, saw dust and sugarcane bagasse, were more effective as observed by Arora and Sandhu (1986). Higher cellulosic content and moisture retention ability of the sugarcane bagasse and *Eicchornia crassipes* biomass may be the major factors resulting into increased enzymatic activities with comparatively lower substrate concentration as observed. The relatively higher cellulose, moisture and the lower lignin contents favour the microbial growth, subsequent enzymatic adsorption and the degradation of the substrate thus leading to the increased levels of enzyme production (Arora and Sandhu, 1986; Madamwar and Patel, 1992). *Aspergillus niger* RK-3 used in this study is a natural isolate, having significant levels of CMCase, FPase and β-glucosidase activities. The enzyme production increased when *Aspergillus niger* RK-3 was co-cultured with *Trichoderma reesei* MTCC-164 (3:1). Increase in activities were 23-33% for CMCase and FPase and 13% in β-glucosidase under co-cultured conditions as compared to the respective maximum activities obtained during single culture conditions with sugarcane bagasse as the substrate. Similar level of increase was detected with *Eicchornia crassipes* biomass as the substrate.

Similarly, the co-cultivation of mutant UNSC-442 along with *Trichoderma reesei* MTCC-164 (3:1) showed a further increase in CMCase, FPase and β-glucosidase activities for sugarcane bagasse and *Eicchornia* biomass substrates. The increase in activities were around 8-10%, 11-23% and 24-29% respectively for CMCase, FPase and β-glucosidase for the treated cellulosic substrates used.

It may be attributed that *Trichoderma reesei* when used alone accumulates cellobiose in the medium due to the lower β-glucosidase in its enzyme complex. This may cause the inhibition of the CMCase and FPase and thus retarding the overall rate of hydrolysis due to catabolite repression. The inhibition may be eliminated to some extent by incorporating the β-glucosidase through *Aspergillus niger* under co-cultured condition. Similar observations with improved cellulolytic activities have been observed by co-cultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus* in SSSF-system (Gupte and Madamwar, 1997; Pandey et al. 1999). The present investigation has also indicated that *Trichoderma reesei* MTCC-164, *Aspergillus niger* RK-3 and its mutant UNSC-442 together is more productive for cellulase production and the levels of the enzymatic activities observed were higher as compared to the enzymatic levels obtained with *Trichoderma reesei* and *Aspergillus phoenicus* combinations by Gutierrez-correa and Tengerdy (1998).

5.5 APPLICATIONS OF CELLULOSE BIOCONVERSION

The enzymatic hydrolyzate was observed as the good source of substrate (i.e. glucose) for single cell protein (baker's yeast) as well as organic acid production particularly gluconic and citric acids by fermentation using *Aspergillus niger* strains. The results were significantly comparable with those when glucose was used as substrate for the production of yeast, gluconic and the citric acid respectively. Lakshminarayana et al. (1975) had reported 80% yield of citric acid by growing *Aspergillus niger* using sugarcane bagasse impregnated with sucrose under solid state fermentation. Similarly citric acid production by *Aspergillus niger*

was also accomplished by using wheat bran hydrolyzate as described (Rohr, 1983). Many other value added products can also be generated by lignocellulosic materials (Chahal et al. 1982; Kuhad and Singh, 1993; Bisaria, 1998). The enzymatic hydrolyzates of sugarcane bagasse and *Eicchornia crassipes* biomass may be accounted as the better substrates than molasses (Lakahani, 1990) because unlike the later, it can directly be used without any pretreatment and is comparatively cheaper and easily available.

Chapter 6

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Microbial bioconversion of lignocellulosic materials is an extremely complex process that requires appropriate strain and precise bioconversion conditions. The major objective of the present investigation was to explore the possibility of achieving the maximum levels of bioconversion using high cellulose containing waste materials. Sugarcane bagasse, generated from sugarcane industries and water hyacinth (*Eicchornia crassipes*) an aquatic biomass occurring in ponds, lakes and rivers are the two lignocellulosic waste materials, available in plenty, have high cellulosic content and with no further application were used for the present study. In an attempt to find a potential strain for bioconversion, twenty-two microbial strains (fungal and bacterial) were isolated from the decomposing cellulosic waste materials. Among these strains a fungal strain RK-3 was finally selected based on it ability of maximum cellulase production. This strain was identified as *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi and designated as *Aspergillus niger* RK-3 for further study.

Production of cellulases by *Aspergillus niger* RK-3 initially was detected using the commercially available synthetic substrates. Carboxymethylcellulose (CMC 1%), led to maximum cellulase activities (CMCase, 1.32 IU ml^{-1}; FPase, 1.15 IU ml^{-1}; β-glucosidase, 0.405 IU ml^{-1}) followed by the phosphocellulose and *Accacia* powder as the substrates. Higher activities as obtained with CMC were more likely due to the greater adsorption of enzymatic proteins and subsequently higher rate of saccharification as compared to the other synthetic substrates. Having determined the cellulolytic ability of the isolated *Aspergillus niger* RK-3 strain with commercially available synthetic substrates, two major lignocellulosic

waste materials, sugarcane bagasse and *Eicchornia crassipes* biomass were evaluated as the substrates. The lignin and hemicellulose counterparts of these materials make these to be more crystalline hence prone towards enzymatic attack. These substrates therefore were subjected for high pressure steaming followed by alkali treatment for decreasing the crystallinity and making the substrates susceptible for enzymatic attack. Scanning Electron Microscopy of the treated substrates demonstrated epidermal peeling and partial maceration of compact tissues following the lignin and hemicellulose removal and making these more susceptible to fungal attack as compared to untreated substrates.

The pretreated bagasse when used as the substrate resulted into 48.5%, 49% and 41.1% higher CMCase, FPase and β-glucosidase activities respectively, as compared to the untreated bagasse. Similarly *Eicchornia crassipes* biomass too was utilized as substrate and CMCase, FPase activities measured were 33-37% higher while a higher (80%) increase for β-glucosidase activity was detected with the treated substrate as compared to the untreated biomass. In concurrence with above, higher level of proteins (35.5%, 10.9%) and cellulose degradation (45.2%, 26%) was observed with treated sugarcane bagasse and *Eicchornia crassipes* biomass respectively as compared to the untreated substrates. It can therefore be inferred that crystalline structure of lignocellulosic substrate after steam–alkali pretreatment was damaged and amorphosity increased which thus promoted higher adsorption and subsequently increased the saccharification.

Analysis of physical, biological and chemical factors affecting cellulase production was carried out in submerged state fermentation. The maximum levels

of production were obtained after 8 days of fungal incubation. A linear rate of fungal growth along with enzyme production was observed during this period of incubation. The other derived conditions for maximum cellulase production included use of 1% of the substrate (sugarcane bagasse or *Eicchornia crassipes* biomass) along with 5% of inoculum, 130 rpm as the agitation rate at the pH 4.8 and a temperature of 30°C for higher biomass accumulation, whereas, 50°C was suitable for measuring the enzymatic activities. The effect of medium nutrients and other chemical factors were analyzed for their influence on cellulase production. The optimal concentration of the every factor after analysis was added as the medium constituent for subsequent analysis.

