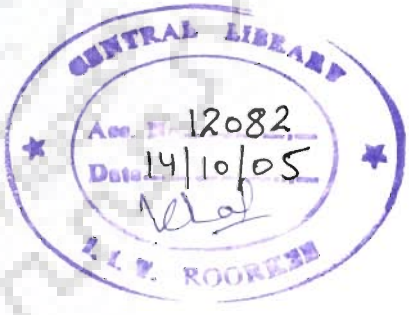
BIOCONVERSION OF CHEAPER CARBOHYDRATE SOURCES FOR LACTIC ACID PRODUCTION

A THESIS

Submitted in fulfilment of the
requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

By

RUMA GANGULY



DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**BIOCONVERSION OF CHEAPER CARBOHYDRATE SOURCES FOR LACTIC ACID PRODUCTION**" in fulfilment of the requirement for the award of the **Degree of Doctor of Philosophy** and submitted in the **Department of Biotechnology of the Indian Institute of Technology**, Roorkee is an authentic record of my own work carried out during the period from January 1999 to 2005 under the supervision of **Dr. R.P. Singh**.

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

Ruma Ganguly

Dated : 7.1. 2005 Signature of the Candidate

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

Dated : 07.1. 2005

Dr. R. P. Singh
Associate Professor
Department of Biotechnology
Indian Institute of Technology Roorkee
Roorkee – 247 667
India

The Ph.D. Viva-Voce examination of Ms Ruma Ganguly, Research Scholar has been held on .....

Signature of Supervisor Signature of HOD Signature of External Examiner

ABSTRACT

The present study was undertaken to isolate a potent microbial strain producing lactic acid and to economize the fermentation process by cutting down the cost of production media by selecting a cheaper raw material. The following strategy was undertaken to fulfill the aim of the present study.

A thorough survey into the various sources for isolating a potent lactic acid producer led to the isolation of a strain designated as RB-5 and identified to be *Rhizopus oryzae*. To maximize the lactic acid production ability of the isolated strain, it was subjected to the physical and chemical mutagenesis. After two stages of UV irradiation, a mutant designated as *Rhizopus oryzae* RBU2-10 was obtained which had the capability of higher levels of lactic acid production. The mutants obtained by chemical mutagenesis did not result in much improvement in lactic acid production ability of the wild type. The distinction between the wild type *R. oryzae* RB-5 and mutant *R. oryzae* RBU2-10 was not limited to their lactic acid production ability but also to their morphological features. The various major parameters were analyzed in submerged condition for increasing the lactic acid production ability of the mutagenized strain *R. oryzae* RBU2-10. Production levels of lactic acid were also evaluated in solid state fermentation (SSF) condition. Of the three supports viz. bagasse, groundnut shell and coconut coir used for the study, bagasse led to higher levels of lactic acid production. But the levels of production obtained under SSF condition were lower as compared to that under submerged condition.

For economization of the fermentation process the synthetic sugars were substituted with cheaper carbohydrate sources mainly with the sugarcane molasses and with various starch based substrates like broken and pest infested rice, partially rotten potatoes, sago and arrowroot starch. The starch based substrates were hydrolyzed prior to their use in the fermentation reactions. Among the substrates used, the enzyme hydrolysed rice starch led to maximum levels of lactic acid production followed by the enzyme hydrolysed potato, arrowroot and sago starch. The various parameters for achieving increased production levels were analysed using enzyme hydrolysed rice starch as the substrate.

Whole cells of *R. oryzae* RBU2-10 were immobilized in order to analyse the system for semicontinuous production of lactic acid. Two types of matrices i.e. calcium alginate and loofa sponge were used for immobilization. The various factors affecting production levels by immobilized mycelia were studied. These observations had indicated that loofa sponge immobilized mycelia had resulted into higher levels of lactic acid production than the calcium alginate trapped cells. In addition, the loofa sponge was considerably more economical and easily available. Extraction of lactic acid was attempted by using various combinations of the carriers mainly tri-n-octylamine, tri-n-pentylamine and the diluents, methyl isobutyl-ketone, 1-octanol. Tri-n-octylamine in methyl isobutyketone was found to be better for the extraction of lactic acid. Extractant when finally treated with the aqueous phase containing 4 N NaOH in the combinations of 1 : 1 and 4 : 1 (extractant : aqueous phase), the earlier combination led into 85-90 % recovery of the lactic acid from the fermentation broth.

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Ruma Ganguly

RUMA GANGULY

TABLE OF CONTENTS

	Page No.
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
LIST OF FIGURES	xiv
LIST OF TABLES	xvii
LIST OF PLATES	xix
GLOSSARY	xx
CHAPTER 1	
INTRODUCTION	1-5
CHAPTER – 2	
LITERATURE REVIEW	6-24
2.1 HISTORICAL BACKGROUND	6
2.2 SYNTHESIS OF LACTIC ACID	7
2.2.1 Chemical Synthesis	7
2.2.2 Fermentation Process	8
2.2.2.1 Carbon source	8
2.2.2.2 Nitrogen, phosphate and trace metals	9
2.2.2.3 pH	11
2.2.2.4 Temperature	11
2.2.2.5 Aeration and Agitation	12
2.3 LACTIC ACID PRODUCTION TECHNOLOGY	13
2.3.1 Microorganism	13
2.3.2 Inoculum Development	14
2.3.3 Raw Materials	15
2.3.4 Fermentation Processes	16

2.3.4.1 Submerged fermentation process 16
2.3.4.2 Solid state fermentation 17
2.3.4.3 Immobilized fermentation 18
2.3.5 Product Recovery 19
2.4 COMMERCIAL PROCEDURE FOR LACTIC ACID PRODUCTION 21
2.5 MANUFACTURING UNITS OF LACTIC ACID 22
2.5.1 Indian Manufacturers 22
2.5.2 Global Manufacturers 23
2.6 PATENTS FOR LACTIC ACID 23
2.7 PROPOSED DEMAND FOR LACTIC ACID BY 2005 AD IN INDIA 24

CHAPTER – 3

MATERIALS AND METHODS 25-45

3.1 MATERIALS 25
3.1.1 Chemicals 25
3.1.2 Microorganisms 25
3.2 METHODS 26
3.2.1 Selection of Strain 26
3.2.1.1 Sources of microbial strain 26
3.2.1.1.1 Soil samples 26
3.2.1.1.2 Partially decomposed fruits and vegetables 26
3.2.1.1.3 Bakery products 26
3.2.1.2 Method of isolation 27
3.2.1.2.1 Screening of lactate producing strains 27
3.2.1.2.1.1 By acid unitage values 27
3.2.1.2.1.2 Qualitative analysis of lactic acid 28
3.2.1.2.1.3 Fermentation studies 28
3.2.2 Identification of Selected Strain 28
3.2.3 Mutagenesis and screening 29
3.2.3.1 Physical mutagenesis 29

3.2.7.3.1 Lactic acid production with calcium alginate entrapped cells of *Rhizopus oryzae* 39
3.2.7.3.2 Lactic acid production with *Rhizopus oryzae* RBU2-10 immobilized in loofa sponge 39
3.2.8 Analysis of Carriers and Diluents for Lactic Acid Extraction 39
3.2.9 Analytical Methods 40
3.2.9.1 Qualitative analysis of lactic acid 40
3.2.9.2 Quantitative analysis of lactic acid 41
3.2.9.2.1 Reagents 41
3.2.9.2.2 Procedure 41
3.2.9.3 Estimation of glucose 42
3.2.9.3.1 Reagents 42
3.2.9.3.2 Procedure 42
3.2.9.4 Dry mycelial weight estimation 43
3.2.9.5 Test for hydrolysis of starch 43
3.2.9.5.1 Rapid test 43
3.2.9.5.1.1 Reagent 43
3.2.9.5.1.2 Method 43
3.2.9.6 Chromatographic analysis 44
3.2.10 Electron Microscopy of the Fungus 44
3.2.11 Statistical Analysis 45

Chapter – 4

RESULTS 46-96

4.1 ISOLATION, PURIFICATION AND SCREENING OF POTENT MICROBIAL STRAIN 46
4.2 MUTAGENESIS OF ISOLATED FUNGAL STRAIN FOR IMPROVING LACTIC ACID PRODUCTION 46
4.2.1 Morphological features 54
4.3 ANALYSIS OF CRITICAL PARAMETERS FOR IMPROVING THE LACTIC ACID PRODUCTION 54

3.2.3.1.1 Second stage UV irradiation 29
3.2.3.2 Chemical mutagenesis 30
3.2.3.2.1 Colchicine treatment 30
3.2.3.2.2 N-methyl-N'-nitro-N-nitrosoguonidine (NTG) treatment 30
3.2.3.3 Screening of Mutants 31
3.2.4 Analysis of Factors Affecting Lactic Acid Production 31
3.2.5 Lactic Acid Fermentation by Free Cells of *Rhizopus oryzae* 32
3.2.5.1 Submerged fermentation 32
3.2.5.1.1 Inoculum preparation 32
3.2.5.1.2 Submerged fermentation process 32
3.2.5.2 Solid state fermentation 33
3.2.5.2.1 Preparation of support 33
3.2.5.2.2 Preparation of inoculum 33
3.2.5.2.3 Solid state fermentation process 34
3.2.6 Processing of Crude Substrates for Lactic Acid Production 34
3.2.6.1 Processing of sugarcane molasses 34
3.2.6.2 Processing of starch based substrates 35
3.2.6.2.1 Acid hydrolysis of starch 36
3.2.6.2.2 Enzymatic hydrolysis of starch 36
3.2.7 Lactic Acid Production with Immobilized *Rhizopus oryzae* 37
3.2.7.1 Immobilization of *Rhizopus oryzae* RBU2-10 in Calcium alginate beads 37
3.2.7.2 Immobilization of *Rhizopus oryzae* RBU2-10 in loofa sponge 37
3.2.7.2.1 Assay of temperature and pH stability of loofa sponge 38
3.2.7.2.1.1 Assay of pH stability 38
3.2.7.2.1.2 Assay of temperature stability 38
3.2.7.3 Semicontinuous lactic acid production with immobilized cells 38

4.3.1 Fermentative Production of Lactic Acid with Sugar Substitutes 56
4.3.2 Lactic Acid Fermentation as a Function of Substrate Concentration Using Glucose 56
4.3.3 Lactic Acid Fermentation as a Function of Temperature, pH, Calcium Carbonate Concentration and Agitation Rate 59
4.3.4 Lactic Acid Production as a Function of Inoculated Spore Concentration and Inoculum Level 59
4.3.5 Lactic Acid Production as a Function of Nitrogen Sources 62
4.4 LACTIC ACID FERMENTATION UNDER SOLID STATE FERMENTATION CONDITION 65
4.5 LACTIC ACID PRODUCTION WITH CHEAPER CARBOHYDRATE SOURCES 73
4.5.1 Lactic acid production as a function of medium composition in rice starch based substrate 75
4.5.2 Lactic acid production in rice starch medium as a function of time 75
4.6 LACTIC ACID PRODUCTION BY IMMOBILIZED *RHIZOPUS ORYZAE* RBU2-10 USING HYDROLYSED RICE STARCH CONTAINING MEDIUM 80
4.6.1 Evaluation and Derivation of Various Conditions for Immobilized Cells of *Rhizopus oryzae* RBU2-10 for Lactic Acid Production 80
4.6.1.1 Lactic acid production as a function of size of immobilized matrices 80
4.6.1.2 Lactic acid production as a function of spore suspension in various matrices 82
4.6.1.3 Lactic acid production as a function of immobilized inoculum level in various matrices 82
4.6.1.4 Lactic acid production by *Rhizopus oryzae* RBU2-10 at varying time intervals 82

4.6.2 Semicontinuous Production of Lactic Acid by Calcium Alginate and Loofa Sponge Immobilized *Rhizopus oryzae* RBU2-10 88

4.7 EXTRACTION OF LACTIC ACID 94

CHAPTER – 5

DISCUSSION 97-108

5.1 ISOLATION AND SCREENING OF THE LACTIC ACID PRODUCING STRAIN 97

5.2 DERIVATION OF CRITICAL PARAMETERS FOR LACTIC ACID PRODUCTION 99

5.3 BIOCONVERSION OF CHEAPER CARBOHYDRATE SOURCES FOR LACTIC ACID PRODUCTION 103

5.4 LACTIC ACID PRODUCTION WITH IMMOBILIZED CELLS 105

5.5 COMPARATIVE ANALYSIS OF EXTRACTION WITH CARRIERS AND DILUENTS COMBINATION 107

CHAPTER – 6

SUMMARY & CONCLUSIONS 109-112

REFERENCES 113-138

RESEARCH PUBLICATIONS

LIST OF FIGURES

S. No.	FIGURES	Page No.
1.	MODEL OF GLUCOSE METABOLISM IN THE FILAMENTOUS FUNGUS *RHIZOPUS ORYZAE*	2
2.	EFFECT OF MUTAGENIC TEATMENTS ON SPORE SURVIVAL AND DISTRIBUTION OF *RHIZOPUS ORYZAE* RB-5	52
3.	PRODUCTION STABILITY OF THE MUTANT *RHIZOPUS ORYZAE* RBU2-10	55
4.	FERMENTATIVE PRODUCTION OF LACTIC ACID WITH VARIOUS SUGAR SUBSTITUTES	57
5.	LACTIC ACID FERMENTATION AS A FUNCTION OF SUBSTRATE CONCENTRATION USING GLUCOSE	58
6.	LACTIC ACID PRODUCTION AT VARYING TEMPERATURES (a), pH (b), CALCIUM CARBONATE CONCENTRATION (c) AND AT VARYING AGITATION RATES (d)	60
7.	LACTIC ACID PRODUCTION AS A FUNCTION OF (a) SPORE CONCENTRATION AND (b) INOCULUM LEVEL	61
8(a)	LACTIC ACID PRODUCTION AS A FUNCTION OF VARIATION IN NITROGEN SOURCES	63

8(b)	EFFECT OF VARYING AMMONIUM SULPHATE CONCENTRATION ON LACTIC ACID PRODUCTION	63
9.	EFFECT OF VARIATIONS OF NUTRIENT CONCENTRATIONS ON LACTIC ACID PRODUCTION (a) POTASSIUM DIHDROGEN PHOSPHATE. (b) MAGNESIUM SULPHATE, (c) ZINC SULPHATE	64
10.	LACTIC ACID FORMATION UNDER DIFFERENT FERMENTATION CONDITIONS	71
11.	LACTIC ACID PRODUCTION AT DIFFERENT TIME PERIODS DURING SOLID-STATE AND SUBMERGED FERMENTATION	72
12.	LACTIC ACID PRODUCTION WITH CHEAPER CARBOHYDRATE SOURCES	74
13.	EFFECT OF VARYING CONCENTRATIONS OF NUTRIENTS ON LACTIC ACID PRODUCTION USING HYDROLYZED RICE STARCH	76
14.	LACTIC ACID PRODUCTION IN RICE STARCH MEDIUM AT DIFFERENT TIME PERIODS	79
15.	LACTIC ACID PRODUCTION AS A FUNCTION OF VARIATION OF SIZE OF IMMOBILIZED MATRICES	81
16.	LACTIC ACID PRODUCTION AS A FUNCTION OF SPORE SUSPENSION IN VARIOUS MATRICES	83

17. LACTIC ACID PRODUCTION AS A FUNCTION OF IMMOBILIZED INOCULUM LEVEL 84

18. LACTIC ACID PRODUCTION AS A FUNCTION OF FERMENTATION TIME 87

19. SEMICONTINUOUS PRODUCTION OF LACTIC ACID BY CALCIUM ALGINATE IMMOBILIZED *RHIZOPUS ORYZAE* RBU2-10 90

20. SEMICONTINUOUS PRODUCTION OF LACTIC ACID BY LOOFA SPONGE IMMOBILIZED *RHIZOPUS ORYZAE* RBU2-10 91

21. SEMICONTINUOUS PRODUCTION OF LACTIC ACID BY FREE CELLS OF *RHIZOPUS ORYZAE* RBU2-10 92

22. EXTRACTION OF LACTIC ACID WITH VARIOUS CARRIERS AND DILUENTS 95

LIST OF TABLES

Table No.	Tables	Page No.
1.	Morphological features of fungal strains and their source of isolation	47
2.	Screening of the fungal strains based on zone formation and lactic acid production under submerged condition	50
3.	Acid unitage value of the mutagenized strains following Ist stage of ultraviolet irradiation	50
4.	Acid Unitage value of the mutants derived following NTG (100 μg/ml) treatment	51
5.	Acid Unitage value of the mutants derived following colchicine treatment	51
6.	Acid Unitage value of the mutagenized strains following IInd stage of ultraviolet irradiation	51
7.	Lactic acid production by the selected mutants obtained after UV and chemical mutagenesis of *Rhizopus oryzae* RB-5	53
8.	Analysis of variance for different physicochemical and biological factors for lactic acid production by *R. oryzae* RBU2-10 using glucose as the substrate	66
9.	Defined set of conditions and factors favourable for lactic acid production in submerged fermentation condition with glucose as a substrate	69
10.	Evaluation of kinetic parameters for lactic acid production using glucose as a substrate under derived conditions of fermentation	70
11.	Analysis of variance for different nutrient concentrations on lactic acid production by *R. oryzae* RBU2-10 using enzyme hydrolyzed rice starch	77

12.	Evaluation of kinetic parameters for lactic acid production by *R. oryzae* RBU2-10 using glucose and enzyme hydrolyzed rice starch as the substrates under derived conditions of fermentation	78
13.	Analysis of variance for multiple parameters for lactic acid fermentation by calcium alginate immobilized cells of *R. oryzae* RBU2-10	85
14.	Analysis of variance for lactic acid production by *R. oryzae* RBU2-10 cells immobilized in loofa sponge	86
15.	Defined set of conditions and factors favourable for lactic acid production by *Rhizopus oryzae* RBU2-10 immobilized in loofa sponge with enzyme hydrolyzed rice starch as a substrate	89
16.	Evaluation of kinetic parameters for lactic acid production by *R. oryzae* RBU2-10 immobilized in loofa sponge cultivated in repeated batch process under derived conditions of fermentation.	93
17.	Effect of the phase ratio on the extraction of lactic acid	96

LIST OF PLATES

S. No.	PLATES	Between pages
1.	FORMATION OF YELLOW COLOURED ZONES BY THE (A) WILD TYPE *RHIZOPUS ORYZAE* RB-5, (B) MUTANT *RHIZOPUS ORYZAE* RBU2-10 THE ZONES FORMED BY THE RESPECTIVE STRAINS WERE OBSERVED AFTER 72 HOURS INCUBATION AT 35°C.	49-50
2.	MORPHOLOGICAL VARIATIONS BETWEEN THE WILD TYPE AND MUTANT STRAINS OF *RHIZOPUS ORYZAE* CULTURED IN P.D.A. MEDIA	54-55
3	SCANNING ELECTRON MICROGRAPHS OF WILD TYPE AND MUTANT STRAINS OF *RHIZOPUS ORYZAE*	54-55
4.	MYCELIAL PELLETS OF MUTANT RBU2-10 (A) AND WILD TYPE RB-5 (B) STRAINS OF *RHIZOPUS ORYZAE*	54-55
5	SOLID SUPPORTS	65-66
6.	LOOFA SPONGE	80-81
7	SCANNING ELECTRON MICROGRAPH OF *RHIZOPUS ORYZAE* RBU2-10 IMMOBILIZED ON LOOFA SPONGE	88-89

GLOSSARY

ANOVA	Analysis of Variance
AU	Acid Unitage
b-θ	Numbers of Block
df	Degree of freedom
dm	Decimeter
DNS	Dinitro Salicylic Acid
gm	Gram
GRAS	Generally Recognized as Safe
hrs	Hours
K-θ	Numbers of Treatment
l	Litre
LDH	Lactate Dehydrogease
ml	Millilitre
mm	Millimeter
N	Normal
n	Number of replicate
NTG	N-methyl-N'-nitro-N-nitrosoguamidine
^{o}C	Degree Celsius
PDA	Potato Dextro Agar
PLA	Poly lactic acid

rpm	Revolutions per minute
SEM	Scanning Electron Microscope
SS	Total sum square
SSB	Block sum square
SSE	Error sum of square
SST	Treatment sum of square
U	Unit
UV	Ultra Violet
V/V	Volume per Volume
W/V	Weight per Volume
µg	Microgram

Chapter 1

Introduction

Lactic acid, a versatile chemical of ubiquitous occurrence in nature is a product of the organisms ranging from microbial sources to human beings. Lactic acid has extensive applications in food, pharmaceutical, cosmetic and textile industries. It is classified as GRAS (generally recognized as safe) for use in food industry as a acidulant, preservative, buffering agent for beverages, bakery products, jams, jellies, processed eggs, soups and pickles. Lactic acid is used as an intermediate in the manufacture of pharmaceuticals and for buffering preparations used in the topical wart medication. The salts of the lactic acid are used as a dietary calcium source and also as a blood coagulant. Technical grade lactic acid is used in the leather tanning industry, for dyeing of the woolen materials and for the brightening of silk and rayon fibres. Lactic acid is also used in electrostatic painting and ethyl lactate is used in the manufacturing of fluorescent tube lights.

