

## **Contents**

List of Tables

List of Figures

Abbreviation

Preface

### **Chapter – 1: Introduction 1 – 15**

1.1	Global fuel scenario	2
1.2	Biobutanol	3
1.3	Global biobutanol scenario	7
1.4	Indian biofuel production scenario	9
1.4.1	Indian market in biobutanol production	10
1.5	Feedstocks for biobutanol production	11
1.5.1	Algal biomass as feedstock	12
1.5.2	Cyanobacteria as sustainable source of energy	13
1.6	Broad objective	15

### **Chapter – 2: Review of Literature 16 – 56**

2.1	Introduction	17
2.2	ABE production through fermentation	18
2.2.1	Feedstocks	18
2.2.2	Pre-processing of feedstocks	25
2.2.2.1	Physical pretreatment	26
2.2.2.2	Chemical pretreatment	27
2.2.2.2.1	Acid pretreatment	27
2.2.2.2.2	Alkali pretreatment	28
2.2.2.3	Other pretreatment techniques	31
2.2.2.4	Enzymatic hydrolysis	33
2.2.3	Microorganism and metabolic pathway	36
2.2.3.1	Butanol toxicity and yield	38
2.2.4	ABE fermentation	39
2.2.4.1	Effect of different operating parameters	39
2.2.4.2	Fermentation modes	40

2.3	Downstream operation	44
2.3.1	Liquid-liquid extraction	44
2.3.2	Perstraction/Membrane-assisted solvent extraction	47
2.3.3	Other butanol recovery techniques	49
2.4	Mathematical models for ABE fermentation process	50
2.5	Economics of butanol production	54
2.6	Challenges in biobutanol production	55
2.7	Specific objective of present work	56

## **Chapter – 3: Materials & Methods** **57 - 89**

3.1	Chemicals and Equipments	58
3.2	Biomass selection	58
3.2.1	Collection, cultivation and characterization of cyanobacterial biomass	58
3.2.1.1	Scanning Electron Microscopic Analysis	60
3.2.2	Batch growth of cyanobacterial biomass	61
3.2.2.1	Carbohydrate analysis	62
3.2.3	Manipulation of culture conditions	63
3.2.3.1	Effect of different operating parameters on growth of cyanobacterial biomass	63
3.2.3.2	Effect of nutrient limitation on cyanobacterial growth	63
3.2.4	Growth kinetics	64
3.2.5	Two-stage growth study	64
3.3	Pretreatment of biomass	65
3.3.1	Preparation and characterization of cyanobacterial biomass	65
3.3.2	Batch pretreatment study	66
3.3.2.1	Optimization of pretreatment conditions through RSM	67
3.3.3	Other pretreatment strategies	68
3.3.3.1	Stepwise pretreatment	68
3.3.3.2	Lipid extraction simultaneous use of cyanobacterial residue for sugar release	69
3.4	Enzymatic hydrolysis	72
3.4.1	Preparation of inoculum for enzyme extraction	72
3.4.2	Solid state fermentation	72
3.4.3	Enzyme assays	73

3.4.3.1	Preparation of standard curve for glucose	75
3.4.3.2	Filter paper assay	75
3.4.3.3	Endoglucanase assay	76
3.4.3.4	$\beta$ -glucosidase assay	76
3.4.3.5	Extracellular protein estimation	77
3.4.4	Thermal stability test of enzyme in the presence of Fe <sub>3</sub> O <sub>4</sub> /Alginate nanocomposite	77
3.4.5	Enzymatic hydrolysis of pretreated cyanobacterial biomass	78
3.5	Butanol fermentation study	79
3.5.1	Preparation of inoculum	79
3.5.2	Effect of various process parameters	80
3.5.3	Preparation of cyanobacterial hydrolysate for fermentation	81
3.5.4	Analysis of end products and metabolites	83
3.5.5	Continuous stirred tank reactor fermentation	84
3.6	Recovery of butanol	86
3.6.1	Liquid-liquid extraction	86
3.6.2	Perstraction /Membrane-assisted solvent extraction	87

## **Chapter – 4: Results & Discussion** **90 – 158**

4.1	Biomass selection	91
4.1.1	Identification and characterization of collected biomass	91
4.1.2	Batch growth study of cyanobacterial biomass	92
4.1.3	Effect of different operating parameters	93
4.1.3.1	Effect of shaking	93
4.1.3.2	Effect of media pH	95
4.1.3.3	Effect of incubation temperature	96
4.1.3.4	Effect of light intensity	97
4.1.4	Effect of nutrient limitation on cyanobacteria growth	99
4.1.4.1	Effect of trace elements concentration	99
4.1.4.2	Effect of nitrogen concentration	100
4.1.4.3	Effect of phosphorus concentration	103
4.1.5	Two-stage growth study	105