Among the nutrients and chemical factors analyzed , ammonium ions (2.5 gl^{-1}); potassium dihydrogen phosphate (2.45 gl^{-1}); vanillin, (0.5 gl^{-1}); succinic acid, (1.0 gl^{-1}) and cellobiose (2.0 gl^{-1}) were capable of stimulating the cellulase levels and hence included as the medium constituents. The use of defined physical, biological condition and the derived chemical constituents (medium R_6m) proved to be very successful as substantial increase in CMCase (83-112%), FPase (59-79%) and β-glucosidase (288-316%) activities were obtained with respect to Mandels and Weber's medium with both the substrates. (sugarcane bagasse, *Eicchornia crassipes* biomass) used.

In an attempt to further increase the bioconversion level of the lignocellulosic substrates used for the study, the wild type *Aspergillus niger* RK-3 was subjected to physical (ultraviolet), chemical (NTG, sodium azide and colchicine) and mixed (physical and chemical) mutagenesis. Mutants obtained were

analyzed by the zone formation using congo-red CMC-agar medium added with glucose (1%) for obtaining a high yielding and end product resistant mutant strain. The strain UNSC-442 was finally selected having greater cellulase activities and found to be a product resistant mutant. The production levels of CMCase, FPase and β-glucosidase was estimated with mutant UNSC-442 strain and compared with that of wild type *Aspergillus niger* RK-3 strain. Levels of CMCase, FPase and β-glucosidase activities observed with mutant UNSC-442 with sugarcane bagasse and *Eicchornia crassipes* biomass were 89-108%, 130-145% and about 54% higher as compared to the activities obtained with wild type strain respectively. A higher rate of cellulose degradation (61.5-67%) was observed under these conditions. Analysis of protein profile after fermentation with wild type *Aspergillus niger* RK-3 and mutant strains RKU-40 and UNSC-442 had denoted increasing levels 95.5 kD, 68 kD and 52.4 kD proteins in the UNSC-442 strain as compared to the wild type RK-3 strain. Zymogram analysis of the partially purified samples indicated increasing activities of CMCase FPase and β-glucosidase corresponding to 91.2 kD, 68 kD and 52.4 kD bands in the mutant strains as compared to the activities from the wild type strain. Mutants UNSC-442 when subcultured for several generation was found to be very stable. Scanning electron microscopy of the mutant UNSC-442 revealed distinct morphological features. The mutant UNSC-442 strain was comparatively fast growing, having extended, branched and swollen mycelia with lesser sporulation as compared to that of the wild type strain.

To further enhance the cellulase production levels and to streamline the bioconversion process, co-cultivation studies using wild type *Aspergillus niger* RK-3, its mutant UNSC-442 along with *Trichoderma reesei* MTCC-164 under semi-solid state fermentation were attempted. Incubation under co-cultured conditions using *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164 resulted a 23-33% increase in the CMCase and FPase activities and 13% increase in β-glucosidase activity over the respective maximum activities observed under single culture condition with sugarcane bagasse (10%) as the substrate. On the other hand a 20-24% increase in CMCase and FPase and a similar increase (13%) in β-glucosidase activity was observed with *Eicchornia crassipes* biomass under similar conditions. Further, co-cultivation of mutant UNSC-442 along with *Trichoderma reesei* MTCC-164 showed a milder increase (8-23%) in CMCase and FPase levels and around 24% increase in β-glucosidase levels with respect to the enzymatic activities as obtained with mutant UNSC-442 strain with sugarcane bagasse as the substrate. About similar results were obtained with *Eicchornia crassipes* biomass under the conditions as referred so for the sugarcane bagasse as the substrate. The high enzyme activities were related to increased soluble protein and total dry mycelial mass under co-cultivation conditions.

The sugarcane bagasse and *Eicchornia crassipes* biomass hydrolyzates as the carbon source generated significant levels (178 mg ml^{-1} and 163 mg ml^{-1}) of the baker's yeast as the source for single cell protein. Similarly, higher levels of gluconic (64.4 mg ml^{-1}; 45.18 mg ml^{-1}) and citric acid (54.8 mg ml^{-1}; 48.3 mg ml^{-1})

were obtained with both the hydrolyzates used. Thus, prime economical gains from the cellulose process technology can be harnessed by designing a system allowing a full scale mineralization of the lignocellulosic waste materials. Further work will however be required to develop it into a commercial reality.

References

REFERENCES

1. Abbi, M. Kuhad, R.C. and Singh, A. Fermentation of xylose and rice straw hydrolysate to ethanol by *Candida shehatae* NCL-3501. *J. Ind. Microbiol.* **17**, 20-23 (1996).

2. Aikane, H.V. and Patil, M.B. Production, isolation and some properties of β-glucosidase of *Chaetomium globosum*. *Indian J.Exp Biol.* **27,** 118-120 (1989).

3. Akhtar, M.W., Duffy, M., Dowds, B.C.A., Sheehan, M.C. and McConnel, D.G. Multigene families of *Cellulomonas flavigena* encoding endo-β-1,4-glucanase (CM-Cellulase). *Gene.* **74**, 549-553 (1988).

4.* Allen, A.L. and Roche, C.D. *Biotechnol. Bioengg.* **33,** 153 (1989). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

5. Anand, L. and Vithayuthil, P.J. Production of extracellular xylanase by thermophilic fungus, *Humicola lanuginosa* (Griffon and Maublance) Bunce. *Indian J. Exp. Biol.* **28**, 434-437 (1990).

6. Aparicio, J.S., Romero, A. and Ripoll, M.M. Crystallization and preliminary X-ray diffraction analysis of a type I β-glucosidase encoded by the bg I A gene of *Bacillus polymyxa*. *J. Mol. Biol.* **240,** 267-270 (1994).

7. Arora, D.S. and Sandhu, D.K. Degradation of lignocellulosic residues by *Polyporous versicolor* and the effect of moisture contents and phenolic compounds. *Acta. Biotechnol.* **6** (3), 293-297 (1986).

8.* Atalla, R.H. *Wood. Agri. Res. (ed. E.L. Soltes), Acad. Press, New York* **59,** *1983).* **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

9. Baik, B.H. and Pack, M.Y. Expression of *Bacillus subtilis* endoglucanase gene in *Lactobacillus acidophillus*. *Biotechnol. Lett.* **12**, 919-924 (1990).

10. Bailey, M. J. The effect of β-glucosidase on some assays for cellulolytic enzymes. *Biotech. Lett.* **12,** 659-700 (1981).

11. Bailey, M.J. and Nevalainen, K.M.H. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. *Enzyme Microbiol. Technol.* **3**, 153-157 (1981).

12. Basu, B., Khan, H.J., Chawla, P. Spectrum. Sci. Rep. *Natl. Inst. Sci. Comm. CSIR.* **37**(7), 39 (2000).

13. Bayer, E.A., Morag, E. and Lamed, R. The cellulosome - a treasuretrove for biotechnology. *Tr. Biotechnol.* **12**, 379-386 (1994).