Lactic acid can be polymerized into polylactic acid (PLA) polymers which are environmentally compatible thermoplastics. These polymers are considered superior because of high strength, biodegradability and are environmentally compatible. Due to extensive applications, the demand of lactic acid is as high as 100,000 metric tones per annum and is increasing year after year.

Production of lactic acid can be accomplished by (i) chemical synthesis or by (ii) fermentation. The chemical synthesis involves reaction of acetaldehyde with HCN to yield lactonitrile which is then hydrolysed to give lactic acid. Some of the other synthetic routes are reaction of nitrous acid on alanine, oxidation of propylene glycol and the reaction of sucrose and calcium oxide at high temperature (240°C). The

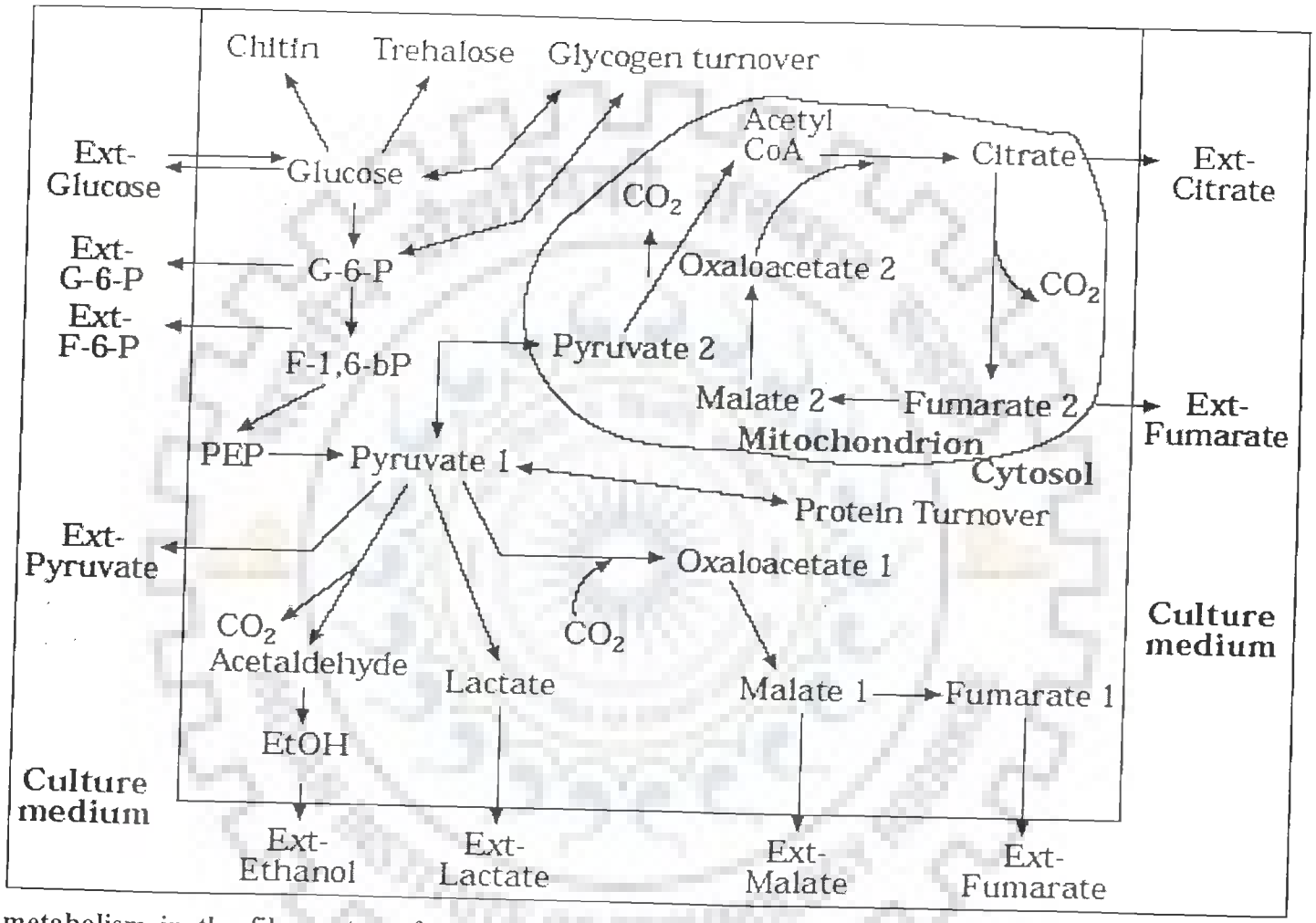

Fig. 1. Glucose metabolism in the filamentous fungus *Rhizopus oryzae*. Ext – Extracellular ; G-6-P, Glucose-6-phosphate ; F-6-P, Fructose-6-phosphate ; F-1,6-bP : Fructose-1,6-bisphosphate (Wright *et al*, 1996)

production of lactic acid by fermentation involves bioconversion of appropriate sugar substrates by the microorganisms.

The chemical synthesis of lactic acid generates a mixture of two isomers whereas biotechnological production yields either of the isomer alone or a mixture of two isomers in varying proportions that mainly depends on the choice of the microbial strain, raw material and the fermentation conditions selected. The microbial strains producing lactic acid can either be bacterial (*Lactobacillus delbrueckii, Lactobacillus amylovorous* etc.) or fungal (*Rhizopus oryzae* etc.). Bacterial pathway for lactic acid production may involve different routes resulting in homo-, hetro-, or mixed acid fermentation. In *Rhizopus oryzae*, in two separately regulated pools of pyruvate metabolism, the cytosolic pyruvate may be channeled into ethanol, malate, oxaloacetate, lactate and fumarate synthesis, whereas through second pool pyruvate may be channeled into the tricarboxylic acid cycle (Wright *et al*, 1996). The same has been depicted in Figure 1.

Bacterial fermentative production of lactic acid (LA) have certain advantages mainly with respect to having the high growth rate and greater productivity whereas the process suffers from the negative trait of producing a racemic mixture i.e. both L(+) and D(-) isomers of the lactic acid. A few strains, however, can produce D(-)LA under specific fermentation conditions. Fungal fermentation on the other hand leads to the exclusive production of L(+) isomers which is the isomer of choice since it is used for the manufacture of polylactic acid. Further, the nutritional requirement for fungal fermentation is comparatively simpler. The mycelial biomass produced at the end of the fermentation can be inexpensively separated from the fermentation broth.

Due to the stringent government regulations for environmental pollution control, there is an increasing concern for the development of suitable fermentation systems for lactic acid production that may be economical, energy efficient and ecofriendly. This has led to a spurt in the research activities in fermentation for the production of this valuable product. More and more industries are interested in developing and using a potent microbial source for lactic acid production and looking for the possibility of selecting the cheaper carbohydrate sources for making the production process economically viable and promising.

India, a country of high diversity, has a variety of natural substances that can be selected for economical production of lactic acid. The criteria for the selection of raw material is not only its cheaper availability but also it's suitability as a carbon substrate for attaining higher levels of production. A major part of Indian economy is dependent on agriculture. During the processing of cereals, grains and other agricultural based materials, various byproducts are generated. Some of these carbohydrate rich materials are broken rice, partially rotten potatoes and molasses etc. The broken and pest infested rice, although have the same nutritional value as that of the whole rice but are considerably cheaper, are not largely used for human consumption and lie unused in godowns. The partially rotten potatoes available in larger quantities from various local and regional vegetable markets, remain unutilized and are discarded as the waste. There are other cheaper, high starch containing substances like sago starch which are not used as a major food material and also the arrowroot that are mainly used for starching of the clothes. Many of these materials are either improperly utilized or go as a waste and contribute to environmental

pollution. These substances if put to proper use and converted into value added products like lactic acid, not only will earn a major share of foreign exchange but would also help in curbing environmental pollution. With this objective in mind the present study was undertaken to evaluate the cheaper carbohydrate sources for lactic acid production. The present study has the following defined outlines of the objectives:

(a) Isolation and screening of potential microbial strains from different sources for lactic acid production and analysis of the various parameters for production.

(b) Genetic manipulation of the isolated strain by physical and chemical mutagens for achieving the maximal improvement in the production ability.

(c) Evaluation of lactic acid production in submerged and solid-state fermentation conditions.

(d) Analysing the feasibility of cheaper carbohydrate substances mainly, the sugar cane molasses and the starch substances i.e. rice, potato, arrowroot and sago for lactic acid production.

(e) Evaluation of different matrices for semicontinuous production and analysis of the suitable approaches for the extraction and recovery of the lactic acid.

The study on the above defined outlines has been detailed in the different sections of this thesis.

Chapter 2

Literature Review

2.1 HISTORICAL BACKGROUND

Lactic acid was first isolated in 1780 by Swedish chemist, Carl Wilhelm Scheele (Goetz, 1994) and was first discovered as a bioconversion product by Blondeau in 1847. It was first produced commercially in 1881 by Charles E. Avery at Littleton, Massachusetts, USA (Vickroy, 1985). The venture was unsuccessful in its attempt to market calcium lactate as a substitute for cream of tartar in baking powder. The first successful use in leather and textile industry began in around 1894 (Garrett, 1930). The production level was close to 5000 kg/yr on a 100% basis (Inskeep *et al*, 1952). The presence of lactic acid bacteria in distilleries was observed in the 1860's and 1870's and their optimum growth temperature was investigated (Vickroy, 1985). A pure culture of *L. delbrueckii* was isolated from soured yeast in 1878 by Leichmann (Garrett, 1930).

Production of lactic acid has been initially attributed to bacteria (Ward *et al*, 1936), but Saito (1911) observed L-lactic acid production by *Rhizopus chinensis* when grown on glucose and in 1919, Ehrlich reported that certain *Rhizopus* species producing fumaric acid had also produced small quantities of D-lactic acid, succinic and L-malic acid. Takahashi and co-workers in 1926, found that certain species of *Rhizopus* fermented 38% of the glucose to L-lactic acid along with the varying quantities of fumaric, succinic and acetic acids. Takahashi & Asai (1933) found that smaller amounts of lactic acid was produced by four species of *Mucor* in addition to acetaldehyde, ethyl alcohol, pyruvic and succinic acids.

Lockwood *et al* (1936) reported that *Rhizopus oryzae* yielded about 43% of lactic acid when grown with D-glucose containing medium in surface culture condition. Snell & Lowery (1964) studied the fermentative production of lactic acid by *Rhizopus* species. They found that yield and fermentation rates of the *Rhizopus species* are comparable to those of *Lactobacillus*. This was elaborately described by them in a US patent (1964).

2.2 SYNTHESIS OF LACTIC ACID

Lactic acid can be obtained by two processes:

2.2.1 Chemical synthesis

2.2.2 Fermentation (bioconversion) process

2.2.1 Chemical Synthesis

Lactic acid production by synthetic route is based on the hydrolysis of lactonitrile by a strong acid such as HCl and leads to the production of a racemic mixture (Vickroy, 1985). Lactonitrile is obtained by base catalysed addition of hydrogen cyanide to acetaldehyde at atmospheric pressure. The crude lactic acid so obtained is esterified with methanol, the ester thus generated is purified by distillation and it's hydrolysis with water in presence of acid catalyst produces lactic acid. The lactic acid obtained is further concentrated and purified. Synthesis of lactic acid via chemical means can be brought about by other methods, such as, alkaline degradation of sugars; synthesis from carbon monoxide, acetaldehyde and water; hydrolysis of chloropropionic acid and by nitric acid oxidation of propylene (TIFAC, 2000). The

synthesis of lactic acid via chemical means results into a mixture of D and L – lactic acids (Ohara & Yahata, 1996) and is therefore disadvantageous.

2.2.2 Fermentation Process

All microorganisms require water, energy source, carbon, nitrogen, minerals and oxygen (if aerobic) for their growth and development. Appropriate combination of the nutrients, presence of the additional factors in the medium and selection of a suitable fermentation system may induce the microbial culture to produce the increasing levels of the desired product. Thus the medium formulation is one of the critical requirement for fermentation reactions.

2.2.2.1 Carbon Source

A wide variety of carbon sources have been used for lactic acid production. The choice of most suitable carbon source not only depends on its price and easy availability but also on its capability to yield high quality product with little or no byproduct formation.

The simplest form of carbon as glucose monohydrate crystals have been readily used for lactic acid production (Ward *et al,* 1936; Waksman *et al,* 1937; Hongo *et al,* 1986; Ishazaki *et al,* 1989; Rosenberg *et al,* 1992b; Soccol *et al,* 1994; Kristofikova *et al,* 1995; Dong *et al,* 1996; Yoo *et al,* 1997; Park *et al,* 1998; Xuemei *et al,* 1999; Krishnan *et al,* 2001; Mirdamadi *et al,* 2002; Martak *et al,* 2003; Bai *et al,* 2003 and Bulut *et al,* 2004). Several other carbohydrates like fructose, ribose, xylose, sucrose, galactose, mannose, cellobiose, maltose, etc have also been utilized for lactic acid production (Rosenberg *et al,* 1994; Yang *et al,* 1995; Monteagudo *et al,* 1997 and

Payot *et al,* 1999). Various substances like cellulose and newspaper (Abe & Takagi 1991), wood (Parajo *et al,* 1996; Woiciechowski *et al,* 1999 and Moldes *et al,* 2001), waste paper (Schmidt *et al,* 1997 and Park *et al,* 2004), corn cob (Melzoch *et al,* 1997; Luo *et al,* 1997; Sreenath *et al,* 2001; Ruenguglikit *et al,* 2003 and Miura *et al,* 2004), wheat straw, alfalfa fiber, soya fiber (Sreenath *et al,* 2001) have been utilized for lactic acid production.

Starch or starchy hydrolysates like barley (Linko *et al,* 1996), corn starch (Zhang & Cheryan, 1991 and Xiadong *et al,* 1997) rice and potato starch (Xiadong *et al,* 1997), wheat flour or wheat starch (Xiadong *et al,* 1997; Hofvendahl *et al,* 1998 and Akerberg *et al,* 2000), sago starch (Nolasco-Hipolito *et al,* 2002), have been used for lactic acid production by bacterial strains belonging to *Lactobacillus* and *Lactococcus* species. In the fungal strains, *Rhizopus* sp has been used for lactic acid production using corn starch (Rosenberg *et al,* 1994 and Yin *et al,* 1998), potato starch (Oda *et al,* 2002) and potato waste water (Huang *et al,* 2003) as the starchy substrates. Methanol and methylamine (Dinarieva *et al,* 1991) and date juice (Nancib *et al,* 2004) have also been used for lactate production.

2.2.2.2 Nitrogen, Phosphate and Trace Metals

Nitrogen sources mainly ammonium sulphate (Hang *et al,* 1989), peptone (Zayed *et al,* 1991), Urea (Yang *et al,* 1995), Yeast extract (Yan *et al,* 2001), ammonium nitrate (Bai *et al,* 2003) had been used for lactic acid production.

Ammonium salts appeared better than nitrates (Ward *et al,* 1936 and Waksman *et al,* 1937). Yeast extract has been used in many fermentation media for bacterial lactic acid production as it is rich in vitamin B, purine and pyrimidines (Amrane &

Pringent, 1994 and Demirci *et al*, 1998). Yeast extract either alone (Zhang & Cheryan, 1991) or in combination with casein or peptone (Parajo *et al*, 1996 and Danner *et al*, 1998) were utilized. A wide variety of other inexpensive nitrogenous sources like malt sprouts, grass extract, appeared suitable for lactic acid production (Hujanen *et al*, 1996).

Phosphate works towards maintaining the hydrogen ion concentration of protoplasm and surrounding fluids and participate in the synthesis of numerous intermediate enzymes, which are important for a variety of metabolic reactions and for other intracellular processes (Mehrotra & Tandon, 1970). Phosphate for lactic acid fermentation has been essentially used in the form of potassium dihydrogen phosphate (Kosakai *et al,* 1997 and Dominguez *et al,* 1999). Varying concentrations of the phosphate for preculture and fermentation medium were required (Kristofikova *et al*, 1991; Yin *et al*, 1997 and Yin *et al*, 1998).

The importance of zinc on growth, acid accumulation and LDH activity was emphasized by Pritchard (1973). He observed that zinc at lower concentrations induced acid production while at higher concentrations, appeared inhibitory. He had further shown that higher concentrations of zinc led to lower lactate dehydrogenase activity, faster glucose utilization and earlier sporulation. Savijoki *et al,* (1997) had observed that Ca^{2+} and Zn^{2+} at lower concentrations had stimulated the L-LDH activity by about two fold in *L. helveticus*. Other ions mainly Cd^{2+}, CO^{2+} and Cu^{2+} (0.1 – 1 mM) had mildly inhibited the activity, whereas, Mg^{2+} and Mn^{2+} had no effect on the enzymatic activity. Bhowmik *et al,* (1993) had reported that Co^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} had inhibitory effect on D-LDH activity in *Lactobacillus helveticus* and Hg^{2+} had effected into maximum inhibition.

2.2.2.3 pH Value

Lactic acid is a strong inhibitor of its own production. So various buffering agents like sodium hydroxide (Friedman & Garden, 1970), ammonium hydroxide (Steiber & Gerhardt, 1995) and $CaCO_3$ (Dominguez *et al,* 1999) are added to maintain the pH of the production process. Schaub (1958) had observed the optimum pH for lactate production was between 5.2 – 5.8. Lopez in 1976 had studied the effect of various additives on lactate production and concluded that its productivity was mainly affected by the buffering ability of the broth. Yu & Hang (1989) had found that the rates of carbon consumption and L(+) lactic acid production were remarkably greater in presence of $CaCO_3$. Soccol *et al,* (1994) had studied the relationships among oxygen consumption, calcium carbonate utilization and lactate production and concluded that oxygen supply together with calcium carbonate both were required for the lactate production. Akerberg *et al,* (1998) had observed that formation of D-lactic acid was higher than its other isomer at pH 4-5. Morphological changes in *Rhizopus oryzae* mycelia were observed by varying the pH of medium by controlled addition of $CaCO_3$ (Du *et al,* 1998). Bai *et al,* (2003) had shown that pH of broth not only affected the fungal morphology and biomass formation but also the lactate production.