4.2	Biomass pretreatment	106
4.2.1	Batch pretreatment	106
4.2.2	Biomass characterization	110
4.2.2.1	Scanning Electron Microscopy	110
4.2.2.2	Fourier Transform Infrared spectroscopy	110
4.2.3	Optimization of pretreatment condition through RSM	112
4.2.4	Other pretreatment strategies	119
4.2.4.1	Stepwise pretreatment	119
4.2.4.2	Lipid extraction and utilization of cyanobacterial residue for sugar release	119
4.3	Enzymatic hydrolysis	120
4.3.1	Enzyme extraction	120
4.3.1.1	Thermal stability of extracted enzyme in the presence of Fe <sub>3</sub> O <sub>4</sub> /Alg nanocomposite	122
4.3.1.2	Enzymatic hydrolysis of pretreated cyanobacterial biomass	124
4.4	Fermentation study	124
4.4.1	Growth pattern of Clostridium strain	124
4.4.2	Butanol fermentation using glucose as the carbon source	126
4.4.2.1	Effect of initial inoculum concentration	128
4.4.2.2	Effect of initial media pH	128
4.4.2.3	Effect of yeast extract concentration	130
4.4.2.4	Effect of initial glucose concentration	132
4.4.2.5	Effect of other parameters	135
4.4.3	Butanol fermentation study with other Clostridial strains	136
4.4.4	Cyanobacterial hydrolysate as carbon source	137
4.4.4.1	Effect of biomass loading	137
4.4.4.2	Fermentation of cyanobacterial hydrolysate prepared from strains grown under normal condition	139
4.4.4.3	Fermentation of cyanobacterial hydrolysate prepared from strains grown under two-stage conditions	141

4.4.5	Continuous stirred tank reactor	144
	4.4.5.1 Mass-balance for CSTR	147
	4.4.5.2 Kinetic modeling of butanol production, bacterial growth & sugar consumption	150
4.5	Recovery of butanol	153
	4.5.1 Liquid-liquid extraction	153
	4.5.2 Membrane-assisted solvent extraction	155
<b>Chapter – 5: Conclusion &amp; Suggestion for Further Work</b>		<b>159 – 161</b>
<b>List of Publications</b>		<b>162</b>
<b>References</b>		<b>163 – 197</b>
<b>Appendix</b>		<b>198 – 201</b>

## **List of Tables**

Table 1.1	Properties of various isomeric forms of butanol	4
Table 1.2	Comparison of properties of different fuels	5
Table 1.3	Production of biobutanol in developed countries	9
Table 2.1	Butanol production from various feedstocks	21
Table 2.2	Cyanobacteria used for various biofuels production	25
Table 2.3	Physical pretreatment of biomass	29
Table 2.4	Chemical pretreatment of biomass	30
Table 2.5	Comparison of different fermentation modes for butanol production	43
Table 2.6	Comparison of different downstream operations for butanol recovery	45
Table 3.1	List of equipment used during experimentation	59
Table 3.2	BG-11 media composition	61
Table 3.3	ATCC 1441 growth media	80
Table 3.4	Fermentation media (P-2 media) composition	82
Table 3.5	Solvent used in liquid-liquid extraction	87
Table 4.1	Characterization of dried cyanobacterial biomass	92
Table 4.2	Specific growth rate at different operating parameters	94
Table 4.3	Specific growth rate of cyanobacterial strains under nutrient limiting conditions	104
Table 4.4	Compositional analysis of cyanobacterial biomass	104
Table 4.5	Biomass and carbohydrate productivity of various cyanobacterial strains	107
Table 4.6	Experimental design for pretreatment of cyanobacterial biomass	113
Table 4.7	Results of the regression analysis of the design	114
Table 4.8	Sugar release from different biomass by various pretreatment agents	118

Table 4.9	Enzymatic activities of commercial and extracted enzymes	121
Table 4.10	Effect of C/N ratio on butanol production	133
Table 4.11	Effect of micronutrients on butanol production	136
Table 4.12	Carbohydrate profiles at different biomass loading	138
Table 4.13	Carbohydrate profiles under two-stage growth conditions	138
Table 4.14	Fermentation in CSTR with glucose and cyanobacterial hydrolysate	146
Table 4.15	Various biokinetic parameters estimated using Mercier model and experimental results obtained during CSTR studies	151

## **List of Figures**

Figure 1.1	Production of biobutanol through fermentation	7
Figure 2.1	General metabolic pathway for ABE fermentation	37
Figure 2.2	Various modes of ABE fermentation	42
Figure 2.3	Techniques for butanol recovery	47
Figure 3.1	Geographic location of different sample collection points	60
Figure 3.2	Two-step pretreatment of cyanobacterial biomass	70
Figure 3.3	Simultaneous recovery of lipid and fermentable sugar from cyanobacterial biomass	71
Figure 3.4	Stepwise incubation and extraction of enzyme	74
Figure 3.5	Anaerobic inoculum preparation and maintenance	79
Figure 3.6	Continuous stirred tank reactor study for butanol fermentation	85
Figure 3.7	Membrane-assisted solvent extraction device	88
Figure 4.1	SEM images of cyanobacterial strains	92
Figure 4.2	Growth pattern of cyanobacterial strains	93
Figure 4.3	Effect of pH on growth of cyanobacterial strains	95
Figure 4.4	Effect of incubation temperature on growth of cyanobacterial strains	97
Figure 4.5	Effect of light intensity on growth of cyanobacterial strains	98
Figure 4.6	Effect of trace elements on growth of cyanobacterial strains	100
Figure 4.7	Effect of N-rich and N-starvation condition on biomass density and appearance	101
Figure 4.8	Effect of nitrogen concentration on the growth of cyanobacterial strains	102
Figure 4.9	Effect of phosphorus concentration on cyanobacterial growth	103