14. Bisaria, V.S. and Ghose, T.K. Biodegradation of cellulosic materials: substrate, microorganism, enzyme and products. *Enzyme Microbiol. Technol.* **3**, 91-104. (1981).

15.* Bisaria, V.S. and Mishra, S. *Crit. Rev. Biotechnol.* **9**, 61 (1989). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

16. Bisaria, V.S. Bioprocessing of agro-residues to value added products. **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

17.* Blackwell, J. *Cellulose and other natural polymer system : Biogenesis, structure and degradation (ed. R. M. Brown), Plenum, New York, 403 (1982).* **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

18. Blanco, A., Juurez, A. and Pastor, F.I.J. Over production of a *Clostridium cellulotyticus* endoglucanase by mutant strain of *Escherichia coli*. *FEMS Microbiol. Lett.* **81**, 221-226 (1991).

19. Borgia, P. and Sypherd, P.S. Control of β-glucosidase synthesis in *Mucor racemosus*. *J. Bacteriol.* **130** (2), 812-817 (1977).

20. Breen, A. and Singlton, F.L. Fungi in lignocellulose breakdown and biopulping. *Curr. Opin. Biotechnol.* **10**, 252-258 (1999).

21. Bronnenmeier, K. and Staudenbauer, W.L. Purification and properties of an extracellular β-glucosidase from the cellulolytic themophilic *Clostridium stercorarium*. *Appl. Microbiol. Biotechnol.* **28,** 380-386 (1988).

22. Cappa, F., Riboli, B., Rossi, F., Callegari M.L. and Cocconcelli, P.S. Construction of novel *Ruminococcus albus* strain with improved cellulase activity by cloning of *Streptomyces rochei* endoglucanase gene. *Biotech. Lett.* **19**(11), 1151-1155 (1997).

23. Castanon, M. and Wilk, C.R. Adsorption and recovery of cellulases during hydrolysis of news paper. *Biotechnol. Bioengg.* **22**, 1037-1053 (1980).

24. Caulfied, D.F. and Moore, W.E Effect of varying crystallinity of cellulose on enzymatic hydrolysis. *Wood Sci.* **6**, 375-379 (1974).

25. Chahal, D.S. and Dhaliwal, B.S. Interrelation of carboxymethylcellulase production and utilization of cellulose by cellulolytic fungi for protein production. *Indian. J. Microbiol.* **13**, 191-204 (1982).

26.* Chahal, D.S., Klueptel D., Morosoli, F. *App. Biochem. Biotechnol.* **51**, 137 (1995). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

27. Chang, Y. The fungi from wheat straw compost. II. Biochemical and Physiological studies. *Transac. Brit. Microbiol. Soc.* **50**, 667-677 (1967).

28. Chaudhary, L.C., Singh, R. and Kamra , D.N. Biodelignification of sugar cane bagasse by *Pleurotus florida* and *Pleurotus cornucopiae*. *Indian J. Microbiol.* **34**, 55-57 (1994).

29. Cowling, E. B. and Kirk, T. K. Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion process. *Biotech. Bioengg. Symp.* **6**, 95-124 (1976).

30. Cowling, E.B. Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials. *Biotechnol. Bioengg. Symp.* 5, 163-181 (1975).

31. Dale, B.E. Lignocellulose conversion and the future of fermentation biotechnolgy. *Tr. Biotechnol.* **5,** 287-293 (1987).

32. Damude, H.G., Ferro, V. and Withers, S.G. Substrate specificity of endoglucanase A from *Cellulomonas fimi*: Fundamental differences between endoglucanases and exoglucanases from Family 6. *Biochem. J.* **315,** 467-472 (1996).

33. Deobald, L.A. and Crawford, D.L. Activities of cellulase and other extracellular enzymes during lignin solubilization by *Streptomyces viridosporus*. *Appl. Microbiol. Biotechnol.* **26**,158-163 (1987).

34. Din, N., Beck, C.F., Miller, R.C., Kilburn, J.D.G and Warran R.A.J. Expression of the *Cellulomonas fimi* cellulase gene Cex and Cen A from the divergent tet promoters of transposon Tn 10. *Arch. Microbiol.* **153**, 129-133 (1990).

35. Doppelbauer, P., Esterbauer, H., Steiner, W., Lafferty, R.M. and Steinmuller, H. The use of lignocellulosic wastes for production of cellulase by *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.* **26,** 485-494 (1987).

36.* Duff, S.J.B. and Murray, W.D. *Biores. Technol.* 55, 1 (1996). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

37. Dunlap, C.E., Thomson, J. and Chiang, L.C. Treatment processes to increase microbial digestibility. *AICHE Symp. Ser.* **72** (15), 58-63 (1976).

38. Eberhart, B.M., Beek, R.S. and Goolsby, K.M. Cellulase of *Neurospora crassa*. *J. Microbiol.* **130**, 181-186 (1977).

39. El-Refai, A.M.H., Atall, M.M. and Safty, E. Microbial formation of cellulases and proteins from some cellulosic residues. *Agri. Wastes*. **11**, 105-113 (1984).

40. Eriksson, K.E. Regulation of endo-1,4, β-glucanase production in *Sporotrichum pulverulentum*. *Eur. J. Biochem.* **90**, 183-190 (1976).

41. Evans, P., Clark, D., Sewell, G.M. *Appl. Environ. Microbiol.* **53**, 2420. **In :** Bioconversion of waste materials to industrial products. *Pub. Blackie Acad. Prof.* London. SE1 8HN. 197-246 (1998).

42* Fan, L.T., Lee, Y. H. and Beardmere, D. H. *Advances in Biochemical Engineering, 101 (1980) (ed. A. Fiechter), Springer-verlag, Berlin.* **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

43. Fan, L.T., Lee, Y.H. and Gharpuray, M.M. The nature of lignocellulosic and their pretreatment for enzymatic hydrolysis. *Adv. Biochem. Engg.* **23**, 157-187 (1982).

44. Ferrer, P., Halkier, T. and Hedegaarde, L. Nucleotide sequence of a β-1,3-glucanase isoenzyme II_A gene of *Oerskovia xanthineolytica* LLG 109 (*Cellulomonas cellulans*) and initial characterization of the recombinant enzyme expressed in *Bacillus subtilis*. *J. Bacteriol.* **178**, 4751-4757 (1996).

45. Fischer, L., Bromam, R., Kengen, S.W.M., Devos, W.M. and Wagner, F. Catalytical potency of β-glucosidase from the extremophile *Pyrococcus furiosus* in glucoconjugate synthesis. *Biotechnol.* **14**, 88-90 (1996).

46. Forsberg, C.W., Schellhorn, H.E. and Gibbins, L.N. The release of fermentable carbohydrate from peat by steam explosion and its use in the microbial production of solvents. *Biotechnol. Bioengg.* **28**, 176-184 (1986).

47. Gabriel, B. L. Biological scanning electron microscopy. *Von Nostrand Reinhold Company*, New York, Toronto, London, Malbourne (1982).