2.2.2.4 Temperature

The temperature desired for lactic acid fermentation depends on the type of microorganism (Patel *et al* 1997). Based on the temperature optima, two major groups of lactic acid bacteria have been categorised; mesophilic, growing at in the temperature range of 28 – 45°C and the thermophilic in the range of 45 – 62°C (Rehm & Reed, 1996). Hujanen *et al,* (1996) had analysed the effect of temperature on lactate

production by *L. casei* and *L. casei* sub sp. *rhamnosus* and observed 37°C as the suitable temperature for lactic acid production by both the strains. Akerberg *et al,* (1998) observed that optimum temperature for lactic acid production by *Lactococcus lactis* ssp *lactis* was between 30° – 35°C.

Varying range of temperature (30-35°C) for lactic acid production by *Rhizopus* sp was observed (Kristofikova *et al*, 1995; Park *et al*, 1998 and Dominguez *et al*, 1999). A maximum level of 21 gdm^{-3} of lactic acid was obtained by *Rhizopus arrhizus* at 30°C (Huang *et al*, 2003) but biomass accumulation continued beyond this temperature.

2.2.2.5 Aeration and Agitation

Bacterial production of lactic acid by *Lactobacillus* strains, mainly by *L. delbrueckii* and *L. casei* require anaerobic conditions (Honda *et al*, 1995 and Gonzalez-vara *et al*, 1996). However, the fungal production by *Rhizopus* sp require normal aeration (Yin *et al*, 1998). A combination of conditions involving higher aeration and higher spore concentration appear unsuitable for production but leads to higher biomass accumulation and visa-versa (Dominguez *et al,* 1999). Similar results were obtained for lactic acid production by *Bacillus coagulans* (Payot *et al,* 1999). The fungal morphology has also been shown to affect the lactate production (Kosakai *et al,* 1997). Increase in agitation led to decrease in the size of fungal pellet but number of pellets increased; while the reverse observation was made with increasing aeration rate (Dominguez *et al*, 1999). Similar observations were also reported by Bai *et al*, (2003) where increase in agitation speed to 300 rpm resulted in the decline of pellet size from 3.9 to 1.4 mm and led to an increase in L-lactic acid production. This

morphological variation was possibly because that increase in agitation rate may lead to the higher shear stress causing the mycelia to grow in smaller pellets. Agitation leading to higher dissolved oxygen resulted into increased biomass.

2.3 LACTIC ACID PRODUCTION TECHNOLOGY

2.3.1 Microorganism

Lactic acid production can be achieved by selecting the suitable strain of bacteria or fungi (Martak *et al*, 2003). Selection of a potent strain is a prerequisite for fermentative production of lactic acid.

The diverse group of bacterial strains producing lactic acid include *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Vagococcus, Clostridium* and *Weissella sp* (Huang *et al*, 2003; Wu *et al*, 2004 and Narayanan *et al*, 2004). Among these strains *Lactobacillus delbrueckii, Lactobacillus helveticus* and *Lactobacillus casei* are considered as the most effective strains for lactic acid production (Amrane *et al*, 1996; Demirci *et al*, 1998 and Hofvendahl *et al*, 2000).

Among the fungal strains, *Rhizopus oryzae, Rhizopus arrhizus, Rhizopus stolonifer* have been observed to have the lactic acid production (Yu *et al*, 1989; Soccol *et al*, 1994; Rosenberg *et al*, 1995; Park *et al*, 1998; Zhou *et al*, 1999; Oda *et al*, 2003; Bai *et al*, 2003 and Miura *et al*, 2004) ability of producing optically pure L(+) lactic acid which is used for the production of polylactic acid (PLA) a new biodegradable material.

Metabolic engineering (McKay, 1990 and Gasson, 1993) and strain improvement by mutation and selection have been used to alter the properties of an

organism. Mutation using physical and chemical agents have been used for enhancing the productivity of fungal metabolites and the enzymes (Grigorov *et al*, 1983; Rowlands, 1983; Tani *et al*, 1988; Jiang *et al*, 1991; Suntornsuk *et al*, 1994; Yang *et al*, 1994; Longacre *et al*, 1997 and Yu *et al*, 2000). Suntornsuk *et al*, (1994) after mutagenesis of the *R. oryzae* NRRL 395 had isolated mutants 1N1, 3N4 and 3N6 that had 57% higher levels of L(+) lactic acid production than the parent strain. Longacre *et al*, (1997) following mutagenesis of *Rhizopus oryzae* had isolated a mutant having higher lactate and lower ethanol yielding ability than the parental strain. Skory *et al*, (1998) had developed a mutant of *Rhizopus oryzae* NRRL 395 having only 5% of the wild type alcohol dehydrogenase activity in limiting oxygen conditions but had ten fold higher lactic acid levels than the parental strain. Bai *et al*, (2004) had studied the metabolic flux of mutant of *R. oryzae* to analyse the changes in the flux profile *in vivo* to engineer an improved strain. Miura *et al*, (2004) had isolated an ammonia tolerant mutant of *Rhizopus* sp which as compared to the parent strain had two fold higher levels of L-lactic acid production in half of the fermentation time under identical culture conditions.

2.3.2 Inoculum Development

Preparation of an appropriate inoculum is essentially needed for a fermentation reaction (Wang *et al*, 1975). Inoculum can either be a spore suspension (Hang *et al*, 1989; Suntornsuk *et al*, 1994 and Hamamci *et al*, 1994) or a vegetative mycelia (Kristofikova *et al*, 1991 and Yin *et al*, 1997). Size of inoculum affects the production as variations in inoculum size resulted into differential levels of production (Soccol *et al*, 1994). Variations of inoculum from 1×10^7 to 3×10^7 spores/ml did not affect the

production (Yu & Hang, 1989). However inoculum size increased from 10^3 to 10^7 spores/ml had affected the fungal morphology and lactic acid production. An inoculum containing 10^6 spores/ml had resulted into pellet shaped structures of fungal biomass and had led to increased levels of lactic acid production (Bai *et al*, 2003).

2.3.3 Raw Materials

Lactic acid production can be achieved from a diverse group of substrates. Pure sugars when used for production prove expensive and the process appears commercially unviable hence, agro-industrial substances which may either be a waste or are the byproducts, may commonly be used (Hofvendahl *et al*, 2000). Whey, a byproduct of dairy industry, which is rich in proteins, salts and lactose is mostly used for fermentative production of lactic acid by lactic acid bacteria (Aeschlimann *et al*, 1991; Amrane *et al*, 1994 and Arasaratnam *et al*, 1996). Byproducts of sugar industry such as molasses have also been used for fermentative production of lactic acid (Aksu *et al*, 1986; Montelongo *et al*, 1993 and Goksungur *et al*, 1997). Starch containing substances such as barley (Yu *et al*, 1989 and Linko *et al*, 1996), cassava (Shamala *et al*, 1988; Yu *et al*, 1989 and Guyot *et al*, 2003), corn (Cheng *et al*, 1991 and Yin *et al*, 1998), sorgum (Richter *et al*, 1994), wheat (Xiadong *et al*, 1997 and Akerberg *et al*, 2000) and potato (Chatterjee *et al*, 1997; Oda *et al*, 2002 and Huang *et al*, 2003) have been used for lactic acid production both by lactic acid bacteria and the fungus of the genus *Rhizopus*.

Starch either alone (Hofvendahl *et al*, 1997 and Akerberg *et al*, 1998) or supplemented with different nutrients like yeast extract (Hofvendahl *et al*, 1997) and ammonium sulphate (Yin *et al*, 1998) have been used. The starchy substances either in

crude (Yu *et al*, 1989; Oda *et al*, 2002 and Huang *et al*, 2003) or in hydrolyzed form (Cheng *et al*, 1991; Linko *et al*, 1996 and Yan *et al*, 2001) were utilized. During the simultaneous saccharification and fermentation of the whole wheat flour by *Lactococcus lactis*, the nutrient limitation was overcome by the protease treatment of the wheat flour (Hofvendahl, 1998). Lignocellulosic substrates like wood (Parajo *et al*, 1996 and Moldes *et al*, 2001), wastepaper (Schmidt *et al*, 1997 and Park *et al*, 2004), corncob (Ruengruglikit *et al*, 2003) have been attempted for bioconversion into lactic acid.

2.3.4 Fermentation Process

Lactic acid is fermentatively produced by submerged (Kristofikova *et al*, 1991; Suntornsuk *et al*, 1994; Soccol *et al*, 1994; Kristofikova *et al*, 1995; Yin *et al*, 1997, Akerberg *et al*, 1998; Dominguez *et al*, 1999 and Huang *et al*, 2003) or by solid state cultivation (Soccol *et al*, 1994 and Xavier *et al*, 1994). Surface fermentative lactic acid production had been reported only in the earlier part of last century (Ward *et al* 1936 and Waksman *et al*, 1937).

2.3.4.1 Submerged Fermentation Process

This process is one of the widely studied processes for lactic acid production (Yu & Hang, 1989; Cheng *et al*, 1991; Rosenberg *et al*, 1992b; Suntornsuk *et al*, 1994; Soccol *et al*, 1994; Kristofikova *et al*, 1995; Parajo *et al*, 1996; Dong *et al*, 1996; Kosakai *et al*, 1997; Yan *et al*, 2001 and Huang *et al*, 2003).

Lhomme *et al*, (1992) found that *R. arrhizus* had followed the anaerobic metabolism for lactic acid synthesis. These conditions were not due to the result of

insufficient oxygen supply but possibly because of the lower rate of oxygen exchange between the culture medium and the cells, resulting due to pellet formation having a compact morphology.

Yang *et al,* (1995) observed that *Rhizopous oryzae* was self immobilized as pellets under submerged condition with xylose as a carbon source. These pellets help in enhancing the mass transfer. Moreover, the pellets can be repeatedly used for lactic acid production by transferring to a fresh medium. Thus the advantage of submerged fermentation is that it may lead into developing a continuous process (Ro & Kim, 1991). The influence of spore concentration on mycelial morphology and L(+) lactic acid production under submerged condition was studied by Yin *et al,* (1998). He had observed the changing mycelial morphology with varying levels of inoculum and found that pellet form of the mycelial morphology produced the higher levels of lactic acid.

2.3.4.2 Solid State Fermentation

Solid state fermentation (SSF) is defined as the fermentation using solid based matrices in absence (or near absence) of free water, however the matrix must possess enough moisture to support the growth and metabolism of microorganism (Durand, 1998; Mitchell *et al*, 2000 and Pandey, 2001). The selection of a suitable matrix is a key factor for SSF (Nagel *et al*, 1999; Hoogschagen *et al*, 2001 and Pandey, 2003). The matrix should have ability to withstand compression or gentle stirring and should be able to be divided into smaller fibrous or granular particles, that should not break or stick to one another, should be porous and have optimal levels of nutrients (Raimbault, 1998).

Studies on lactic acid production by lactic acid bacteria or by filamentous fungi

(such as *Rhizopus*) have mainly been performed under submerged condition as described earlier, but Soccol *et al*, (1994) had reported that higher rate of glucose utilization by *Rhizopus oryzae* was observed during solid state cultivation. Xavier *et al*, (1994) had obtained higher levels of lactic acid production by *Lactobacillus casei* ssp *casei* CFTRI 2002 during SSF using sugarcane press mud as the substrate.

2.3.4.3 Immobilized Cell Fermentation Process

Immobilization of cells is one of the preferred approaches for achieving higher and economical levels of the production. Higher productivity, operational stability and decreased contamination of product are some of the advantages that immobilized systems offer. It has been observed that use of filamentous organism for immobilization generally leads to decrease in medium viscosity. This condition enables enhanced nutrient and oxygen transfer, helping therefore into development of a suitable system for repeated batch and continuous production (Webb *et al*, 1986; Webb, 1989; Vassilev *et al*, 1992 and Federici, 1993). Lactic acid production has been attempted by immobilizing the culture cells in a variety of matrices like agar (Tuli *et al*, 1985 and Roukas *et al*, 1991), alginate beads (Hang *et al*, 1989; Roukas *et al*, 1991; Norton *et al*, 1994; Yoo *et al*, 1996; Roukas *et al*, 1998; Xuemei *et al*, 1999 and Yan *et al*, 2001), K-carrageenan (Audet *et al*, 1989 and Roukas *et al*, 1991), polyacrylamide (Roukas *et al*, 1991), ceramic particles, beads of sintered glass (Goncalves *et al*, 1992), polyurethane foam (Sun *et al*, 1999), polypropylene matrix (Krishnan *et al*, 2001) and loofa sponge (Roble *et al*, 2003).

Amongst the various approaches for cell immobilization for lactic acid production, calcium alginate based immobilization has been widely studied both for

lactic acid bacteria (Boyaval *et al*, 1988; Guoqiang *et al*, 1991; Champagne *et al*, 1992 and Wang *et al*, 1995) and for *Rhizopus* (Hang *et al*, 1989; Hamamci & Ryu 1994 and Xuemei *et al*, 1999). Entrapment using calcium alginate oftenly suffers limitations like insufficient oxygen supply due to the diffusional resistance of the gel matrices. This may lead to the decreased fermentation efficiency (Sun & Furusaki, 1998).

Search for alternative carrier for immobilization had shown the loofa sponge (*Luffa cylindrica*) as a suitable matrix for immobilization (Iqbal & Zafar, 1994) of microorganisms, i.e., *Candida brassicae* (Ogbonna *et al*, 1994; Ogbonna *et al*, 1996 and Ogbonna *et al*, 1997), *Aspergillus niger* (Slokoska *et al*, 1998) and *Lactococcus lactis* sp *lactis* (Roble *et al*, 2003). Natural biostructure of *Luffa cylindrica* with an open network of fibrous support which provides it with strength and enables the instant contact of immobilized cells to the surrounding aqueous medium is an remarkably advantageous feature for this immobilization matrix.

2.3.5 Product Recovery

Lactic acid fermentation is a typical example where product accumulation causes inhibition of bioconversion reaction (Ohara *et al*, 1993 and Kwon *et al*, 1996). There are recovery approaches that help in separation of the product from the culture broth and allow the fermentation reaction to continue further. Classical methods are the precipitation of calcium lactate using calcium hydroxide. Precipitate is recovered by filtration and converted to lactic acid by addition of sulfuric acid. The dilute acid product is then sequentially purified using activated carbon, evaporated and crystallized (Planas *et al*, 1999). This separation and purification process accounts for up to 50% of the production cost (Chaudhuri & Pyle, 1992 and Eval & Bressler,

1993). This approach not only is expensive but also environmentally unfavourable as it adds to a large amount of $CaSO_4$ sludge as solid waste (Shreve & Brink, 1977).

Apart from the above classical approach, various other methods have been studied which are mainly the liquid surfactant membrane extraction (Sirman *et al*, 1991), direct distillation (Cockrem & Johnson, 1991), chromatographic methods (Hauer & Marr, 1994), ultrafiltration (Hauer & Marr, 1994), membrane bioreactor (Juang & Huang, 1997 and Tong *et al*, 1998), electrodialysis (Vonktaveesuk *et al*, 1994; Siebold *et al*, 1995; Lee *et al*, 1998; Bailly, 2002 and Choi *et al*, 2002), solvent extraction (Tamada *et al*, 1990a, b; Yabannavar & Wang, 1991; Siebold *et al*, 1995; Tik *et al*, 2001; Wasewar *et al*, 2002, Pai *et al*, 2002 and Matsumoto *et al*, 2003), adsorption (Kaufman *et al*, 1994 and Sosa *et al*, 2001), reverse osmosis (Timmer *et al*, 1994). Liquid – liquid extraction has the advantage that lactic acid can be removed easily from the fermentation broth, preventing the lowering of pH. Also the lactic acid can be re-extracted and the extractant recycled to the fermentation broth (Wasewar *et al* 2002).

The liquid-liquid extraction consists of two separate phases where organic acid is transferred from one phase to another based on the solubility difference between the phases. The efficiency of transfer can be improved by a carrier compound which is usually a secondary or a tertiary amine soluble or diluted in an organic solvent. The carrier reacts with lactic acid called carrier-facilitated transport or reactive extraction, which is kept in the organic phase until it encounters a stripping solution such as sodium carbonate solution (Demirci *et al*, 1999). The reactive extraction is particularly attractive due to the possibility of high selectivity for desired compounds (Scheler *et al*, 1996).

Among the secondary and tertiary amines for extraction tertiary amines (Tung *et al*, 1994; Ye *et al*, 1996; Juang & Huang, 1997 and Frieling *et al*, 1999) are preferred. The secondary amines suffer the drawback of gel formation which interferes with phase separation.

The extractability of tertiary amines increases with chain length of amine in polar diluents, whereas in nonpolar diluent such as n-heptane, the extractability of tertiary amines decreases with chain length. The efficiency of stripping seems to increase when a combination of polar and nonpolar diluents have been used (Han & Hong, 1996; Chaudhury *et al*, 1998 and Hong & Hong, 1999). Matsumoto *et al*, (2003) performed the synergistic extraction of lactic acid with tributylphosphate and alkylamines to define an effective synergistic extraction system. Wasewar *et al*, (2003) had studied the kinetics of extraction of lactic acid from aqueous solutions using alamine 336 in methyl isobutyl ketone.

2.4 COMMERCIAL PROCEDURE FOR LACTIC ACID PRODUCTION

A successful plant for lactic acid production was initially established by Boehringer Ingelheim, Germany in 1895 (Buchta, 1983). The production of lactic acid can be accomplished from a variety of carbohydrates. Apart from the availability of raw materials at cheaper prices, the other criteria which are taken into consideration are achieving the faster fermentation rate with higher yield of lactic acid and with negligible byproduct formation, ability of the substrate to be fermented with little or no pretreatment and year around availability of the substrate (Vickroy, 1985 and Rehm & Reed, 1996).

The major processes for manufacturing of lactic acid utilize different

substrates. Whey in concentrated form has been used without any pretreatment by Sheffield Product Company, Norwich, NY, USA (Vickroy, 1985). American Maize Products Company had utilized an important byproduct of its corn wet milling process for lactic acid production. This by-product was the dextrose which formed pills during the dextrose drying operation. The company produces approximately 160,000 pounds of lactic acid per month (TIFAC, 2000). Bowmans Chemicals Limited is probably first all glass continuous solvent extraction and purification plant for lactic acid which is in operation at Bowmans Chemicals Ltd., Moss Bank Works, Widnes. The plant has a capacity of producing 2400 tonnes of pure acid per year (TIFAC, 2000).

Ecological Chemical Products Co. has a commercial plant in Afdell, Wisconsin, that produces high purity lactic acid used for the food and pharmaceutical industry. Using the cheese whey permeate, the company produces 20 million pounds of lactic acid annually (TIFAC, 2000).

2.5 MANUFACTURING UNITS OF LACTIC ACID

2.5.1 Indian Manufacturers (TIFAC, 2000)

(i) Lactochem Ltd.,
Chengai, MGR District

(ii) Munirabad Chemical Company,
Raichur, Karnataka

(iii) Sangita Bio-Chem Ltd.
Balasore, Orissa

(iv) Prathista Industries Ltd.
Temple Alwal, Secunderabad, AP

2.5.2 Global Manufacturers (TIFAC, 2000)

(i) Cargill Inc.,
Minneapolis USA

(ii) Purac America,
USA

(iii) Galactic SA,
Belgium

(iv) Musashino Chemical Laboratory Ltd.,
Japan

2.6 PATENTS FOR LACTIC ACID

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8. Tsai *et al*, US Patent, 1995, US P 5,464,760.
9. Carlson *et al*, US Patent, 2002, US P 6,475,759.
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2.7 PROPOSED DEMAND FOR LACTIC ACID BY 2005 AD IN INDIA (TIFAC, 2000)

S. No.	Sector	Projected demand (in tonnes)
1.	IV Fluid	690
2.	Derivatives of lactic acid/pharmaceuticals	315
3.	Biscuit Sector	55
4.	Beer Sector	45
5.	Others	90
	Total	1195

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 Chemicals

The various chemicals and biochemicals used for the study were of highest purity and quality available from the national and international manufacturers. Lactic acid, α-amylase, glucoamylase were obtained from M/s Fluka Chemie GmbH, Switzerland; N-methyl-N′-nitro-N-nitrosoguanidine (NTG) and colchicine were obtained from M/s Sigma-Aldrich, USA; Tri-n-octylamine, 1-octanol, methyl isobutyl ketone and other solvents were procured from M/s Spectrochem India Ltd, rest of the other chemicals were obtained from M/s E Merck, Germany and from M/s Sisco Research Laboratories, India.