Figure 4.10	Two-stage growth study of <i>L. limnetica</i> and <i>O. obscura</i> biomass	106
Figure 4.11	Effect of operating parameters on sugar release from different strains	109
Figure 4.12	SEM images of <i>L. limnetica</i>	111
Figure 4.13	FTIR spectra of <i>L. limnetica</i>	111
Figure 4.14	Combined effect of treatment temperature and time on sugar release at constant acid concentration of 1.5 M	114
Figure 4.15	Combined effect of treatment temperature and acid concentration on sugar release at constant treatment time of 37.5 min	115
Figure 4.16	Combined effect of treatment temperature and time on sugar release optimized by the software	117
Figure 4.17	Combined effect of treatment temperature and acid concentration on sugar release optimized by the software	118
Figure 4.18	Sequential recovery of lipid and carbohydrate	120
Figure 4.19	Effect of Fe <sub>3</sub> O <sub>4</sub> /Alg concentration on the activity of extracted enzyme	122
Figure 4.20	Thermal stability of enzyme in terms of relative activity	123
Figure 4.21	Growth curve of <i>C. beijerinckii</i> ATCC 35702	125
Figure 4.22	Clostridial strain (a) Gram staining, (b) SEM image of Clostridial spores, (c) SEM image of growing Clostridial cells	125
Figure 4.23	Serum bottles with crimped cap for anaerobic fermentation study	126
Figure 4.24	Gas chromatogram of (a) Standard hydrogen gas (b) Gas mixture from batch reactor	127
Figure 4.25	Standard plot for gas chromatographic analysis	127
Figure 4.26	Effect of inoculum concentration on butanol production	131
Figure 4.27	Effect of initial media pH on butanol production	131

Figure 4.28	Effect of yeast extract concentration on butanol production	134
Figure 4.29	Effect of initial glucose concentration on butanol production	134
Figure 4.30	Cyanobacteria grown, pretreated & fermented at optimized conditions	140
Figure 4.31	Fermentation of cyanobacterial hydrolysate supplemented with glucose	140
Figure 4.32	Two-stage grown cyanobacteria, pretreated & fermented at optimized conditions	142
Figure 4.33	Fermentation of two-stage grown cyanobacterial hydrolysate supplemented with glucose	142
Figure 4.34	Comparison of butanol productivity from cyanobacterial hydrolysate, fermented under various conditions	144
Figure 4.35	Comparison of shake-flask and CSTR butanol production	145
Figure 4.36	Proposed fermentation pathway for butanol production	147
Figure 4.37	C-balance at optimized conditions using glucose as the main C-source	149
Figure 4.38	C-balance at optimized conditions using <i>L. limnetica</i> hydrolysate as the main C-source	150
Figure 4.39	Comparison of experimental data with model prediction	152
Figure 4.40	Butanol extraction	154
Figure 4.41	Butanol extraction using various extractants	154
Figure 4.42	Membrane-assisted solvent extraction from aqueous butanol solution	156
Figure 4.43	Membrane-assisted solvent extraction with glucose and cyanobacterial hydrolysate media	157

## Abbreviation

AB	Acetone-butanol
ABE	Acetone-butanol-ethanol
Ac	Acetone
Alg	Alginate
BBD	Box-Behnken design
BG	Bacterial growth
$\beta$ G	$\beta$ -glucosidase
BGPY	Billion gallons per year
But	Butanol
CC	Carbohydrate content
CSTR	Continuous stirred tank reactor
d	day
DB	Drybiomass
DNS	dinitrosalicylic acid
EB	Enzyme-buffer
EG	Endoglucanase
ES	Enzyme-substrate
FP	Filter paper cellulase
FTIR	Fourier Transform Infrared
g/g db	g/g of drybiomass
GP	Gas production
HPLC	High Performance Liquid Chromatography
IU/gds	International unit per gram dry substrate
LLH	<i>L. limnetica</i> hydrolysate
LLHS	<i>L. limnetica</i> hydrolysate supplemented with glucose
MPM	Modified P-2 media
NC	Nanocomposite
NCT	Nanocomposite treated
NR	Nitrogen-rich
NREL	National Renewable Energy Laboratory
NS	Nitrogen-starvation

PABA	para-aminobenzoic acid
PDA	Potato Dextrose Agar
pNPG	<i>p</i> -nitrophenyl glucopyranoside
PR	Phosphorus-rich
PS	Phosphorus-starvation
RID	Refractive Index Detector
RSM	Response Surface Methodology
SB	Substrate-buffer
SEM	Scanning Electron Microscopy
SF	Shake-flask
SSF	Solid state fermentation
TE	Trace elements
WhTE	Without trace elements
WTE	With trace elements