48* Gardner, K.H. and Blackwell, J. Biopolym. **13**, 1975 (1974). **In** : Bioconversion of waste materials to industrial products. *Pub. Blackie. Acad. Prof.* London, SE 1 8HN. 197-246 (1998).

49. Garg, S.K. and Neelkantan, S. Effect of nutritional factors on cellulase enzyme and microbial protein production by *Aspergillus terreus* and its evaluation. *Biotechnol. Bioengg.* **24**, 109-125 (1982).

50. Ghose, T.K. and Bisaria, V.S. Studies on the mechanism of enzymatic hydrolysis of cellulosic substrates. *Biotechnol. Bioengg.* **21**, 131-146 (1979).

51. Giorda , R., Ohmachi, T., Shaw, D.R. and Ennis, H.S. A shared internal threonine-glutamic acid-threonine-proline repeat defines a family of *Dictyostelium discoideum* spore germination specific proteins. *Biochem.* **29**, 7264-7269 (1991).

52. Goel, S.C. and Ramachandran, K.B. Studies on adsorption of cellulase on lignocellulosics. *J. Ferment. Technol.* **3**, 281-286 (1983).

53. Gong, C.S., Ladisch, M.R. and Tsao, G.T. Cellobiase from *Trichoderma viride*: purification, properties, kinetics and mechanisms. *Biotechnol. Bioengg.* **19**, 959-981 (1977).

54. Gough, C.L., Dow, G.L., Keen, J., Henrissat, B. and Daniels M.J. Nucleotide sequence of the eng *XCA* gene encoding the major endoglucanase of *Xanthomonas campestris*. *Gene.* 53-59 (1990).

55. Gunju, R.K., Vithayuthil, P.J. and Murthy, S.K. Factors influencing production of cellulases by *Chaetomium themophile*. Var. *coprophile. Indian. J. Exp. Biol.* **12,** 259-264 (1990).

56. Gupte, A. and Madamwar, D. Solid state fermentation of lignocellulosic waste for cellulase and β-glucosidase production by co-cultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus. Biotechnol. Prog.* **13**, 166-169 (1997).

57. Gutierrez-correa, M. and Tengerdy, R.P. Xylanase production by fungal mixed culture solid substrate fermentation on sugarcane bagasse. *Biotechnol. Lett.* **20** (1), 45-47 (1998).

58. Halliwel, G. The action of cellulolytic enzymes from *Myrothecium verrucaria*. *Biochem. J.* **79**, 185-192 (1961).

59. Hamamoto, T., Shoseyov, O. Foong, F. and Doi, R.H. A *Clostridium cellulovorans* gene, eng D. codes for both endo-β-1,4-glucanase and cellobiosidase activities. *FEMS Microbiol. Lett.* **72**, 228-288 (1990).

60. Hartree, M.M., Yu, E.K.C., Reid, I.D. and Saddler, J.N. Suitability of aspen wood biologically delignified with *Pheblia tremellosus* for fermentation to ethanol or butanol. *Appl. Microbiol. Biotechnol.* **26**, 120-125 (1987).

61. Hatakka, A. I. and Pirhonen, T.I. Cultivation of wood rotting fungi on agricultural lignocellulosic materials for the production of crude protein. *Agri. Wastes.* **12**, 81-97 (1985).

62. Henrissat, B., Claeyssens, M., Tomme, P., Lemesele, L. and Mornon, J.P. Cellulase femilies by hydrophobic cluster analysis. *Gene.* **81**, 83-95. (1989).

63. Himmel, M.T., Ruth, M.F. and Wymom, C.E. Cellulase for commodity products from cellulosic biomass. *Curr. Opi. Biotechnol.* **10**, 358-364 (1999).

64. Ismail, A.S., Hamdy, N.N. and El-Refai, A.H. Enzymatic saccharification of Egptian sugarcane bagasse. *Agri. wastes.* **12**, 99-109 (1985).

65. Jackson, L.S., Joyce, T.W., Heitmann, J.A., and Giesbrecht, F.G. Enzyme activity recovery from secondary fiber treated with cellulase and xylanase. *J. Biotechnol.* **45**, 33-44 (1996).

66. Jourin, B. and Gramstrom, M. β-glucosidase gene of naturally occurring and cellulolytic *Streptomyces* species characterization of two such genes in *Streptomyces lividans*. *Appl. Microbiol. Biotechnol.* **30**,502-508 (1989).

67. Ju, L.K. and Afolabi, O.A. Waste papers hydrolysate as soluble inducing substrate for cellulase production in continuous culture of *Trichoderma reesei*. *Biotechnol. Prog.* **15**, 91-97 (1999).

68. Jurasek, L. Enzymatic hydrolysis of aspen wood. *Dev. Ind. Microbiol.* **20**, 177-183 (1978).

69. Kaya, F., Heitmann, J.A., Joyce, T.W., Effect of shear fields on hemicellulase binding of pulp fibers . *J. Bitechnol.* **45**, 23-31 (1996).

70* Kersten, P.J. *Processdings of the National Academy of Science of the USA*, **87**, 2936 (1990). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

71. Kim, D.W., Jang, Y.H. and Jeong, Y.K. Adsorption behaviors of two cellobiohydrolases and their core proteins from *Trichoderma reesei* on avicel PH 101. *Biotechnol. Lett.* **9**, 893-897 (1997).

72. Kim, D.W., Yang, J.H. and Jeong, Y.K. Adsorption of cellulase from *Trichoderma viride* on microcrystalline cellulose. *Appl. Microbiol. Biotechnol.* **28,** 148-154 (1988).

73. Klyosov, A.A., Mitevich, D.V. and Sinitsyn, A.P. Role of the activity and adsorption of cellulases in the efficiency of the enzymatic hydrolysis of amorphous and crystalline cellulose. *Biochem.* **25**, 540-542 (1986).

74. Knowles, J., Lehtovaara, P. and Teeri, T. Cellulase families and their genes. *Tr. Biotechnol.* **5**, 255-260 (1987).

75.* Koeings, J.W. *Arch. Microbial.* **99**, 1296 (1974). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

76. Kubicek, C. P., Eveleigh, D.E., Esterbauer, H., Steiner, W. and Kubicek-Pranz, E. M. *Trichoderma* cellulases : Biochemistry, physiology, genetics and applications. *Royal Soc. Chem. Cambridge U.K.* (1990).

77. Kubicek, C.P., Mach, R.L and Gruber, F. Regulation of cellulase biosynthesis. *Crit. Rev. Biotechnol.* **9**, 1-8 (1998).

78. Kuhad, R.C. and Johri, B.N. Fungal decomposition of paddy straw: Light and scanning electron microscopic study. *Indian J. Microbiol.* **32** (3), 255-258 (1992).

79. Kuhad, R.C. and Singh, A. Lignocellulose biotechnology: Current and future prospects. *Crit. Rev. Biotechnol.* **13**,151-172 (1993).

80. Kuhad, R.C., Kumar, M. and Singh, A. A hypercellulolytic mutant of *Fusarium oxysporum*. *Lett. Appl. microbiol.* **19**, 397-400 (1994).