The various culture media components were obtained from M/s Hi Media, India. Sago starch (*Metroxylon sago*) and arrowroot (*Maranta arundinacea*) were procured from local market. Broken rice (*Oryza sativa*) was obtained from a local rice mill, M/s Goyal Rice Mill, Bahadarabad, Haridwar (Uttaranchal) and the pest infested rice was obtained from various godowns of local market. Partially rotten potatoes (*Solanum tuberosum*) were procured from local Mandi (wholesale vegetable market) and loofa sponge (*Luffa cylindrica*) used for immobilization was procured from the Haridwar city (Uttaranchal) market. Sugarcane molasses were obtained from M/s Mahalakshmi Sugar Mills, Iqbalpur, Haridwar (Uttaranchal), India.

3.1.2 Microorganisms

Rhizopus oryzae RB-5, the parental strain used for the study was isolated from garden soil, while the mutagenized strain RBU2-10 was developed in the laboratory by the two stage UV-irradiation of the wild type strain.

3.2 METHODS

3.2.1 Selection of Strain

3.2.1.1 Source of microbial strain

Following samples from local and adjoining areas were collected for obtaining the desired type of microbial strain.

3.2.1.1.1 Soil samples

A thorough survey of forest and gardens of the adjoining areas in and around Roorkee of Uttaranchal State (Laksar, Bahadarabad, Haridwar, Dehradun, Rishikesh, Behat) and Saharanpur, Uttar Pradesh was carried out. Several soil samples from the survey area were collected for analysis and selection of strains.

3.2.1.1.2 Partially decomposed fruits and vegetables

Partially decomposed fruits like apple, mango, grapes and various other types of fruits and the decomposing vegetable materials like potato, tomato, pumpkin were collected in sterilized polythene bags from the local market of Roorkee and nearby places.

3.2.1.1.3 Bakery products

The bakery products like decomposing bread, bun, cake pieces were collected from the rejected material of the bakeries of various places in sterilized polythene bags for isolation of the microbial strain.

3.2.1.2 Method of isolation

The suspension of all the three samples i.e. garden and forest soil, decomposing fruits and vegetable materials were made in 0.85 % saline. The sample suspension were then centrifuged at 5000 g for 20 min. The clear supernatant thus obtained was subjected to serial dilutions and then plated on Potato Dextrose Agar (PDA) medium. The plates were then incubated for 5-7 days at 35°C. The various distinct colonies obtained were picked up and restreaked on PDA plates to pick up the respective single colony. The isolated pure cultures were stored at 4°C in PDA slants for further study.

3.2.1.2 Screening of Lactate Producing Strains

3.2.1.2.1 By Acid Unitage values

The various isolated cultures were subjected to primary screening for lactic acid production by determining the acid unitage values. Briefly, a loopful of the inoculum of the various strains was added to sterile double distilled water to a ten fold dilution. Single spore was then inoculated into petriplates containing mineral agar acid indicator medium as described (Suntornsuk *et al,* 1994) with slight modifications. The medium used was as described by Park *et al,* (1998) and contained (g/l) : glucose, 120; $(NH_4)_2SO_4$, 3.02; $MgSO_4.7H_2O$, 0.25; $ZnSO_4.7H_2O$, 0.04; KH_2PO_4, 0.15; agar, 20; bromocresol green, 0.2 and Triton X-100 1.5 ml/l in distilled water (pH 5.5). The mineral acid indicator medium plates inoculated with single spore were incubated at 35°C for 48-72 hrs. After 72 hrs, the acid unitage (AU) value of the colonies were determined by dividing the diameter of the yellow zone (lactic acid production) by the diameter of the zone of the colonies. The colonies having notable acid unitage values were picked up and stored at 4°C in PDA slants for further study.

3.2.1.2.1.2 Qualitative analysis of lactic acid

Following preliminary screening, lactic acid production from the isolated strains was analysed according to the method of Smith (1958). Briefly, a suitable aliquot of the fermentation broth were spotted on Whatman No.4 paper and developed in a solvent system using ultra pure lactic acid as standard. After drying, the spots were developed for analysis.

3.2.1.2.1.3 Fermentation studies

The final step in screening for potent lactic acid producing strain was carried out by fermentation studies. A loopful of the inoculum containing 10^7 spores/ml was inoculated into 25 ml of fermentation medium (Park *et al*, 1998) in 250 ml Erlenmeyer flask. The medium contained (g/l): glucose, 120; $(NH_4)_2 SO_4$, 3.02; Mg SO_4. $7H_2O$, 0.25; $ZnSO_4.7H_2O$, 0.04; KH_2PO_4, 0.15.

Flasks were incubated at 35°C in an orbital shaker (Sanyo Gallenkemp, UK) at 150 rpm for 96 hrs. The pH of the medium was maintained by adding 1 gm of $CaCO_3$ to each flask after 24 hrs of inoculation. The levels of the lactic acid produced, glucose consumed and dry cell weight of the mycelial mass produced was estimated at regular periodical intervals. The strain showing maximum levels of lactic acid production was selected and maintained at 4°C in PDA slant for further study.

3.2.2 Identification of Selected Strain

Culture showing maximal levels of lactic acid production was subjected to morphological, microbiological studies according to Alexopoulas *et al*, (1979) and appeared to belong to genus *Rhizopus*. The culture was further identified as *Rhizopus*

oryzae at Indian Agricultural Research Institute (IARI), New Delhi and denoted as *Rhizopus oryzae* strain RB-5.

3.2.3 Mutagenesis and Screening

Improvement in the lactic acid producing ability of parental strain, *Rhizopus oryzae* RB-5 was attempted by physical and chemical mutagenesis of the parental strain.

3.2.3.1 Physical Mutagenesis

Mutagenesis of the parental strain RB-5 was done by ultra violet (UV) exposure according to Petruccioli *et al*, (1995). Spore suspensions (10^7 spores/ml) were prepared in 50 mM potassium phosphate buffer (pH 7.0). Three milliliters of this suspension in sterilized petriplates were exposed to UV light (2.48 W m^{-2}, distance 0.70 m) for different time periods ranging from 2 to 20 minutes. Following UV treatment, the petriplates with spores were stored in dark at 4°C for 24 hours to avoid photo repair. The treated spores along with control suspension (untreated) were then screened initially by plating aliquots of spore suspension on PDA and incubated at 35°C for 7 days in order to have 1-2 colonies per plate. The surviving spores were then screened for detecting their acid production ability by culturing on mineral salt agar acid indicator medium for 72 hours at 35°C.

3.2.3.1.1 Second Stage UV Irradiation

The mutagenized strain with maximum lactic acid production ability following the first stage of UV-irradiation was subsequently treated with UV irradiation using the similar method as described earlier. The PDA plates were seeded with treated and

control spore suspension and incubated at 35°C for 7 days so as to have 1-2 colonies per plate. The surviving spores were then screened for detecting their acid production ability by incubating on mineral salt agar acid indicator medium for 72 hours at 35°C.

3.2.3.2 Chemical Mutagenesis

3.2.3.2.1 Colchicine Treatment

The mutagenesis of RB-5 using colchicine was performed according to the method of Claudimara *et al,* (1996). Spore suspension (10^7 spores/ml) was prepared in double distilled water and mutagenesised by treating with colchicine at varying concentrations (0.01 – 2.0 µg/ml) for 24 hours.

Spores were then washed twice with double distilled water, spread on PDA and incubated at 35°C in order to have 1-2 colonies per plate. The surviving spores were then spotted on mineral acid agar indicator medium and incubated at 35°C for 72 hours. A set of control spore suspension was also treated similarly excluding the colchicine treatment.

3.2.3.2.2 N-methyl-N'-nitro-N-nitrosoguanidine (NTG) Treatment

The NTG treatment was performed as described (Kuhad and Singh, 1994). Briefly, spore suspension having 10^7 spores/ml of RB-5 was added with NTG (100 µg/ml) in saline for 10-60 minutes. The spores following treatment were washed twice and seeded on PDA plates and incubated at 35°C for 7 days in order to have 1-2 colonies per plate. The surviving spores were then incubated on mineral salt agar acid indicator medium at 35°C for 72 hours for detecting their acid production ability. The untreated spore suspension were also identically cultured.

3.2.3.2.3 Screening of Mutants

The survival rate of the spores after every treatment was determined. The surviving spores were picked up from PDA plates and plated on mineral salt acid indicator medium. The acid unitage value was determined as described earlier.

The colonies showing higher AU values were maintained on PDA slants at 4°C for analysis of lactic acid production under submerged fermentation. The fermentation medium (25 ml) as described previously was inoculated with 10^7 spores / ml of the selected mutants in 250 ml of Erlenmeyer flask. The flasks were incubated at 35°C, 150 rpm for 96 hrs and levels of lactic acid produced were measured.

3.2.4 Analysis of Factors Affecting Lactic Acid Production

The effects of various physical, chemical and biological factors affecting lactic acid production by *Rhizopus oryzae* RBU2-10 was studied in parallel with the wild type strain *Rhizopus oryzae* RB-5. The submerged fermentation was performed initially by using the medium and conditions according to Park *et al*, 1998 as described earlier.

The effect of temperature (20°C – 40°C), pH (3.5 – 7.5), agitation (90 rpm – 170 rpm) and fermentation time were analysed. Besides, production levels at varying spore concentrations (10^4 – 10^8 spores/ml) and the inoculum level (1% - 5%) were also determined. Various sugars such as glucose, fructose, sucrose, lactose, galactose, mannose and xylose were used as carbon source to identify the suitable carbon source for lactate production.

Among other nutrients, various sources of nitrogen mainly sodium nitrate,

ammonium chloride, ammonium sulphate, peptone and urea and other nutrient supplements, mainly PO_4^{3-}, Mg^{2+} and Zn^{2+} were analysed for determining their concentrations needed for achieving maximum production. The suitable concentration of calcium carbonate which is an important buffering agent for the fermentation process was also studied. These analyses thus have enabled to define the most suitable conditions and the medium constituents for achieving maximal levels of production. The suitable concentration of a nutrient as detected at one stage was used for detecting the required concentration of other nutrient during subsequent stages.

3.2.5 Lactic Acid Fermentation by Free Cells of *Rhizopus oryzae*

Production of lactic acid using free cells of *Rhizopus oryzae* RBU2-10 and RB-5 was determined using submerged and solid state fermentation conditions.

3.2.5.1 Submerged Fermentation

The submerged fermentation was performed under the derived medium and the conditions.

3.2.5.1.1 Inoculum Preparation

The mutagenized RBU2-10 and the wild type strain RB-5 were subcultured from the slants on PDA plates at 30°C for 7 days. After 7 days a loopful of the inoculum was transferred in the sterilized double distilled water and a uniform suspension was prepared. Then 1% (v/v) inoculum containing 1.0 X 10^6 spores/ml was used as inoculum for the bioconversion process.

3.2.5.1.2 Submerged fermentation process

Bioconversion studies under submerged condition were carried out in 50 ml of

defined fermentation medium in 500 ml Erlenmeyer flasks. The medium as derived from the earlier studies was used for fermentation. The medium was inoculated with 1% (v/v) inoculum having 1X 10^6 spores/ml of the mutant and the wild type strains and incubated in an orbital shaker (Sanyo Gallenkemp, UK) with constant shaking 150 rpm at 30°C for 96 hours. 4% $CaCO_3$ was added after 24 hrs of fermentation to maintain the pH to 5.5. After completion of fermentation, the medium was filtered for separation of biomass, filtrate was centrifuged (5000 g, 15 min.) and the clear supernatant obtained was subjected to analysis.

3.2.5.2 Solid State Fermentation

The solid state fermentation was carried out according to the method of Soccol *et al*, (1994).

3.2.5.2.1 Preparation of Support

Three matrices, i.e. bagasse, groundnut shell and coconut coir fibre were used as the solid support for fermentation process. These materials were chopped into small pieces of about 0.8 – 2 mm. The finely chopped pieces of bagasse, groundnut shell and coir fibre were dipped in 2N HCl overnight followed by thorough washing with distilled water so as to remove traces of acid. The solid supports were dry sterilized at 110°C for 24 hours (Soccol *et al*, 1994).

3.2.5.2.2 Preparation of Inoculum

The parental strain *Rhizopus oryzae* RB-5 and the highest lactate producing selected mutant *Rhizopus oryzae* RBU2-10 were subcultured on PDA plates and incubated for 7 days at 30°C. After 7 days a loopful of the inoculum was transferred

into double distilled water and serially diluted. Preculture medium (20 ml) containing (g/l) : glucose, 60; $(NH_4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.25; KH_2PO_4, 0.15; $ZnSO_4.7H_2O$, 0.05 at pH 5.5 in 250 ml Erlenmeyer flask was inoculated with spore suspension (10^6 spores/ml) and the flask was incubated under shaking (50 rpm) for 24 hrs at 30°C.

3.2.5.2.3 Solid State Fermentation Process

The fermentation medium (40 ml) as derived from earlier studies contained (g/l) : glucose, 120; $(NH_4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.25; KH_2PO_4, 0.15; $ZnSO_4.7H_2O$, 0.050 was added with 10 ml inoculum, 5 gm of solid support and 5 gm $CaCO_3$ was then added into the inoculated medium in a 500 ml Erlenmeyer flask. The $CaCO_3$ was sterilized separately before use. The mixture was mixed well with a sterilized glass rod to obtain a homogeneous mixture. The flasks were inoculated at 30°C for 5 days under stationary conditions. The flasks were periodically aerated for circulation of air. The fermented material of each flask was collected, squeezed to collect the concentrated liquid. The samples thus collected were centrifuged (5000 g, 15 min) and the supernatant thus obtained was used for further analysis.

3.2.6 Processing of Crude Substrate for Lactic Acid Production

Different naturally available cheaper carbohydrates were selected to evaluate their suitability as substrates for lactic acid production.

3.2.6.1 Processing of Sugarcane Molasses

Sugarcane molasses is cheaper and widely available throughout the country as a byproduct of sugarcane industry. Molasses though contains 50 % sugars, but suffers from certain drawbacks like higher concentrations of heavy metals which are

detrimental for the bioconversion reaction Thus molasses was subjected to pretreatment for removal of heavy metals before its use for fermentation reaction.

The crude molasses following dilution (4-5 times) was passed through a bed of activated charcoal for decolourization. The decolourized molasses was then treated with hexacyanoferrate (0.125 %) at pH 4.0 – 4.5 followed by heating at 90°C for 15 minutes. The resulting precipitate containing heavy metals was removed by filtration. The filtrate thus obtained was acidified with 1N HCl to a pH of 1.0 – 1.5 and heated at 80°C for 1 hour for converting the sugar sacchrides into simple sugar. Treated molasses was then added to fermentation medium to a final concentration equivalent to 12% of reducing sugar. The pH of the medium was set to 5.5.

3.2.6.2 Processing of Starch Based Substrates

This region being the rice belt of the country has a number of rice mills. During processing of rice about 10-15% of the total rice are generated as broken/small grain rice that has almost no commercial value and is generally used as animal feed and available at considerably lower prices. Similarly, around 10% of the rice during storage in the godowns become pest infested and are wasted. Rotten potatoes are available free of cost from the numerous regional vegetable markets, whereas sago and arrowroot starch are available at considerably cheaper prices.

The pest infested rice grains were exposed to the sun in a solar cooker for about an hour and then washed repeatedly in double distilled water to get rid of any dead pests. These grains were then dried in the sun and ground to fine powder. The broken rice similarly was also ground to fine powder. The rotten part of the potatoes were removed and the remaining part of the potatoes was peeled and cut into very small

pieces and then homogenized. The substrates were then subjected for acid or enzymatic hydrolysis. Sago and arrowroot starch were purchased (Rs. 14/kg and Rs. 10/kg respectively) from the local market and used.

3.2.6.2.1 Acid Hydrolysis of Starch

The acid hydrolysis of starch was carried out as described (Yin *et al,* 1997) with certain modifications. Briefly, 100 ml of double distilled water was added to 450 gm of the starchy substrate and the mixture was stirred well. While stirring, 500 ml of boiling water was added to the mixture following boiling for 5 minutes to ensure gelatinization of starch. The gelatinized starch was cooled to room temperature and more water was added to make up the volume to 1 liter. The slurry was then treated with 2N HCl to a pH of 1.0 – 1.5 and then autoclaved at 121°C for 20 minutes. Clear supernatant is collected after filtration and reducing sugar content was estimated. Its pH was then adjusted to 5.5 and then used as substrate for fermentation reaction.

3.2.6.2.2 Enzymatic Hydrolysis of Starch

The enzymatic hydrolysis of starch was performed as described (Akerberg *et al*, 2000) with modifications.

To 450 gm of the starchy substrate, 100 ml of double distilled water was added with stirring continuously. 500 ml of boiling double distilled water was then added to this mixture and then boiled for 5 minutes for gelatinization of starch. It was then cooled to room temperature and volume was made up to 1 litre with double distilled water. Gelatinized starch was then added with 1 M $CaCl_2$ to a final concentration of 0.45 mM and the pH was set to 6.9. α-amylase (1 U/gm of substrate) was added and

the solution was incubated at 70°C for 2 hours for liquefaction of the starch. The liquefied starch was cooled to room temperature and the pH was adjusted to 4.8. The mixture was then added further with glucoamylase (1 U/gm of substrate) and incubated at 60°C for 72 hours. Following incubation, the hydrolysed starch was cooled to room temperature and filtered. The filterate was diluted so as to have reducing sugar equivalent to 12%, pH was adjusted to 5.5 and the substrate was then used for fermentation reaction.

3.2.7 Lactic Acid Production with Immobilized *Rhizopus oryzae*

3.2.7.1 Immobilization of *Rhizopus oryzae* RBU2-10 in Calcium Alginate Beads

Immobilization of *Rhizopus oryzae* RBU2-10 was carried out as described (Xuemei *et al*, 1999). The mutant *Rhizopus oryzae* RBU2-10 was subcultured at 30°C for 7 days. The spores were aseptically harvested in 250 ml of distilled water. The spore suspension (250ml) was then mixed with 750 ml of sodium alginate (40 gm/l) to obtain a mixture consisting 30 gm/l of sodium alginate and a final spore concentration of 10^6 spores/ml. The mixture was added dropwise into sterilized 20 g/l solution of $CaCl_2$ under aseptic condition at 10°C. The beads were then kept at 10°C for 2 hrs. The beads (3 mm dia) were then washed separately and added to the production medium.

3.2.7.1.2 Immobilization of *Rhizopus oryzae* RBU2-10 in Loofa Sponge

For immobilizing the fungus in loofa sponge some of the characteristics to assess the suitability of loofa sponge as immobilizing matrix was studied as described (Ogbonna *et al*, 1994).

3.2.7.2.1 Assay of Temperature and pH Stability of Loofa Sponge

3.2.7.2.1.1 Assay of pH stability

The stability of the sponge was determined by soaking the sponge in the buffer solutions of different pH (1.1 – 14) for two weeks and observing the change in shape, structure and texture of the sponge.

3.2.7.2.1.2 Assay of Temperature stability

The temperature stability of the sponge was observed by repeatedly autoclaving the sponge at 121°C for varying lengths of time (20-40 min).