81. Kundu, P. and Das, A. Utilization of Cheap carbohydrate sources for production of calcium gluconate by *Penicillium funiculosum* mutant MN 238. *Indian J. Exp. Biol.* **22**, 279-81 (1984).

82.* Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature.* **227**, 680-685 (1970).

83. Lakhani, A.K., Bioconversion of lignocellulosic wastes. *Ph.D. Thesis.* University of Roorkee, Roorkee, (1990) India.

84.* Lakshminarayan, K., Chaudhary, K. Ethiraj, S. and Tauro *Biotechnol. Bioengg.* **17**, 291 (1975). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

85. Lamed, R., Setter, E. and Bayer, E.A. *J. Bacteriol.* **156**, 828-836 (1983). **In :** Bayer, E.A. The cellulosome a treasuretrove for biotechnology. *Tr. Biotech.* **12**, 376-386 (1994).

86. Liang, C.H.Y. and Marchessault, R.H. Infrared spectra of crystalline polysaccharides I hydrogen bonds in native cellulose *J. Poly. Sci.* **37**, 385-395. (1959).

87. Lien, O.G. Determination of gluconolactone, galactonolactone and their free acids by the hydroxamate method. *Anal. Chem.* **31** (8) 1363-1366 (1959).

88.* Lipinsky, E.S. Perspective on preparation of cellulose for hydrolysis. In: Hydrolysis of cellulose mechanisms of enzymatic and acid catalysis (R.D. Brow and C. Jurasek eds.). *Adv. Chem. Ser.* **181**,1-23 (1979).

89. Lotfi, C.F.P., Santelli, G.M.M. Comparative analysis of colchicine induced micronuclei in different cell types in vitro. *Mut. Res.* **349**, 77-83 (1996).

90. Lowry, O.H., Rosebrough, N. J., Farr, A.L. and Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275 (1951).

91. MacLeod, A.M., Tull, D., Rupitz, K., Warren, R.A.J. and Withers, G. Mechanistic consequences of mutation of active site carboxylates in a retaining β-1,4-glycanase from *Cellulomonas fimi*. *Biochem.* **35**, 13165-13172 (1996).

92. Macris, B.J., Kekos, D. and Evangelidou, E. A simple and inexpensive method for cellulase and β-glucosidase production by *Neurospora crassa*. *Appl. Microbial. Biotechnol.* **31**, 150-151 (1989).

93. Madamwar, D. and Patel, S. Formation of cellulases by co-culturing of *Trichoderma reesei* and *Aspergillus niger* on cellulosic waste: *In Industrial Biotechnology*, (V.S. Malik, and P. Sridhar. eds.) Publ. IBH, Oxford, New Delhi. 471-478 (1992).

94. Maheshwari, D.K. Jahan, H., Paul, J. and Varma, A. Wheat straw, a potential substrate for cellulase production using *Trichoderma reesei*. *World J. Microbiol. Biotechnol.* **28**, 12-16 (1992).

95. Maheshwari, D.K., Gohade, S., Paul, J., Varma, A. A paper mill sludge as a potential source for cellulase production by *Trichoderma reesei* QM 9123 and *Aspergillus niger* using mixed cultivation. *Carbohyd. poly.* **23**, 161-163 (1994).

96. Mandels, M. and Reese, E.T. Induction of cellulase in fungi by cellobiose. *J. Bacteriol.* **79**, 816-826 (1960).

97. Mandels, M. and Weber, J. The Production of cellulases. *Advances in Chemistry.* **95**, 391-414 (1969).

98. Mandels, M. Microbial sources of cellulases. *Biotech. Bioengg. Symp.* **5**, 81-105 (1975).

99. Mandels, M., Kastick, J.A. and Purizele, R. The use of adsorbed cellulase in the continuous conversion of cellulose to glucose. *J. Polym. Sci.* **36**, 445-459 (1971).

100.* Mandels, M., Sternberg, D. and Andreotti, R.E. Growth and cellulase production by *Trichoderma*. **In :** Symposium on enzymatic hydrolysis of cellulose. (M. Bailey, T.M. Eneri, and M. Linko eds) 231-254 (1975).

101. Marier, J. R. and Boulet, M. Direct determination of citric acid in milk with improved pyridine acetic anhydride method. *J. Dairy Sci.* **41**, 1683-1688 (1958).

102. Mason, J.C., Richards, M., Zimmermann, W. and Broda, P. Identification of extracellular proteins from actinomycetes responsible for the solubilization of lignocellulose. *Appl. Microbiol. Biotechnol.* **28**, 276-280 (1988).

103. McCarthy. Lignocellulose degrading actinomycetes. *FEMS Microbiol. Rev.* **104**, *145-163 (1987).* **In** : Tuncer, M., Ball, A.S., Rob, A., Wilson, M.T. Optimization of extracellular lignocellulolytic enzyme production by a thermophilic actinomycetes *Thermomonospora fusca* BD 25. *Enzyme. Microbiol. Technol.* **25**, 38-47 (1999).

104. Medve, J., Karlsson, J., Lee, D. and Tjerneld, F. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei* : Adsorption, sugar production pattern and synergism of the enzymes. *Biotechnol. Bioengg.* **59** (5), 621-634 (1998).

105. Meldgaard, M. and Svendsen, I.B. Different effects of N-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)-β-glucanases secreted from yeast. *Microbiol.* **140**, 159-166 (1994).

106. Menon, K., Rao, K.K. and Pushalkar, S. Production of β-glucosidase by *Penicillium rubrum* O stall. *Indian. J. Exp. Biol.* **32**, 706-709 (1994).

107. Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31**, 426-428 (1959).

108. Monem, A.H., Refai, EI., Mohamad, M., Atalla, H. A. and Safty, E. Microbial formation of cellulases and proteins from some cellulosic residues. *Agri. Wastes.* **11**,105-113 (1984).

109.* Montenecourt, B.S. *Trends Biotechnol.* **1**, 156 (1983). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

110. Mukhopadhyey, S. and Nandi, B. Optimization of cellulase production by *Trichoderma reesei* ATCC 26921 using a simplified medium on water hyacinth biomass. *J. Sci. Ind. Res.* **58**, 107-111 (1999).

111. Muller, H.W., Trosch, W. and Kuibe, K.D. Effect of phenolic compounds on cellulose degradation by some white rot basidiomycetes. *FEMS Microbiol. Lett.* **49**, 87-93 (1988).

112. Muniswaran, P.K.A. and Charyulu, N.C.L.N. Solid substrate fermentation of coconut coir pith for cellulase production. *Enzyme Microbial. Technol.* **16**, 436-440 (1994).

113. Neely, W.C. Factors affecting the pretreatment of biomass with gaseous ozone. *Biotechnol. Bioengg.* **25**, 59-65 (1984).

114. Neill. G., Gob, S.H., Warren, R.A.J., Kilburn, D.G. and Miller R.C. Structure of the gene encoding the exoglucanase of *Cellulomonas fimi*. *Gene.* **44**, 325-330 (1996).

115. Nelson, N. and Oliver, D. W. Study of cellulose structure and its relation to reactivity. *J. Polym. Sci. Part C.* **36**, 305-320 (1971).

116. Norkrans, B. Growth and cellulolytic enzymes in *Trichoderma symbolae. Bot Upsalienses* **11**, 5-126 (1950).

117. Olivier, S.P. and Toit, P.J.D. Sugarcane bagasse as a possible source of fermentation carbohydrates. II. Optimization of the xylose isomerase reaction for isomerization of xylulose in laboratory scale units. *Biotechnol. Bioengg.* **28**, 684-699 (1986).

118. Palczar, M.J., Chan, E.C.S and Krieg, M.R. Microbiology, (5 th ed.). *McGraw-Hill Pub. USA*. 1-903 (1986).

119. Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P. Solid state fermentation for the production of industrial enzymes. *Curr. Sci.* 77, 1-10 (1999).

120. Parish, R.W. Inhibition of *Dictyostelium discoideum* β-glucosidase by purines. *J. Bacteriol.* **129** (3), 1642-1644 (1977).

121. Paul, J. and Varma, A. Influence of sugars on endoglucanase and β-xylanase activities of a *Bacillus* strain. *Biotech. Lett.* **22**, 61-64 (1990).

122. Pentilla, M., Lehtovaara, P., Nevalainen, H., Bhikhambhai, R. and Knowles, J. Homology between cellulase genes of *Trichoderma reesei*: Complete nucleotide sequence of the endoglucanase I *Gene*, 325-330 (1986).

123. Petit, M.A., Joliff, G., Mesas, J.M., Klier, A., Rapoport, G and Ehrlicb, S.D. Hypersecretion of a cellulase for *Clostridium thermocellum* in *Bacillus subtilis* by induction of chromosomal DNA amplification. *Biotechonol.* 555-562 (1990).

124. Phillips, D.H. Oxygen transfer in mycelial pellets. *Biotechnol. Bioengg.* **8**, 456-460 (1966).

125. Pons, J., Planas, A. and Qucral, E. Contribution of a disulfide bridge to the stability of 1,3,4-β-D-glucon 4 D-gluconohydrolase from *Bacillus licheniformis*. *Protein Engg.* **8**, (a) 939-945 (1995).

126. Puri, V.P. and Mamers, H. Explosive pretreatment of lignocellulosic residues with high pressure carbon dioxide for the production of fermentation substrates. *Biotech. Bioengg.* **25** (12), 3149-3161. (1983).

127. Puri, V.P. and Pearce, G.R. Alkali explosion pretreatment of straw and bagasse for enzymatic hydrolysis. *Biotech. Bioengg.* **28**, 480-485 (1986).

128. Rajaram, S. and Verma, A. Production and characterization of xylanase from *Bacillus thermoalkalophilus* growth on agriculture wastes. *Appl. Microbiol. Biotechnol.* **34**, 141-144 (1990).

129. Rajendran, A., Gunasekaran, P. and Lakshmanan, M. Cellulase activity of *Humicola fuscoatra*. *Indian J. Microbiol.* **34**, 289-295 (1994).

130. Reese, E.T. and Mandels, M. Enzymatic hydrolysis of β-glucanase. **In:** Advances in enzymatic hydrolysis of cellulose and related material (ed.) E.T. Reese Rergamon press, New York, 197-234 (1963).

131.* Reese, E.T., Segal, L., Tripp, V.W. The effect of cellulase on the degree of polymerization of cellulose and hydrocellulose. *Textile Res. J.* **27**, 626-632 (1957).

132. Reinikainen, T., Teleman, O. and Teeri, T.T. Effects of pH and high ionic strength on the adsorption and activity of native and mutated cellobiohydrolase I from *Trichoderma reesei*. *Proteins: Str. Func. Genet.* **22**, 392-403 (1995).

133. Reyes, L. M. and Noyola, T.P. Isolation of a hyperxylanolytic *Cellulomonas flavigena* mutant growing on continuous culture on sugarcane bagasse. *Biotechnol. Lett.* **20** (5), 443-446 (1998).

134. Rivers, D.B. and Emert, G.H. Lignocellulose pretreatment: A comparison of wet and dry ball attrition. *Biotechnol. Lett.* **9** (5), 365-368 (1987).

135. Robson, L. and Chambliss, G.H. Cellulases of bacterial Origin. *Enzyme Microb. Technol.* **11**, 626-644 (1989).

136.* Rohr, M., Kubicek, C.P. and Kominek, *J., Biotechnol.* **3** (ed. H. Dellweg), *Verlag Chemie, Deer field Beach,* FL. 419 (1983). **In :** Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

137.* Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C. and Jones, T.A. *Science **249**, 380-386 (1990).* **In :** Sakon, J., Adney, W.S., Himmel, M.E.,

Thomas, S.R. and Karplus, P.A. Crystal structure of thermostable family 5 endocellulase E1 from *Acidothermus cellulolyticus* in complex with cellotetrose. *Biochem.* **35**, 10648-10660 (1996).

138.* Rowland, S.P. and Roberts, J. J. *J. Poly. Sci. Part A-1, 10, 2447 (1972).* **In :** Bioconversion of waste materials to industrial products. *Pub. Blackie. Acad. Prof.* London, SE 1 8HN. 197-246 (1998).

139. Ryu, D.D.Y. and Mandels, M. Cellulases; biosynthesis and applications. *Enzyme Microbiol. Technol.* **2**, 91-102 (1980).

140. Saarilahti, I., Henrissat, B. and Polva, E.T. Cels : a novel endoglucanase identified from *Erwinia carotovora* sub sp. *caratovora. Gene.* 9-14 (1990).

141. Sakon, J., Adney, W.S., Himmel, M.E., Thomes, S.R. and Karplus, P. A. Crystal structure of thermostable family 5 endocellulase E1, from *Acidothermus cellulolyticus* in complex with cellotetraose. *Biochem.* **35**, 10648-10660 (1996).

142. Schwarz, W.H., Jausis, S. and Kouba, M. Cloning and expression of *Clostridium stercorarium* cellulase genes in *Escherichia coli. Biotechnol. Lett.* **11**, 461-466 (1989).

143. Segal, L., Creely, J.J., Martin, J. and Corrad, C.M. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Textile Res. J.* **29**, 786-794 (1959).

144. Sexena, S., Bahadur, J. and Varma, A. Production and localization of carboxymethylcellulase, xylanase and β-glucosidase from *Cellulomonas* and *Micrococcus* sp. *Appl. Microbiol. Biotechnol.* **34**, 668-670 (1991).

145. Sharma, D.K. and Sahgal, P.N. Production of furfural from agricultural wastes by using pressurised water in a batch reactor. *J. chem. Tech. Biotechnol.* **32**, 666-668 (1982).

146. Sharma, S., Sandhu, D.K. and Bagga, P.S. Physical characterization of isozymes of endo-β-1,4-glucanase and β-1,4-glucosidase from *Aspergillus* species. *FEMS Microbiol. Lett.* 7, 99-104 98 (1991).