Immobilization of the *Rhizopus oryzae* in loofa sponge was carried out as described by Slokoska *et al*, (1998) with some modifications. Loofa sponge (*Luffa cylindrica*) were cut into pieces (16 x 18 x 3.5 mm), soaked in double distilled water and continuously rinsed. The pieces were then dried in an oven at 105°C. A sponge piece weighing about 0.10 gm was autoclaved (121°C, 30 min), in 100 ml of double distilled water. The loofa sponge was then added to the 50 ml of fermentation medium containing rice starch equivalent to 6% of the reducing sugar as described earlier. The matrix was then inoculated with spores of *Rhizopus oryaze* RBU2-10 (10^6 spores/ml) and the flasks were incubated under shaking (50 rpm) at 30°C. After 24 hours the immobilized mycelia were washed and the medium was replaced with fresh rice starch medium containing 12% reducing sugars for desired period.

3.2.7.3 Semicontinuous Lactic Acid Production with Immobilized Cells

Submerged fermentation with immobilized matrices were carried out in the defined rice starch medium for the production of lactic acid.

3.2.7.3.1 Lactic Acid Production with Calcium Alginate Entrapped Cells of *R. oryzae*

50 ml fermentation medium in 500 ml Erlenmeyer flask was inoculated with 100 beads (weighing about 10 gm) containing immobilized spores and incubated (150 rpm, 30°C) for 24 hours. 2 gm $CaCO_3$ was then added to each flask to bring the pH to about 5.5 ± 0.1. The fermentation was continued for desired periods.

3.2.7.3.2 Lactic Acid Production with *Rhizopus oryzae* RBU2-10 Immobilized in Loofa Sponge

A piece of sponge (1.6 x 1.8 x 0.35 cm size) immobilized with *Rhizopus oryzae* RBU2-10 spores was added to 50 ml of fermentation medium in 500 ml conical flask. 2 gm $CaCO_3$ was added to bring the pH to 5.5 ± 0.1. The flasks were incubated (150 rpm, 30°C) for desired periods.

Following analysis of various factors for enhanced production of lactic acid using immobilized *Rhizopus oryzae* RBU2-10, fed-batch fermentation for lactate production was carried out. At the end of each fermentation cycle the immobilized matrices were retrieved by sieving, washed with sterile water and then transferred to the similar volume of fresh medium for the next cycle of cultivation. The cycles were repeated again and again till the production of lactic acid dropped to considerably lower level. Following each cycle of fermentation, the broth was analysed for lactic acid production.

3.2.8 Analysis of Carriers and Diluents for Lactic Acid Extraction

Lactic acid is an important commercial product and extraction of the same is a

growing requirement in fermentation based industries. The conventional method of lactic acid extraction is expensive and environment unfriendly. Solvent extraction of lactic acid appear a promising technique. So a combination of carriers and diluents were analysed for lactic acid extraction in the present study. 25% (v/v) and 50% (v/v) solutions of tri-n-octylamine, and tri-n-pentylamine were prepared using methyl isobutylketone and 1-octanol as the diluents. Procedure for extraction was as described by Wasewar *et al*, (2002). Briefly, 50 ml of the clear fermentation broth and organic phase (50 ml) (containing mixture of carriers and diluents) were gently mixed in a shaker bath at 30°C for 24 hours. The two phases were allowed to settle for 30 minutes for a complete phase separation. The phase containing lactic acid was separated and concentration of lactic acid was determined colorimetrically. The acid concentration in organic phase was calculated by mass balance (Wasewar *et al*, 2002). Combinations of the solvent and aqueous phases were taken for achieving an improvement in lactic acid extraction. The recovery of lactic acid from the organic phase was carried out by treating the organic phase with 4 N NaOH in different combinations. (Choudhury *et al*, 1998).

3.2.9 Analytical Method

3.2.9.1 Qualitative Analysis of Lactic Acid

The qualitative analysis of lactic acid was carried out as described by Smith (1958). Samples from fermentation broth along with standard lactic acid were chromatographically analysed. A solvent system consisting of n-Butanol:glacial acetic acid : H_2O (12:3:5) was used for separation using Whatman No. 4 paper at a temperature of 20 ± 2°C. Following chromatography, the Whatman No. 4 paper was sprayed with aqueous $KMnO_4$ 1% (w/v) for detection.

3.2.9.2 Quantitative analysis of lactic acid

Quantitative estimation of lactic acid was performed by Barker-Summerson method as described (Pyrce, 1969). The lactic acid in fermentation broth is converted into acetaldehyde by heating with sulphuric acid. The acetaldehyde then reacts with *p*-hydroxybiphenyl to yield coloured complex. Addition of copper catalyses the conversion of lactic acid into acetaldehyde. Initially the copper sulphate and calcium hydroxide is added to remove the carbohydrate, since it may interfere in colour development. The *p*-hydroxybiphenyl is dissolved in dimethylformamide instead of alkali as the reagent mixes better and is stable for indefinite period.

3.2.9.2.1 Reagents

(i) Concentrated sulphuric acid (analytical grade)

(ii) Colour reagent – prepared by dissolving 1.5 gm of *p*-hydroxybiphenyl in 100 ml of dimethylformamide

(iii) 20% $Cu\ SO_4 . 5H_2O$

(iv) 3% $Cu\ SO_4 . 5\ H_2O$

3.2.9.2.2 Procedure

The fermentation broth was centrifuged (5000 g, 15 min) to remove the cells. To 1 ml of diluted supernatant, 1 ml of 20% $CuSO_4.5H_2O$ and 8 ml of double distilled water was added. Reaction mixture was then added with 1 gm of $Ca(OH)_2$, shaken vigorously and then set aside for 30 min at room temperature. Reaction mixture was then centrifuged (5000 g, 15 min). 1 ml of supernatant thus collected was added to a test tube containing 0.05 ml of 3% $CuSO_4.5H_2O$ solution. The tube was chilled on ice

and added with 5 ml of concentrated H_2SO_4 slowly and with mixing. The tubes were placed in boiling water bath for 5 min and cooled to room temperature. Reaction mixture was added with 0.1 ml of *p*-hydroxybipheyl solution and incubated (30°C) for 30 minutes. The colour which developed was measured at 565 nm in Spectrophotometer (Perkin Elmer, Lambda Bio 40, USA) and concentration of the lactic acid was determined using a standard curve.

3.2.9.3 Estimation of Glucose

The residual glucose was measured by DNS method (Miller, 1959). The method is based on the reduction of the 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid while the aldehyde group gets oxidized to carboxyl group.

3.2.9.3.1 Reagents

(1) DNS reagent	(g/l)
3,5-dinitrosalicylic acid	10
Phenol	2
Sodium sulphate	0.5
Sodium potassium tartarate	200

All the components are dissolved in 1% NaOH solution.

3.2.9.3.2 Procedure

To a 3 ml test solution, similar volume of DNS reagent was added. The mixture was heated for 5 minutes in a boiling water bath then cooled. The absorbance was measured at 575 nm in a spectrophotometer (Perkin Elmer, Lambda Bio 40, USA).

3.2.9.4 Dry Mycelial Weight Estimation

The dry weight of mycelium was measured as described (Rosenberg *et al*, 1992b). The fermentation broth was filtered through Whatman No. 4 filter paper. Filtered biomass was then washed free of adsorbed $CaCO_3$ in the mycelia by washing the myceliam with acidified double distilled water (pH 2.5 with 4 N HCl). The mycelia thereafter was washed again with distilled water until the washing showed the neutral pH. Mycelia was then dried to a constant weight at 85°C.

3.2.9.5 Test for the Hydrolysis of Starch

3.2.9.5.1 Rapid Test

The method is based on the principle where starch at different stages of hydrolysis after reaction with iodine show gradual change in colour. The procedure was described by Jain (1990) with modifications as below.

3.2.9.5.1.1 Reagent

Iodine solution – 5 gm/l

3.2.9.5.1.2 Method

To a drop of iodine on a slide, a drop of starch solution was added. Three conditions were observed (a) development of blue colour indicated the presence of starch, (b) development of reddish brown colour indicated partial degradation of starch and (c) if the solution remained colourless, that indicated a complete hydrolysis of starch.

3.2.9.6 Chromatographic Analysis

The method as described by Yahiro *et al*, (1997) was followed. The hydrolysed starch solution was spotted on filter paper No. 50 at room temperature using glucose and maltose as the standard in a solvent system containing 60% v/v 1-propanol. The sugars were detected by spraying the chromatogram with 0.75% silver nitrate dissolved in acetone.

3.2.10 Electron Microscopy of the Fungus

The detailed analysis of morphological structure of the wild type and mutant fungal strains were carried out by Scanning Electron Microscope (SEM) (LEO-435, VP, UK). Scanning electron microscopy was performed according to the method as described (Gabriel, 1982). The fungal mat was taken and primary fixation was done using 3% glutaraldehyde and 2% formaldehyde in a ratio of 4:1 for two hours. The samples after primary fixation were washed with double distilled water and dehydrated by treating through successive alcohol gradient (30%, 50%, 70%, 80%, 90% and 100%) in a stepwise manner. The retention time of samples in each alcohol series up to 70% alcohol gradient was 15 minutes, but later, the time period was increased to 30 minutes for alcohol concentrations ranging from 80% to 100%. After complete dehydration, samples were air dried and coated with gold by gold shadowing technique. The samples were then viewed under SEM and photomicrograph was taken at the desired magnification.

3.2.11 Statistical Analysis

The experimental data obtained in the present study was statistically analysed.

The method of statistical analysis (ANOVA) was performed as described by Somasegaran and Hoben (1994). ANOVA tables were used for calculating the sum of squares between and within the treatments. 1% and 5% levels for ANOVA test was used for comparing the calculated F ratio by using standard tables.

Table for Analysis of Variance

Source of Variation	Sum of Square	Degree of Freedom (d.f.)	Mean Square (MS)	F. Ratio
Between the table (T)	SST	k-1	SST/k-1	$\frac{SST}{SSE} x \frac{bkn-k-b+1}{(k-1)}$
Within 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		

d.f., Degree of freedom; SST, Treatment sum of squares;
SSB, Block sum of squares; SSE, Error sum of squares;
SS, Total sum of squares; k, Number of treatment; b, Number of blocks (number of replicates per treatment); n, Number of replicate per treatment per block.

Chapter 4

Results

4.1 ISOLATION, PURIFICATION AND SCREENING OF POTENT MICROBIAL STRAIN

A total of 63 fungal strains were isolated including 22, 13, 11, 17 strains isolated respectively from the forest, garden soils and from the decomposing food and horticultural materials. The morphological features of the isolated strains, after purification were studied on PDA plates following incubation at 35°C (Table 1). These strains were initially screened on the basis of zone formation and by determining the Acid Unitage (AU) value on acid indicator medium plates. Out of 63 strains, 17 strains having the AU value in the range of 1.3 to 6.0 were subjected to second phase of screening by evaluating their lactic acid production ability in submerged condition. This had demonstrated strains RB-5, RB-4 and RB-3 producing relatively higher levels of lactic acid (Table 2) and a maximal of (36.25 g/l) production was obtained from RB-5 strain. The strain RB-5 was identified to be *Rhizopus oryzae* by Indian Agricultural Research Institute, New Delhi and was stored at 4°C on PDA for further use.

4.2 MUTAGENESIS OF THE ISOLATED FUNGAL STRAIN FOR IMPROVEMENT OF LACTIC ACID PRODUCTION

The improvement in the lactic acid production ability of fungal strain *R. oryzae* RB-5 isolated from the garden soil was attempted by physical (UV irradiation) and chemical mutagenesis. The spore suspensions of *R. oryzae* RB-5 were exposed to UV or to the chemical mutagens. The percentage of spore survival was determined. Based on the AU values the percentage distribution i.e. the positive, negative and the

Table 1. **Morphological features of fungal strains and their source of isolation**

Culture strain	Colony colour	Source of isolation	Place of isolation
RB-1	Greenish	Decomposing food	Roorkee
RB-2	Bluish	Decomposing food	Roorkee
RB-3	Whitish grey	Garden soil	Roorkee
RB-4	Dirty white	Garden soil	Roorkee
RB-5	White	Garden soil	Roorkee
RB-6	White	Garden soil	Roorkee
RB-7	Black	Forest soil	Roorkee
RB-8	Grey colour	Forest soil	Roorkee
RB-9	Whitish pink	Forest soil	Roorkee
RB-10	Blue green	Forest soil	Roorkee
RB-11	Pinkish brown	Forest soil	Roorkee
RB-12	Grey	Forest soil	Roorkee
RB-13	Yellow	Forest soil	Roorkee
RB-14	Greyish green	Forest soil	Roorkee
RB-15	Pinkish black	Forest soil	Roorkee
RB-16	Dirty white	Forest soil	Roorkee
RB-17	Yellowish green	Horticultural waste	Roorkee
RB-18	Creamish	Horticultural waste	Roorkee
RB-19	Rose coloured	Horticultural waste	Roorkee
RB-20	Pinkish	Horticultural waste	Roorkee
RB-21	Greyish	Horticultural waste	Roorkee
RB-22	Whitish grey	Horticultural waste	Roorkee
RB-23	Greenish white	Garden soil	Haridwar
RB-24	Greenish	Garden soil	Haridwar
RB-25	Greenish yellow	Decomposing food	Haridwar
RB-26	Yellow	Decomposing food	Haridwar
RB-27	Green	Decomposing food	Haridwar
RB-28	Dark green	Forest soil	Haridwar
RB-29	Pinkish brown	Forest soil	Haridwar
RB-30	Dirty white	Garden soil	Laksar
RB-31	White	Garden soil	Laksar
RB-32	Pinkish brown	Garden soil	Laksar
RB-33	Yellowish green	Garden soil	Laksar
RB-34	Creamish	Garden soil	Laksar
RB-35	Creamish	Forest soil	Bahadarabad
RB-36	Dirty white	Decomposing food	Bahadarabad

RB-37	White	Decomposing food	Bahadarabad
RB-38	Greyish white	Horticultural waste	Bahadarabad
RB-39	Blue	Horticultural waste	Bahadarabad
RB-40	Greenish	Horticultural waste	Bahadarabad
RB-41	Yellow	Decomposing food	Dehradun
RB-42	Pinkish	Forest soil	Dehradun
RB-43	Creamish	Forest soil	Dehradun
RB-44	Greyish	Forest soil	Dehradun
RB-45	Whitish black	Forest soil	Dehradun
RB-46	Light creme	Forest soil	Dehradun
RB-47	Blue	Decomposing food	Rishikesh
RB-48	Rose colour	Horticultural waste	Rishikesh
RB-49	Green	Horticultural waste	Rishikesh
RB-50	Blackish white	Forest soil	Rishikesh
RB-51	Dark green	Forest soil	Rishikesh
RB-52	Dirty white	Decomposing food	Saharanpur
RB-53	White	Decomposing food	Saharanpur
RB-54	Pinkish	Horticultural waste	Saharanpur
RB-55	Yellow	Horticultural waste	Saharanpur
RB-56	Brown	Horticultural waste	Saharanpur
RB-57	Blue	Forest soil	Behat
RB-58	Green	Garden soil	Behat
RB-59	Whitish grey	Garden soil	Behat
RB-60	Yellow	Horticultural waste	Behat
RB-61	Blue	Horticultural waste	Behat
RB-62	Pink	Horticultural waste	Behat
RB-63	Yellow	Horticultural waste	Behat

corresponding (unaltered) colonies were also determined. Of the 85% colonies that survived following 3 minutes of UV irradiation, 20% had the higher AU values than parent strain and were termed as positive mutant, whereas 12% of the colonies had AU value less than parent strain and were denoted as negative mutants, 68% of the mutants had AU value (6.0) that corresponded to that of the parent strain, hence termed as the corresponding mutants (Fig. 2a). The mutant exhibiting the highest AU value (12.8) (Table 3) was denoted as *R. oryzae* RBU1-3 (48.15 g/l lactic acid) (Table 7).

Wild type strain *R. oryzae* RB-5 was also subjected to NTG and colchicine mutagens. Mutant RBN-40 generated by NTG treatment (100 µg/ml, 40 min) had the maximum AU value of 10.8 (Table 4). Of the total mutants obtained, 20%, 10%, and 70% respectively were the negative, positive and corresponding mutants (Fig. 2c), whereas, mutant RBC-0.1 generated by colchicine treatment (0.1 µg/ml) had an AU value of 8.6 (Table 5). 7% of the mutants obtained following treatment were negative, 13% positive and 80% were the corresponding mutants (Fig. 2d). Prolonged treatment with NTG or colchicine generally led to the decreased number of the corresponding mutants while the negative mutants increased.

Among the strains obtained following initial stage of mutagenesis using UV, NTG and colchicine treatments, mutant RBU1-3, obtained following UV irradiation had the maximum AU value and was subjected to a second round of UV irradiation in an attempt for a further improvement in the lactic acid production ability of the strain. The mutant thus generated after 10 minutes of UV exposure had the highest AU value (19.0) (Plate-I) and was termed as *R. oryzae* RBU2-10 (Table 6). Among the strains

PLATE 1. FORMATION OF YELLOW COLOURED ZONES BY THE (A) WILD TYPE *RHIZOPUS ORYZAE* RB-5, (B) MUTANT *RHIZOPUS ORYZAE* RBU2-10 THE ZONES FORMED BY THE RESPECTIVE STRAINS WERE OBSERVED AFTER 72 HOURS INCUBATION AT 35°C.

A

B

PLATE - 1

Table 2. Screening of the fungal strains based on zone formation and lactic acid production under submerged condition

S. No.	Fungal Strain	AU Value	Lactic acid (g/l)
1	RB-3	5.1	19.02
2	RB-4	5.7	25.14
3	RB-5	6.0	36.25
4	RB-6	4.2	16.17
5	RB-21	3.7	13.25
6	RB-22	1.5	3.12
7	RB-23	2.0	5.33
8	RB-30	3.3	10.09
9	RB-31	2.6	6.85
10	RB-36	3.6	10.16
11	RB-37	3.8	11.29
12	RB-39	1.7	4.34
13	RB-44	4.0	15.07
14	RB-45	3.1	9.25
15	RB-50	2.2	5.48
16	RB-52	1.3	3.72
17	RB-53	1.9	4.24

Table 3. Acid unitage value of the mutagenized strains following Ist stage of ultraviolet irradiation*

S. No.	Time of exposure (min)	Mutant	AU value
1	2	RBU1-2	7.0
2	3	RBU1-3	12.8
3	4	RBU1-4	7.8
4	5	RBU1-5	10.2
5	6	RBU1-6	8.5
6	7	RBU1-7	9.4
7	10	RBU1-10	8.1
8	15	RBU1-15	7.8
9	20	RBU1-20	6.7

* Intensity, 2.48Wm^{-2}

Table 4. Acid Unitage value of the mutants derived following NTG (100 μg/ml) treatment

S. No.	Time of exposure (min)	Mutant	AU value
1	10	RBN-10	7.1
2	20	RBN-20	7.6
3	30	RBN-30	8.2
4	40	RBN-40	10.8
5	50	RBN-50	9.1
6	60	RBN-60	7.3

Table 5. Acid Unitage value of the mutants derived following colchicine* treatment

S. No.	Colchicine (μg/ml)	Mutant	AU value
1	0.01	RBC-0.01	6.2
2	0.02	RBC-0.02	7.1
3	0.1	RBC-0.1	8.6
4	0.2	RBC-0.2	7.5
5	0.5	RBC-0.5	6.4
6	1.0	RBC-1.0	6.2

* Duration of treatment, 24 hr

Table 6. Acid Unitage value of the mutagenized strains following IInd stage of ultraviolet irradiation*

S. No.	Time of exposure (min)	Mutant	AU value
1	2	RBU2-2	15.1
2	3	RBU2-3	14.7
3	4	RBU2-4	15.8
4	5	RBU2-5	16.2
5	6	RBU2-6	15.0
6	7	RBU2-7	17.4
7	10	RBU2-10	19.0
8	15	RBU2-15	13.2
9	20	RBU2-20	12.0

* Intensity, 2.48 Wm^{-2}

Fig. 2. EFFECT OF MUTAGENIC TREATMENTS ON SPORE SURVIVAL AND DISTRIBUTION OF *RHIZOPUS ORYZAE* RB-5.

(a) Ist Stage UV irradiation, (b) IInd Stage UV irradiation, (c) N-methyl-N'-nitro-N-nitrosoguanidine treatment, (d) Colchicine treatment

(▧, Corresponding mutant; ▩, Negative mutant; ▤, Positive mutant; •, Percentage survival)

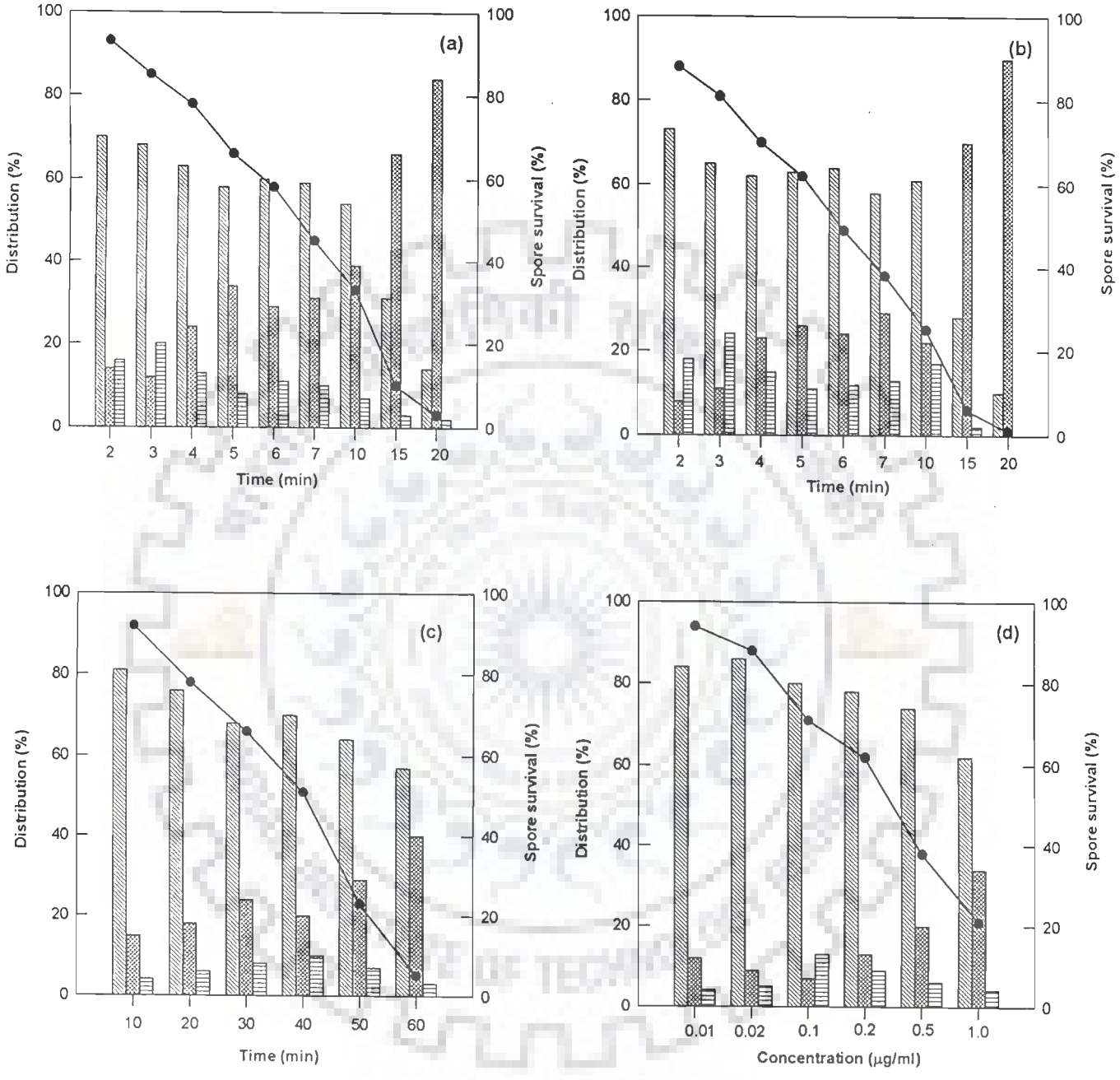

(a)
(b)
(c)
(d)
Distribution (%)
Spore survival (%)
Time (min)
Concentration (μg/ml)
0
20
40
60
80
100
2
3
4
5
6
7
10
15
20
30
50
60
0.01
0.02
0.1
0.2
0.5
1.0

Fig. 2

Table 7. **Lactic acid production by the selected mutants obtained after UV and chemical mutagenesis of *Rhizopus oryzae* RB-5**

Mutation	Strain of *Rhizopus oryzae*	Lactic acid (g/l)	Yield (%)	Biomass (g/l)
1st stage UV	RBU1-3	48.15	44.4	9.5
2nd stage UV	RBU2-10	59.34	53.6	8.4
NTG	RBN-40	44.29	42.3	7.7
Colchicine	RBC-0.1	42.53	39.6	7.3

generated after this treatment 22% were negative, 17% positive and 61% were the corresponding mutants (Fig. 2b).

The strains with maximum AU values were selected and analysed for lactic acid production under submerged condition. Among these, strain RBU2-10 obtained after two stages of UV irradiation had the maximum levels of production (59.34 g/l) (Table 7), which was 63.69 % higher than that of the wild type RB-5 strain. The mutant *R. oryzae* RBU2-10 exhibited stable levels of lactic acid production during successive generations with minor variations (Fig. 3).

4.2.1 Morphological Features

The wild type *R. oryzae* RB-5 strain had white mycelia which sporulated into black coloured spores whereas, mutant *R. oryzae* RBU2-10 had dirty white mycelia and developed into greyish spores, further, the colony of the mutant strain had compactly growing mycelia which were aerially directed compared to the colony of wild type strain (Plate 2). In addition, the wild type *R. oryzae* RB-5 had smoother hyphae and oval spores, whereas, mycelia of the mutant *R. oryzae* RBU2-10 were rough and had flattened oval spores (Plate 3). Upon culturing, RBU2-10 grew as multiple, small sized pellets, whereas, wild type RB-5 had larger pellets but lesser in number. The pellets of wild type *R. oryzae* RB-5 had loosely arranged mycelia while mutant *R. oryzae* RBU2-10 had pellets with compact mycelia (Plate 4).

4.3 ANALYSIS OF CRITICAL PARAMETERS FOR IMPROVING THE LACTIC ACID PRODUCTION

The fermentation medium and conditions as defined by Park *et al,* (1998) were

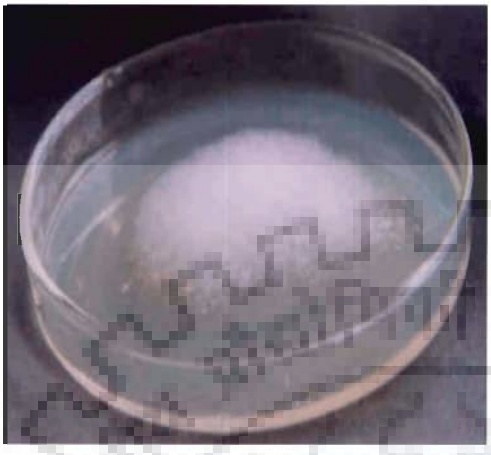
PLATE 2. MORPHOLOGICAL VARIATIONS BETWEEN THE WILD TYPE AND MUTANT STRAINS OF *RHIZOPUS ORYZAE* CULTURED IN P.D.A. MEDIA

A_1: WILD TYPE *RHIZOPUS ORYZAE* RB-5 AFTER 48 HOURS

A_2: MUTANT *RHIZOPUS ORYZAE* RBU2-10 AFTER 48 HOURS

B_1: SPORULATING WILD TYPE *RHIZOPUS ORYZAE* RB-5 AFTER 120 HOURS

B_2: SPORULATING MUTANT *RHIZOPUS ORYZAE* RBU2-10 AFTER 120 HOURS



A_1 A_2

B_1 B_2

PLATE - 2

PLATE 3. SCANNING ELECTRON MICROGRAPHS OF WILD TYPE AND MUTANT STRAINS OF *RHIZOPUS ORYZAE*

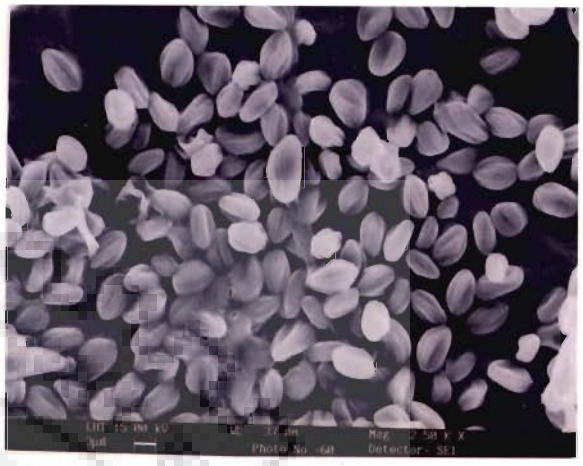
A_1: MYCELIA OF WILD TYPE *RHIZOPUS ORYZAE* RB-5 (2 KX)

A_2: SPORES OF WILD TYPE *RHIZOPUS ORYZAE* RB-5 (2.5 KX)

B_1: MYCELIA OF MUTANT *RHIZOPUS ORYZAE* RBU2-10 (1.99 KX)

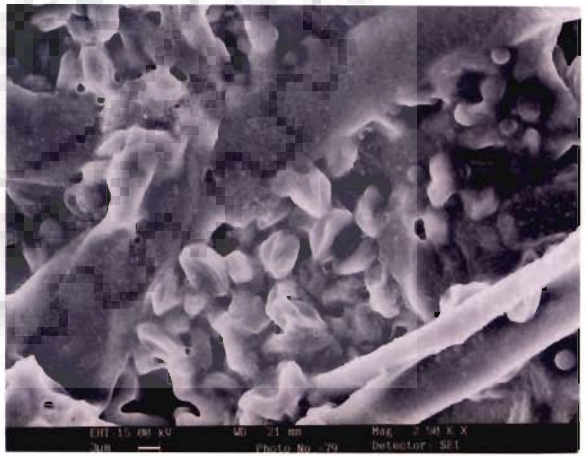
B_2: SPORES OF MUTANT *RHIZOPUS ORYZAE* RBU2-10 (2.5 KX)

A_1



A_2

B_1



B_2

PLATE - 3

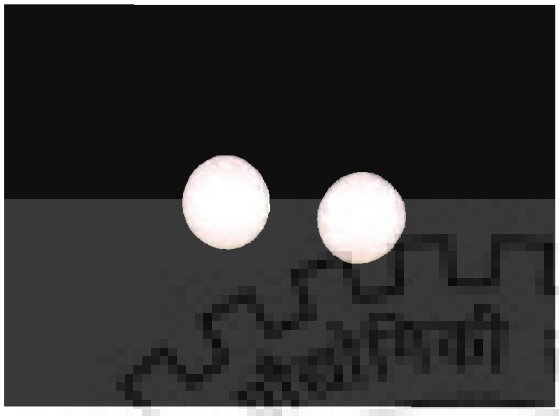
PLATE 4. MYCELIAL PELLETS OF MUTANT RBU2-10 (A) AND WILD TYPE RB-5 (B) STRAINS OF *RHIZOPUS ORYZAE*

A_1: SURFACE VIEW OF PELLETS

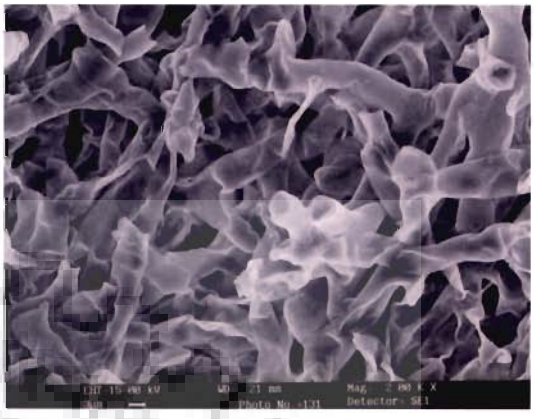
A_2: SCANNING ELECTRON MICROGRAPH OF SECTIONAL VIEW OF PELLETS (2 KX)

B_1: SURFACE VIEW OF PELLETS

B_2: SCANNING ELECTRON MICROGRAPH OF SECTIONAL VIEW OF PELLETS (2 KX)

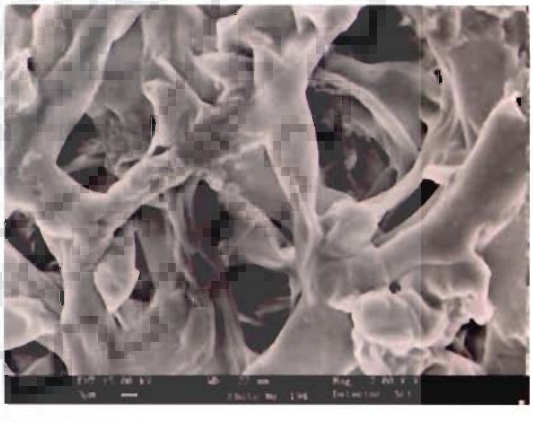


A_1



A_2

B_1



B_2

PLATE - 4

Fig. 3. PRODUCTION STABILITY OF THE MUTANT *RHIZOPUS ORYZAE* RBU2-10

Lactic acid production and biomass generation was determined following transfer of the culture every 15 days for a total period of 10 months.

Lactic acid production : ▨, *R. oryzae* RBU2-10; □, *R. oryzae* RB-5

Biomass : ■, *R. oryzae* RBU2-10; •, *R. oryzae* RB-5

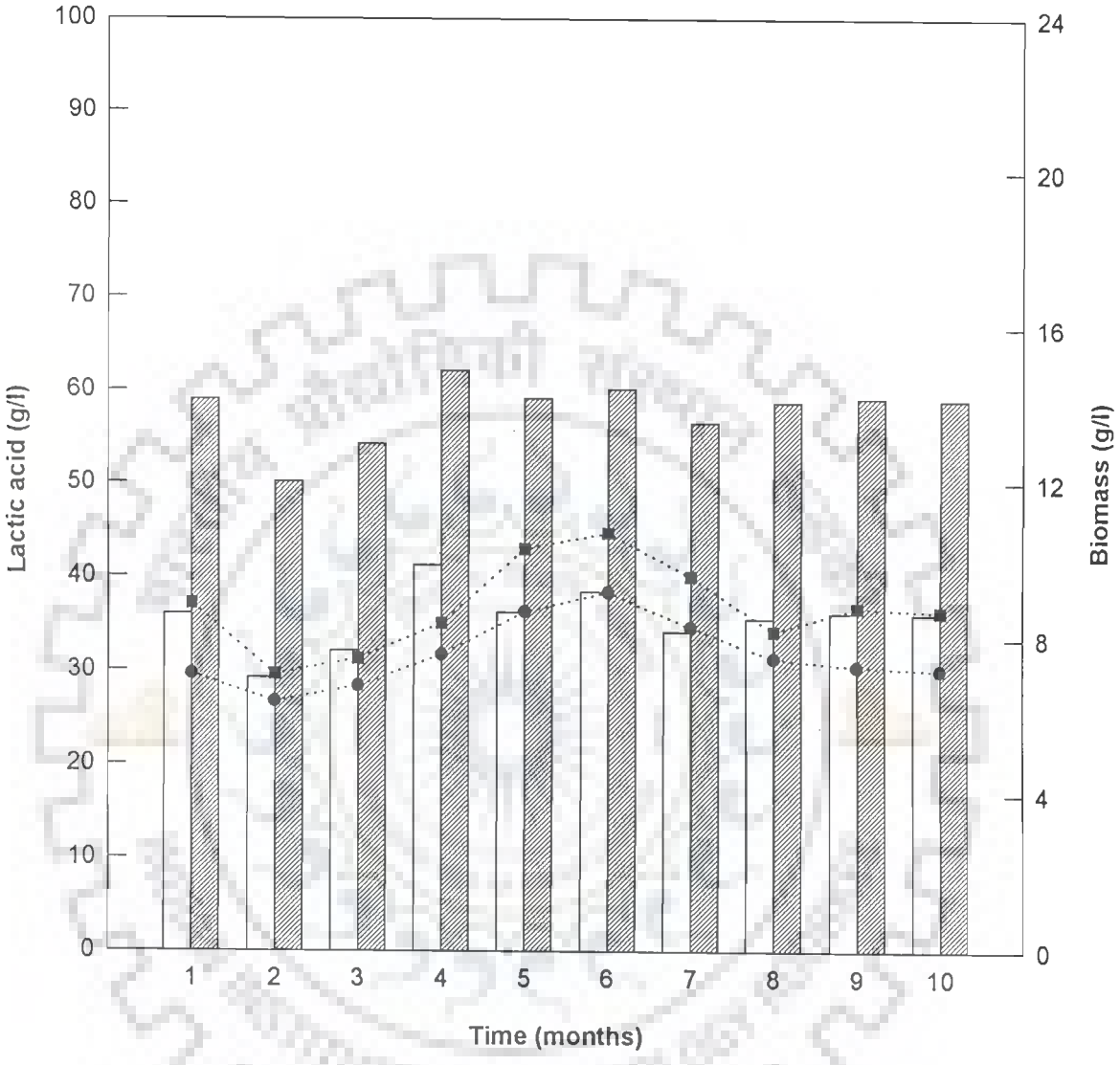

Lactic acid (g/l)
Biomass (g/l)
Time (months)
100
90
80
70
60
50
40
30
20
10
0
24
20
16
12
8
4
0
1
2
3
4
5
6
7
8
9
10

Fig. 3

initially followed. Using this medium and the conditions, major physicochemical and biological factors were analysed for determining the most favourable conditions for achieving enhanced levels of lactic acid production.

4.3.1 Fermentative Production of Lactic Acid with Sugar Substitutes

Among the various sugars substitutes analysed for supporting the lactic acid production, glucose followed by fructose were the most favourable substrates and had resulted into 59.34 and 57.21 g/l of lactic acid by mutant *R. oryzae* RBU2-10 (Fig. 5). This was followed by mannose and sucrose that had yielded into 52.15 and 38.29 g/l of lactic acid respectively.The galactose, lactose and xylose were observed to be least suitable substrates (Fig. 4). A significant variation in lactic acid production was observed between the sugar substitutes ($P < 0.01$) (Table 8).

4.3.2 Lactic Acid Fermentation as a Function of Substrate Concentration Using Glucose

To define the concentration that results into maximal production of lactic acid, varying concentrations of the glucose were used as substrate under submerged condition. Glucose at 12% (w/v) concentration led to the maximum levels of production i.e. 59.34 g/l and 36.15 g/l respectively by both mutant *R. oryzae* RBU2-10 and wild type strain *R. oryzae* RB-5. Lactic acid concentration increased up to 12% (w/v) glucose concentration whereas further addition did not lead to any further increase in production (Fig. 5a) ($P < 0.01$, Table 8). Higher levels of sugar utilization by RBU2-10 and higher biomass accumulation was observed under these conditions (Fig. 5b).

4 PRODUCTION OF LACTIC ACID WITH VARIOUS SUGAR SUBSTITUTES

Wild type *Rhizopus oryzae* RB-5 and the mutant *Rhizopus oryzae* RBU2-10 were grown under submerged condition containing sugar substrates (12%) as described in the methods. Lactic acid was estimated after 72 hours at 35°C.