147. Sheweita, S.A. Ichi-ishi, A., Park, J.S., Liu, C., Malburg, L.M. and Doi, R.H. Characterization of eng F, a gene for a non-cellulosomal *Clostridium cellulovorans* endoglucanase. *Gene.* **182**, 163-167 (1996).

148. Shiang, M., Linden, J.C., Mohagheghi, A., Grohmam, K. and Himmel, M.E. Regulation of cellulase synthesis in *Acidothermus cellolyticus. Biotechnol. Prog.* **7**, 315-322 (1991)

149. Sineiro, J., Dominguez, H., Nunez, M.J. and Lema, J.M. Inhibition of cellulase activity by sunflower polyphenols. *Biotechnol. Lett.* **19** (6), 521-524 (1997).

150. Singh, A., Abidi, A.B., Agarwal, A.K., Darmwal, N.S. and Srivastawa, S. Utilization of cellulosic substrate for the production of single cell protein and cellulase enzyme. *Indian J. Bio. Res.* **6**, 1-6 (1988a).

151. Singh, A., Abidi, A.B., Agrawal, A.K. and Darmwal, N.S. Evaluation of alkali treatment for biodegradation of corncobs by *Aspergillus niger* AS101. *Folia Microbiol.* **34**,479-484 (1989a).

152. Singh, A., Abidi, A.B., Darmwal N.S. and Agrawal, A.K. Production of protein and cellulase by *Aspergillus niger* AS101 in solid state culture. *MIRCEN Journal.* **5**, 451-456 (1989b).

153. Singh, A., Abidi, A.B., Darmwal, A.S. and Agrawal, A.K. Saccharification of cellulosic substrates by *Aspergillus niger* cellulase. *World J. Microbiol Biotechnol.* **6**, 333-336 (1990).

154. Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K. Effect of sugars, organic acids and phenolics on cellulase production by *Aspergillus niger* AS101. *Agri. Biol. Res.* **4** (2), 63-66 (1988d).

155. Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K. Evaluation of chemical pretreatment for biodegradation of lignocellulosic wastes by *Aspergillus niger* AS101. *MIRCEN Journal.* **4**, 473-479 (1988c).

156. Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K. Fermentation of corn cobs by *Aspergillus niger* AS101 for the production of cellulase and single cell protein. *Biomemb.* **14**, 153-157 (1988b).

157. Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K. Influency of nutritional factors on cellulase production from natural cellulosic residues by *Aspergillus niger* AS101. *Agri. Biol. Res.* 7, 19-27 (1991).

158. Smith, D.C. and Wood, T.M. Xylanase production by *Aspergillus awamori*, development of a medium and optimization of the fermentation parameters for the production of extracellular xylanase and β-xylosidase while maintaining low protease production. *Biotechnol. Bioengg.* **38,** 883-890 (1991).

159. Somasegaran, P. and Hoben, H. J. **In :** Hand book for Rhizobia : Methods in legume-*Rhizobium* technology, *Pub. Springer-Verlag*, New York 402-408 (1994).

160. Sprey, H. and Bochem, P. Formation of cross-fractures in cellulose microfibril structure by an endoglucanase-cellobiohydrolase complex from *Trichoderma reesei. FEMS Microbiol. Lett.* **106**, 239-244 (1991).

161. Srivastava, S.K., Gopalkrishnan, K.S. and Ramachandran, K.B. The production of β-glucosidase in shake-flasks by *Aspergillus wentii. J. Ferment. Technol.* **65** (1), 95-99 (1987).

162. Stahlberg, J., Johansson, G. and Pettersson, G. A new model for enzymatic hydrolysis of cellulose based on the two domain structure of cellobiohydrolase I. *Biotechnol.* **9**, 286-290 (1991).

163. Steiner, J., Saccha, C. and Enzyaguirre, J. Culture conditions for enhanced cellulase production by a native strain of *Penicillium purpurogenum*. *World J. Microbiol. Biotechnol.* **10**, 280-284 (1993).

164. Sternberg, D. and Mandels, G.R. Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose *J. Bacteriol,* **139**, 761-769 (1979).

165. Stewart, B.J. and Leatherwood. Derepressed synthesis of cellulase by *Cellulomonas. J. Bacteriol.* **128**, 2609-615 (1976).

166. Stone, J. E., Scallan, A. M., Donefer, E. and Ahlgren, E. Digestibility as a simple function of a molecule of similar size of cellulase enzyme. *Adv. Chem. Ser.* **95**, 219-241 (1969).

167. Stutzenberger, F. Production and activity of *Thermomonospora curvate* cellulases on protein-extracted Lucerne fibers. *Appl. Microbiol. Biotechnol.* **28**, 387-393 (1988).

168. Stutzenberger, F. Regulation of cellulolytic activity. *Annv. Rep. Ferment. Processes.* **8**, 111-154 (1985).

169. Sugden, C. and Bhat, M.K. Cereal straw and pure cellulose as carbon sources for growth and production of plant cell-wall degrading enzymes by *Sporotrichum thermophile. World J. Microbiol. Biotechnol.* **10**, 444-451 (1994).

170.* Szabo, I.J., Johansson, G. and Pettersson, G.J., *Biotechnol.* **48**, 221 (1996). **In** : Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

171. Takashima, S., Likura, H., Nakamura, A., Hidaka, M., Masaki, H. and Vozumi, T. Overproduction of recombinant *Trichoderma reesei* cellulases by *Aspergillus oryzae* and their enzymatic properties. *J. Bacteriol.* **65**, 163-171 (1998).

172. Tanahashi, M., Takada, S., Aoki, J., Goto, J., Higuchi, T. and Hanai, S. Dehydrogenative polymerization of monolignols by peroxidase and hydrogen peroxide in a dialysis tube. II. Estimation of molecular weight by thermal softening method. *Wood Res.* **69**, 36-40 (1982).

173. Tanaka, M., Song, G.J., Matsuno, R. and Kamikubo, T. Evaluation of effectiveness of pretreating rice straw with N-butylamine for improvement of sugar yield. *Appl. Microbial. Biotechnol.* **22**, 19-25 (1985).

174. Tansey, M. R. Agar- diffusion assay of cellulolytic ability of themophilic fungi. *Arch. Microbiol.* **77**, 1-11 (1971).

175. Teunissen, M.J., Lahaye, D.H.T.P., Veld, J.H.J.H and Vogels, G.D. Purification and characterization of an extracellular β-glucosidase from the anaerobic fungus *Piromyces* sp. Strain E_2. *Arch. Microbiol.* **158**, 276-181 (1992).

176. Tomme, P., Kwan, E., Gilkes, N.R., Kilburn, D.G. and Warren, R.A.J. Characterization of Cen C, an enzyme from *Cellulomosas fimi* with both endo- and exoglucanase activities. *J. Bacteriol.* **178**(14), 4216-4223 (1996).

177. Tomme, P., Warren, R.A.J. and Gilkes, N.R. Cellulose hydrolysis by bacteria and fungi. *Adv. Microbiol. Physiol.* **37**, 1-18 (1995).

178. Toyama, N. and Ogowa, K. Sugar production from agricultural woody waste by saccharification of *T. viride* cellulase. *Biotechnol. Bioengg. Symp.* **5**, 225-244 (1976).

179. Tuncer, M., Ball, A.S., Rob, A. and Wilson, M.T. Optimization of extracellular lignocellulolytic enzyme production by a thermophilic actinomycete *Thermonospora fusca* BD 25. *Enzyme Microb. Technol.* **25**, 38-47 (1999).

180. Updegraph, D.M. Semimicro determination of cellulose in biological materials. *Anal. Chem.* **32**, 420-424 (1969).

181. Van-Wyk, J.P.H. Cellulase adsorption-desorption and cellulose saccharification during enzymatic hydrolysis of cellulose materials. *Biotech. Lett.* **19** (18), 775-778 (1997).

182. Vatsala, T.M. Degradation of cellulose by phototrophic bacterium *Rhodospirillus rubrum. Indian J. Exp. Biol.* **7**, 963-966 (1989).

183. Veen, P.V.D., Ruijter, G.J.G. and Visser, J. An extreme cre A mutation in *Aspergillus nidulans* has severe effects on D-glucose utilization. *Microbiol.* **141**, 2301-2306 (1995).

184.* Veness, R..G. and Evans, C.S. *J. Gen. Microbiol*, **135**, 2799 (1989). **In :** Bioconversion of waste materials to industrial products (ed. A. M. Martin) *Pub. Blackie. Acad. Prof. London, SE1 8 HN.* 197-246 (1998).

185. Vipan, Singh, A., Dhillon, G.S. and Kaur, R. Factors affecting saccharification of delignified rice straw by cellobiase supplemented cellulases. *Indian J. Microbiol.* **34** (4), 297-301 (1994).

186. Waki, T., Suga, K. and Ichikowa, K. Production of Cellulase in fed-betch culture. **In :** Proceeding II international symposium on bioconversion and fed back control on cellulase biosynthesis. *Biochemicals engineering, BERC, IIT Delhi* India, 359-364 (1976).

187. Walker, J. M. SDS-Polyacerylamide gel electrophoresis of proteins. **In :** The protein protocols handbook (ed. J. M. Walker). *Humana Press Inc.999,* Totowa, New Jersey. 55-61 (1996).

188.* Walseth, C.S. The influence of the fine structure of cellulose on the action of cellulase. *Tappi.* **35**, 233-238 (1952).

189. Walter, S. and Schrempf, H. The synthesis of the *Streptomyces reticuli* cellulase (avicelase) is regulated by both activation and repression mechanisms. *Mol. Gen. Genet.* **251**, 186-195 (1996).

190. Weil, J., Westgate, P., Kohlman, K. and Ladish, M.R. Cellulose pretreatments of lignocellulosic substrates. *Enzyme Microbiol. Technol.* **16**, 1002-1004 (1994).

191. Whelan, H.A. and Prembroke, J.T. The effect of protease on stability of cellulase and xylanase from *Cellulomonas flavigena. Biotechnol. Lett.* **11**, 891-894 (1989).

192. Wojtczak, G., Breuil, C., Yamuda, J. and Saddler, J.N. A comparison of the thermostability of cellulase from various thermophilic fungi. *Appl. Microbiol. Biotechnol.* **27**, 82-87 (1987).

193. Wood, T.M. and McCrae, S.I. Purification and some properties of a (1,4)-β-D-glucan glucohydrolase associated with the cellulase from the fungus *Penicillium funiculosum. Carbohy. Res.* **110**, 291-303 (1982).

194.* Wood, W.E., Neubauer, D.G. and Stutzenberger, F. *Biotechnol. Bioengg. **160, 1047- 1054 (1984).*** **In** : Robson, L.M. and Chambliss, G.H. Cellulases of bacterial origin. *Enzyme Microb. Technol.* **11**, 626-644 (1989).

195. Wu, J. and Ju, L.K. Enhancing enzymatic saccharification of waste news print by surfactant addition. *Biotech. Prog.* **14**, 649-652 (1998).

196. Wyatt, A.M. and Broda, P. Informed strain improvement for lignin degradation by *Phanerochaete chrysosporium*. *Microbiol.* **141**, 2811-2822 (1995).

197. Yadav, J.S., Sondhi, H.S. and Neekantan, S.N. Production of cellulase microbial protein and reducing sugars on wheat straw by cellulolytic fungus *Aspergillus terreus*. *Indian J. Exp. Biol.* **26**, 546-548 (1988).

198. Yeoh, H.H., Tan, T.K. and Koh, S.K. Kinetic properties of β-glucosidase from *Aspergillus ornatus*. **25**, 25-28 (1986).

199. Zohrer, E., Albertini, S., Gocke, E., Knasmuller, S. Mutation induction and mutation spectra of *S. typhimurium* TA 100 after exposure to isohistidine. *Mut. Res.* **356**, 155-161 (1996).

200. Zurbriggen, B., Bailey, M.J., Pentilla, M.E., Poutanen, K. and Linko, M. Pilot scale production of a heterologous *Trichoderma reesei* cellulase by *Saccharomyces cerevisiae*. *J. Biotechnol.* **13**, 267-278 (1990).

---

* *Original references are not consulted.*

RESEARCH PUBLICATIONS

1. **Singh, R.P. and Kumar, R.** Adavances in cellulose biotechnology. **In :** *Innovative Approaches in Microbiology*. Maheshwari and Dubey (ed.), *BSMPS Publ. Dehra Dun,* India. 321-342 (2000).

2. **Kumar, R. and Singh, R.P.** Semi-solid state fermentation of *Eicchornia crassipes* biomass as a lignocellulosic biopolymer for cellulase and β-glucosidase production by co-cultivation of *Aspergillus niger* RK-3 and *Trichoderma reesei* MTCC-164. ***Appl. Biochem. Biotechnol.*** (Accepted).

3. **Kumar, R. and Singh, R.P.** Synthesis of carboxymethylcellulase (CMCase) and β-glucosidase by *Aspergillus niger* RK-3 using natural and commercially available cellulosic substrates. **In : *Proc. Natl. Symp. Biochem. Environ. Agri.*** Punjab Agricultural University, Ludiana, India.(2000) (In Press).

4. **Kumar, R Singh, R.P.** A novel substrate for CMCase, FPase and β-glucosidase production by *Aspergillus niger* RK-3 strain. ***In: BIOHORIZON-99.*** Indian Institute of Technology, New Delhi, March, 6-7, 12-13 (1999)

5. **Kumar, R. and Singh, R.P.** Production of carboxymethylcellulase, β-glucosidase and microbial proteins using wheat straw and sugarcane bagasse by cellulolytic fungi. **In : *Natl. Symp. Biotech.; New Trends and prospects.*** Gurukul Kangri University, Hardwar, India. Dec. 26-28. 47-48 (1996)

6. **Kumar, R. and Singh, R.P.** Production of cellulase, β-glucosidase and microbial proteins by different cellulolytic fungi. ***In : 5th APSI. Natl. Semi. Scope of Microbiology in Agriculture and Industry.*** Gurukul Kangri University, Hardwar, India. Oct. 4-5, 8-9 (1996).