G, F, S, Ga, M, L and X represent Glucose, Fructose, Sucrose, Galactose, Mannose, Lactose and Xylose respectively;

□ and ▨ ; represent lactic acid production respectively by *R. oryzae* RB-5 and *R. oryzae* RBU2-10; ● and ■; represent biomass generation from the *R. oryzae* RB-5 and *R. oryzae* RBU2-10 strains respectively.

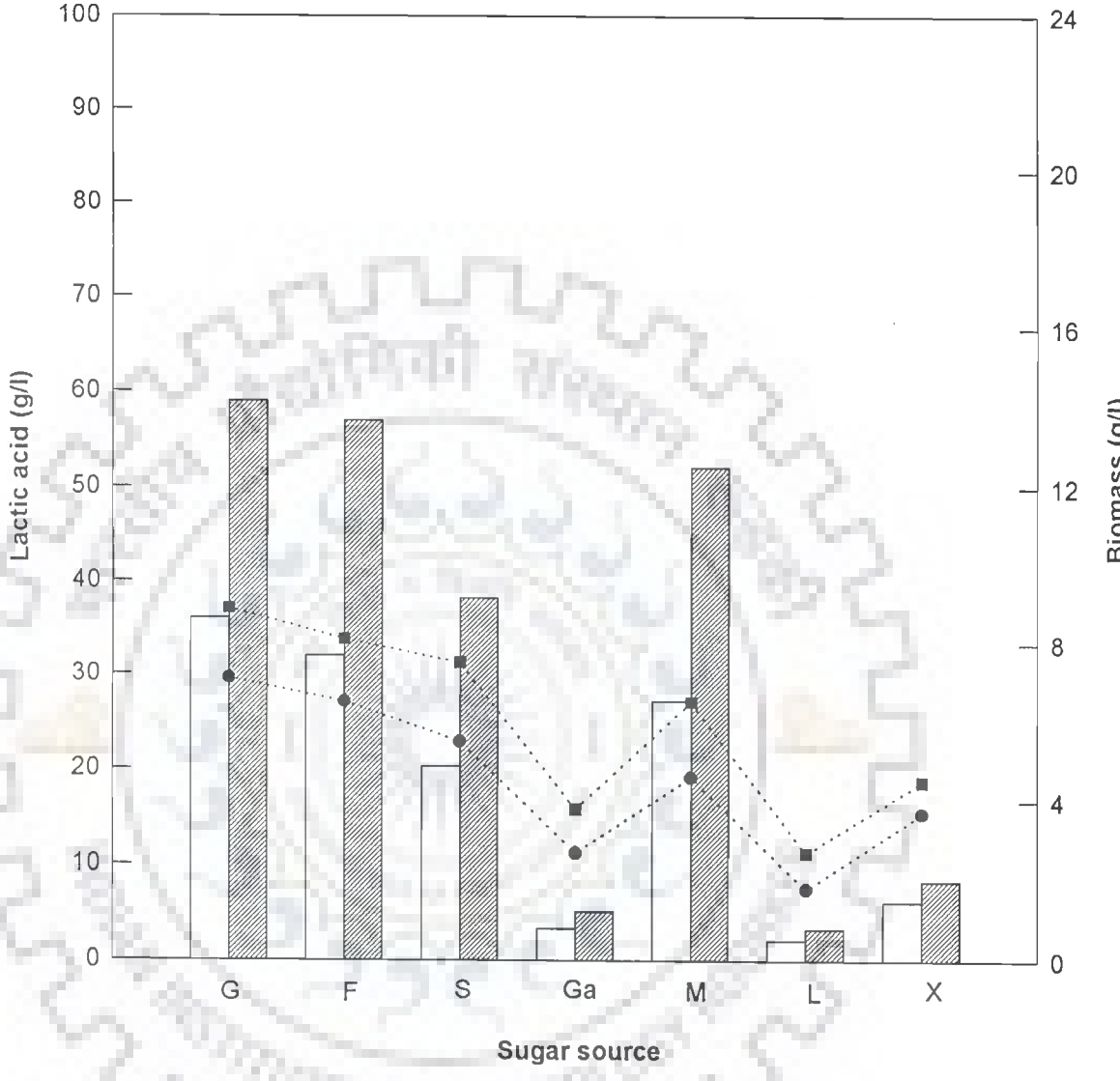

100
90
80
70
60
50
40
30
20
10
0
Lactic acid (g/l)
24
20
16
12
8
4
0
G
F
S
Ga
M
L
X
Sugar source

Fig. 4

5. LACTIC ACID FERMENTATION AS A FUNCTION OF SUBSTRATE CONCENTRATION USING GLUCOSE

(a) : ☐ and ▨ ; represent Lactic acid production by *R. oryzae* RB-5 and *R. oryzae* RBU2-10 respectively.

(b) : ☐ and ▨ ; represent residual sugar for *R. oryzae* RB-5 and *R. oryzae* RBU2-10 strains respectively; ● and ■ ; represent biomass for *R. oryzae* RB-5 and *R. oryzae* RBU2-10 strains respectively.

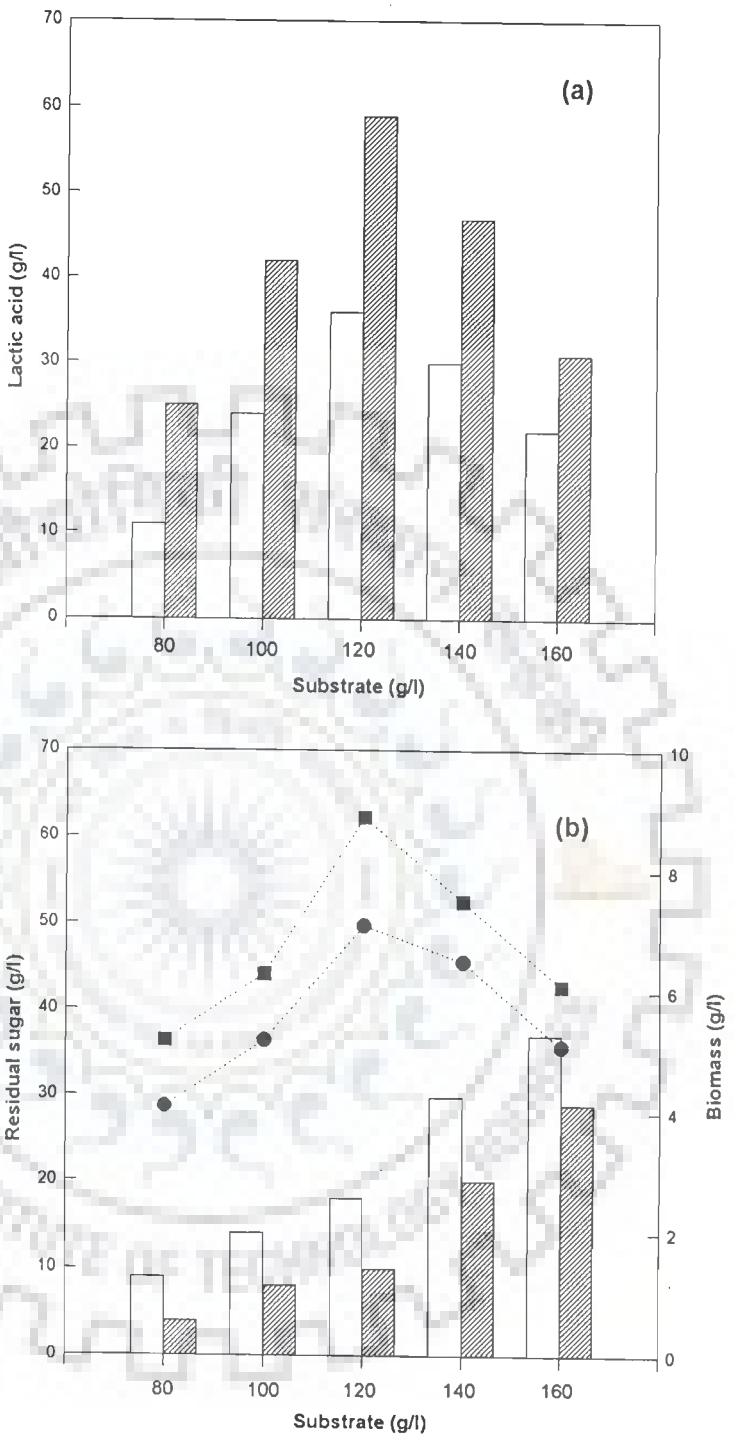

(a)
Lactic acid (g/l)
70
60
50
40
30
20
10
0
80
100
120
140
160
Substrate (g/l)
(b)
Residual sugar (g/l)
Biomass (g/l)
10
8
6
4
2
0
Substrate (g/l)

Fig. 5

4.3.3 Lactic Acid Production as a Function of Temperature, pH, Calcium carbonate concentration and Agitation rate

The most preferable temperature for lactic acid production by the mutant strain *R. oryzae* RBU2-10 was 30°C that led to 64.15 g/l of lactic acid with a yield of 59.39 % (Fig. 6a) ($P < 0.01$, Table 8). Lower and higher range of temperatures were less preferable. Although the levels of lactic acid declined beyond 30°C but the biomass continued to increase. The pH range of 4.5 to 6.5 appeared suitable for lactic acid production, but most suited pH was 5.5 (Fig. 6b).

The $CaCO_3$ was desired to regulate pH for achieving higher levels of production. $CaCO_3$ when added to 4% led to maximum levels of production (64.15 g/l) by mutant strain RBU2-10 (Fig. 6c). Further addition of $CaCO_3$ was detrimental for both, to the fungal growth and to acid production. Upon variation of the calcium carbonate concentration, significant variation in lactic acid production ($P < 0.01$) was observed (Table 8).

Varying the agitation rates (90 – 170 rpm), had indicated that though the biomass continued to increase upon increase in agitation but no more accumulation of lactic acid was observed beyond an agitation of 150 rpm for both mutant *R. oryzae* RBU2-10 and for the wild type *R. oryzae* RB-5 strains (Fig. 6d). At various agitation rates a significant difference ($P < 0.01$, Table 8) in lactic acid production was observed.

4.3.4 Lactic Acid Production as a Function of Inoculated Spore Concentration and Inoculum Level

It was detected that the inoculated spore concentrations not only influenced the

Fig. 6. LACTIC ACID PRODUCTION AT VARYING TEMPERATURES (a), pH (b), CALCIUM CARBONATE CONCENTRATION (c) AND AT VARYING AGITATION RATES (d).

Lactic acid production in the medium containing 12% glucose was analysed under submerged condition as described in the methods. The optimal condition as derived in the each stage was followed for detecting the optimal conditions in the subsequent stages.

●–● , ■–■ ; ◇ ◇ , ○ ○ ; △–△ , ▽–▽ ; represent lactic acid yield, lactic acid concentration and biomass obtained by mutant *R. oryzae* RBU2-10 and the wild type *R. oryzae* RB-5 respectively.

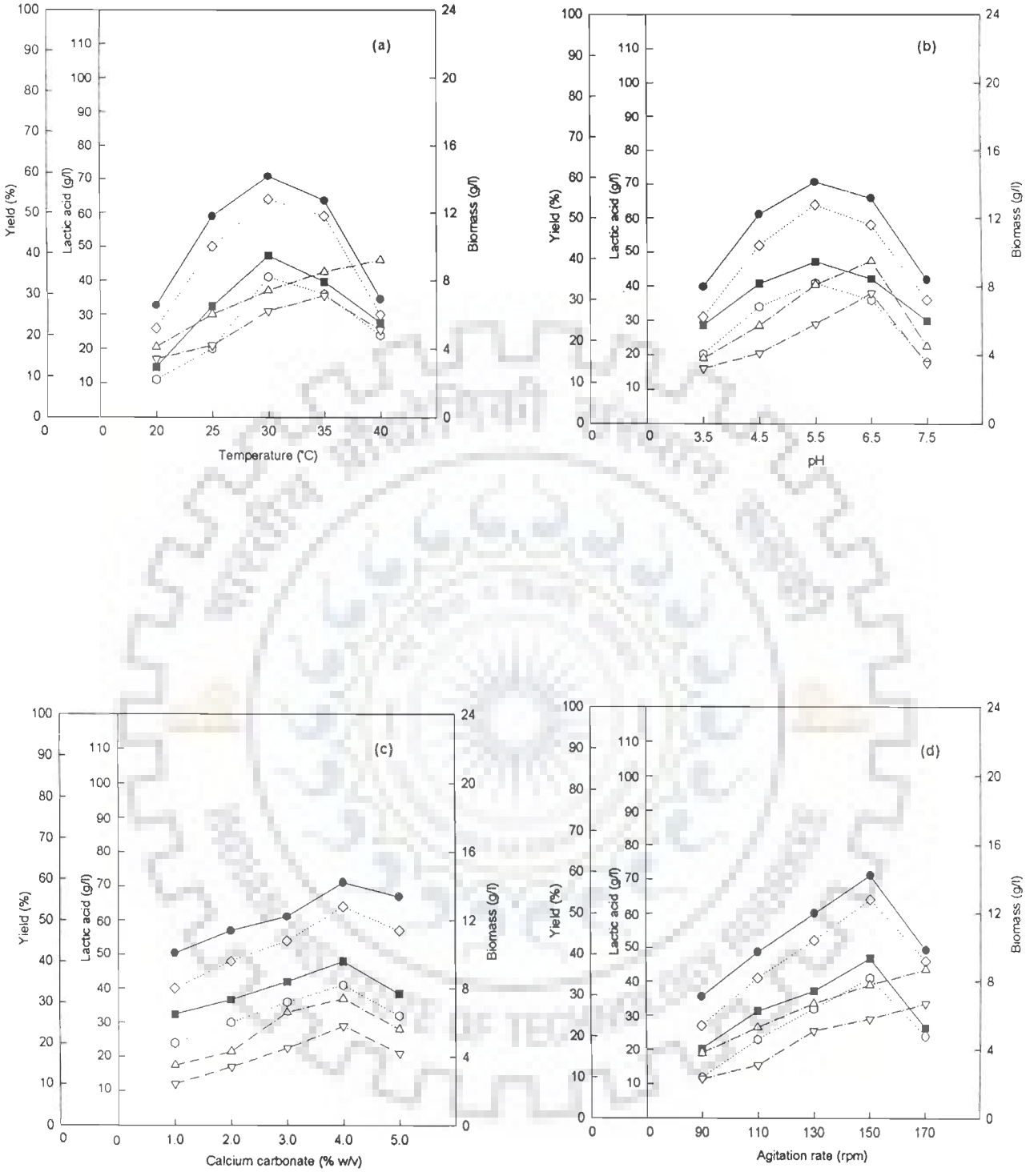

(a)
Yield (%)
Lactic acid (g/l)
Biomass (g/l)
Temperature (°C)
(b)
pH
(c)
Calcium carbonate (% w/v)
(d)
Agitation rate (rpm)

Fig. 6

Fig. 7 LACTIC ACID PRODUCTION AS A FUNCTION OF (a) SPORE CONCENTRATION AND (b) INOCULUM LEVEL.

Level of production under submerged condition at 30°C and at 150 rpm was analysed. Calcium carbonate (4%) after 24 hour was added to maintain pH at 5.5. Varying inoculum levels containing 10^6 spores/ml were used for deriving the suitable inoculum levels.

●–●, ■–■; ◇···◇, ○···○ ; △–··△, ▽–··▽ ; represent lactic acid yield, lactic acid concentration and biomass obtained by mutant *R. oryzae* RBU2-10 and wild type *R. oryzae* RB-5 respectively.

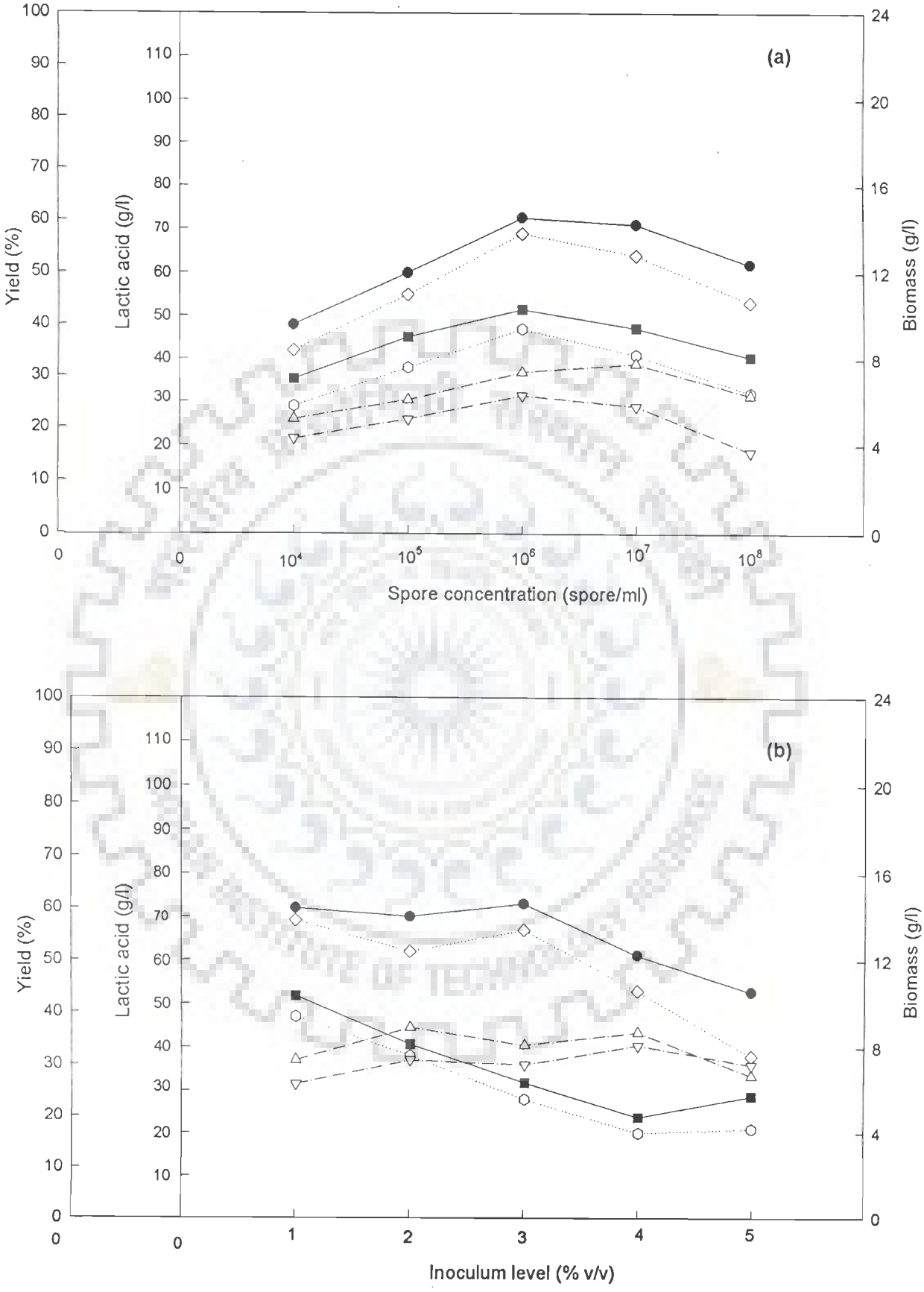

(a)
Yield (%)
Lactic acid (g/l)
Biomass (g/l)
Spore concentration (spore/ml)
10⁴
10⁵
10⁶
10⁷
10⁸
(b)
Yield (%)
Lactic acid (g/l)
Biomass (g/l)
Inoculum level (% v/v)
1
2
3
4
5

Fig. 7

mycelial morphology but also the lactic acid production ability of both mutant *R. oryzae* RBU2-10 and the wild type *R. oryzae* RB-5. Spores at a concentration of 10^6 spores/ml led to higher levels of lactic acid production (69.01 g/l) by mutant *R. oryzae* RBU2-10 (Fig. 7a) and resulted into mycelial pellet formation. Under these conditions the pellets of mutant *R. oryzae* RBU2-10 were small sized and numerous whereas the pellets of wild type strain were larger but relatively smaller in number. Upon variation of the inoculated spore concentration a significant variation in lactic acid production ($P < 0.01$) (Table 8) was observed. An inoculum level of 1-3% of above derived spored concentration appeared suitable for lactic acid production by the mutant *R. oryzae* RBU2-10 and the wild type *R. oryzae* RB-5 strains (Fig. 7b). Therefore 1% inoculum level was chosen for both the strains.

4.3.5 Lactic Acid Production as a Function of Nitrogen Sources

The concentration of nitrogen source initially selected was 0.30% (w/v) as per the defined concentration in the Park's medium (Park *et al*, 1998). Among the nitrogen sources selected, ammonium sulphate led to maximum production (69.01 g/l) followed by ammonium chloride (63.29 g/l). Peptone and urea resulted into lower levels of production whereas sodium nitrate was least preferable nitrogen source (Fig. 8a). Significant variation ($P < 0.01$) in lactic acid production was observed between different nitrogen sources and within ($P < 0.05$) the nitrogen sources (Table 8).

Variations in the ammonium sulphate concentration indicated 0.2% w/v as the ideal concentration for maximum levels of acid production (75.21 g/l) (Fig. 8b). A significant variation ($P < 0.01$) was observed between the various ammonium sulphate concentrations and within the concentration ($P < 0.05$) (Table 8).

Fig. 8(a). LACTIC ACID PRODUCTION AS A FUNCTION OF NITROGEN SOURCES

An inoculum level of 1% containing 10^6 spores/ml was used. Other conditions were as described in the legends for Fig. 7.

●–●, ■–■ ; ◇ ◇, ○ ○ ; △–△, ▽–▽ ; represent lactic acid yield, lactic acid concentration and biomass obtained by mutant *R. oryzae* RBU2-10 and wild type *R. oryzae* RB-5 respectively.

Fig. 8(b). EFFECT OF VARYING CONCENTRATION OF AMMONIUM SULPHATE ON LACTIC ACID PRODUCTION

Conditions were as described in the legends for Fig. 8(a).

●–●, ■–■ ; ◇ ◇ , ○ ○ ; △–△ , ▽–▽ ; represent lactic acid yield, lactic acid concentration and biomass obtained by mutant *R. oryzae* RBU2-10 and wild type *R. oryzae* RB-5 respectively.

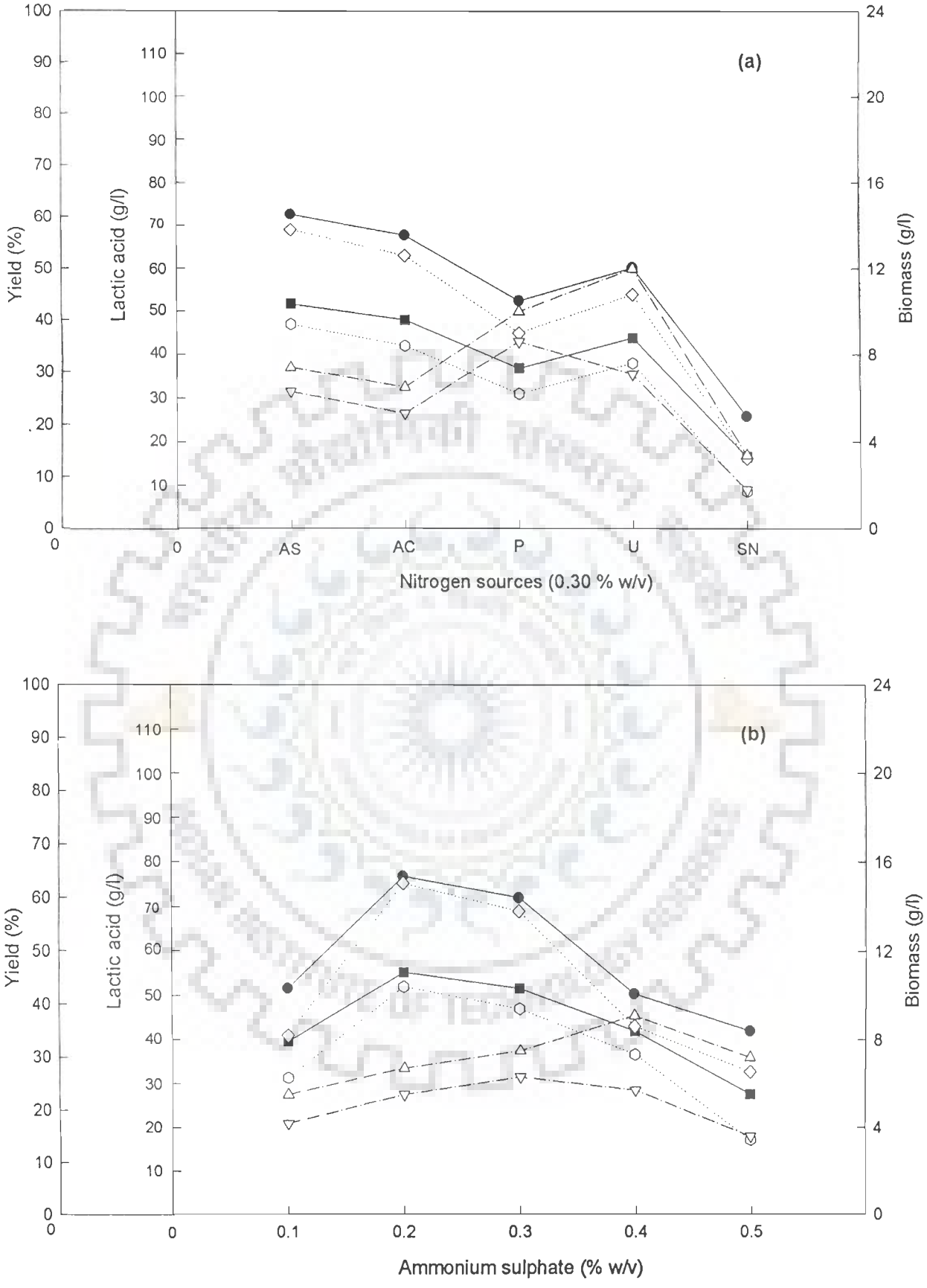

(a)
Yield (%)
Lactic acid (g/l)
Biomass (g/l)
AS
AC
P
U
SN
Nitrogen sources (0.30 % w/v)
(b)
Yield (%)
Lactic acid (g/l)
Biomass (g/l)
0.1
0.2
0.3
0.4
0.5
Ammonium sulphate (% w/v)

Fig. 8

Fig. 9. EFFECT OF VARIATIONS OF NUTRIENT CONCENTRATIONS ON LACTIC ACID PRODUCTION (a) POTASSIUM DIHYDROGEN PHOSPHATE, (b) MAGNESIUM SULPHATE, (c) ZINC SULPHATE

Conditions were as described in the legends for Fig. 8(b). Optimal concentration of the nutrient as detected in one stage was followed for the subsequent stages.

●–●, ■–■ ; ◇ ◇ , ○ ○ ; △–△ , ▽–▽ ; represent lactic acid yield, lactic acid concentration and biomass obtained by mutant *R. oryzae* RBU2-10 and wild type *R. oryzae* RB-5 respectively.

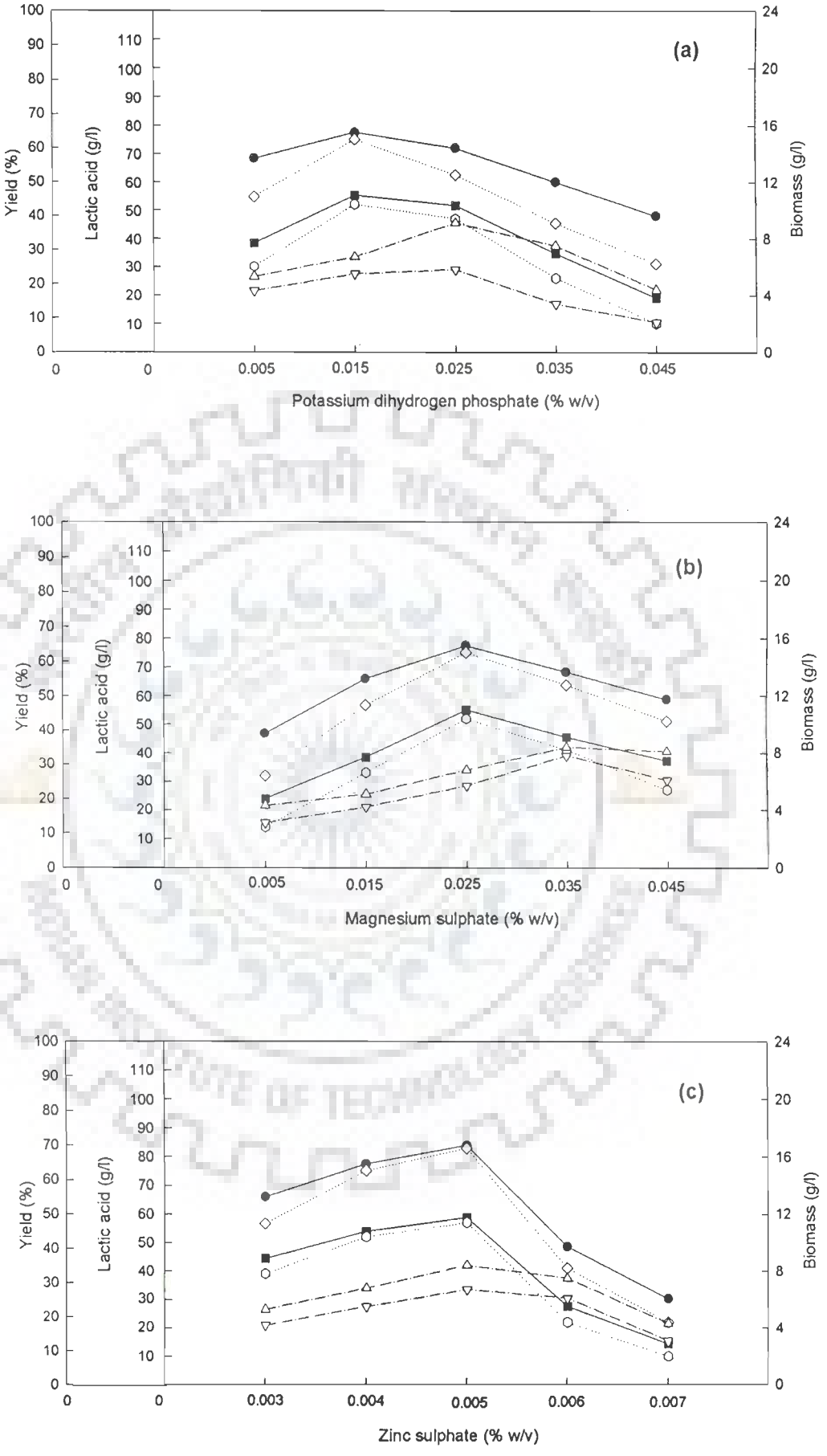

(a)
Yield (%)
Lactic acid (g/l)
Biomass (g/l)
Potassium dihydrogen phosphate (% w/v)
0.005
0.015
0.025
0.035
0.045
(b)
Magnesium sulphate (% w/v)
(c)
Zinc sulphate (% w/v)
0.003
0.004
0.005
0.006
0.007

Fig. 9

Among other essential nutrients, KH_2PO_4, $MgSO_4$ and $ZnSO_4$ at 0.015, 0.025 and 0.005 % w/v appeared to be the optimal concentrations for maximum (83.15 g/l) production (Fig. 9a-c). Upon varying the concentrations of these salts, significant variation in lactic acid production ($P<0.01$) was observed (Table 8). Thus the most favourable conditions for lactic acid production have been defined (Table 9).

Kinetics of lactic acid production was also analysed. Higher degree of conversion (0.585 g/g) and production rate (1.154 g/l/h) had denoted that the mutant strain RBU2-10 was more efficient than wild type strain RB-5 for its lactic acid prodution ability (Table 10).

4.4 LACTIC ACID FERMENTATION UNDER SOLID STATE FERMENTATION CONDITION

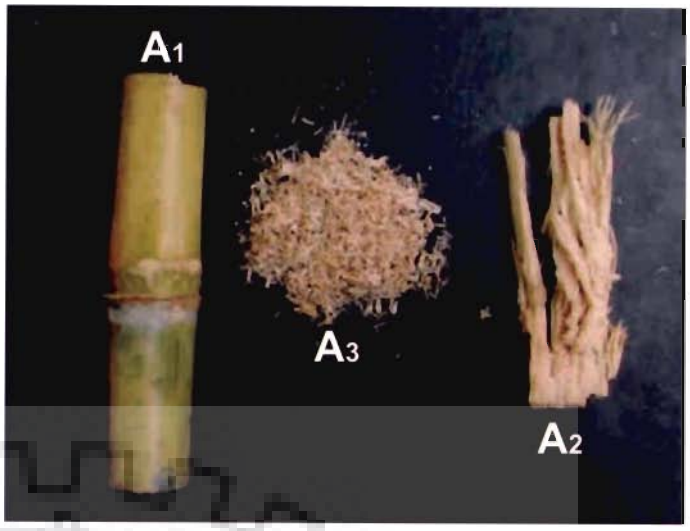
Following submerged fermentation, the production levels were also evaluated under solid state fermentation (SSF) condition. Three solid matrices i.e. bagasse, coconut coir and groundnut shells which are available in plenty in this part of India and are cheaper (Plate 5) were used. Solid supports were impregnated with the fermentation medium as derived and indicated in Table 9. Of the three supports, sugarcane bagasse had resulted into maximum production (65.05 g/l) of lactic acid by mutant *R. oryzae* RBU2-10 (Fig. 10). Following bagasse, groundnut shell (54.71 g/l) and coconut coir (47.16 g/l) were the other preferable solid supports. The production level observed under SSF condition was however lower compared to the production under submerged condition. Production was further evaluated for different time periods to detect the suitable time for achieving the maximum levels of production. Solid-state fermentation was performed up to 120 hours, maximum levels

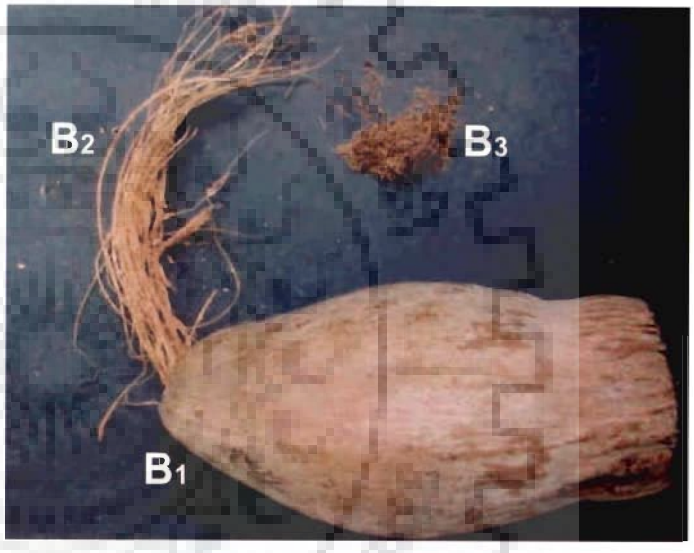
PLATE 5. SOLID SUPPORTS

A_1, A_2, A_3 SHOWS RESPECTIVELY SUGARCANE PIECES, BAGASSE AND BAGASSE CUT INTO SMALL PIECES

B_1, B_2, B_3 SHOWS RESPECTIVELY COCONUT, COCONUT COIR AND COCONUT COIR CUT INTO SMALL PIECES

C_1, C_2, C_3 SHOWS RESPECTIVELY GROUNDNUT, GROUNDNUT SHELL AND GROUNDNUT SHELL CUT INTO SMALL PIECES





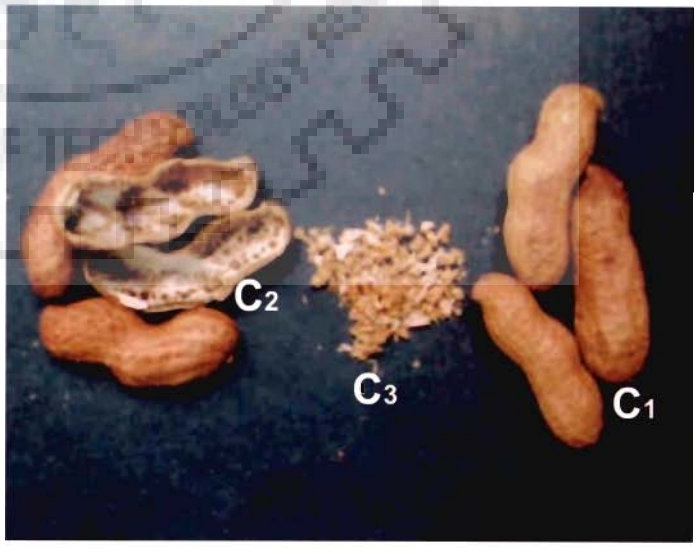


PLATE - 5

Table 8. Analysis of variance for different physicochemical and biological factors for lactic acid production by *R. oryzae* RBU2-10 using glucose as the substrate

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Sugar substrate	Variation in lactic acid production between sugar substrates	7	11729.254	1675.607	3724.09**
	Variation in lactic acid production within the sugar substrates	2	0.174	0.087	0.195
	Error	14	6.232	0.445	
Sugar concentration	Variation in lactic acid production between different sugar concentrations	4	2142.049	535.51	239.06**
	Variation in lactic acid production within the sugar concentration	2	16.101	8.0505	3.59
	Error	8	17.92	2.24	
Temperature	Variation in lactic acid production between different temperatures	4	3550.644	887.661	319.53**
	Variation in lactic acid production within temperature	2	7.331	3.6655	1.319
	Error	8	22.23	2.778	
pH	Variation in lactic acid production between different pH	4	2207.462	551.8905	436.10**
	Variation in lactic acid production within the pH	2	4.105	2.0525	1.620
	Error	8	10.132	1.2665	
$CaCO_3$	Variation in lactic acid between different concentrations of $CaCO_3$	4	993.6	248.4	11.09**

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
	Variation in lactic acid production within different concentration of $CaCO_3$	2	2.316	1.158	0.413
	Error	8	22.38	2.798	
Spore concentration	Variation in lactic acid production between different spore concentrations	4	2307.6	576,9	1017.01**
	Variation in lactic acid production within spore concentration	2	1.072	0.536	0.9449
	Error	8	4.538	0.567	
Inoculum level	Variation in lactic acid production between different inoculum levels	4	1928.4	482.1	636.01**
	Variation in lactic acid production within the inooculum level	2	2.196	1.098	1.448
	Error	8	6.064	0.758	
Nitrogen sources	Variation in lactic acid production between different nitrogen sources	4	5175.6	1239.9	9762.9**
	Variation in lactic acid production within the nitrogen source	2	1.404	1.381	10.874*
	Error	8	1.016	0.127	
Ammonium sulphate concenteration	Variation in lactic acid production between different concentrations of ammonium sulphate	4	4160.32	1040.08	3525.69**
	Variation in lactic acid production within each concentration of ammonium sulphate	2	11.484	5.92	20.06*
	Error	8	2.36	0.295	

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Potassium dihydrogen phosphate	Variation in lactic acid production between different concentrations of potassium dihydrogen phosphate	4	3331.59	832.8	666.24**
	Variation in lactic acid production within the different concentration of potassium dihydrogen phosphate	2	3.345	1.675	1.34
	Error	8	10.028	1.25	
Magnesium sulphate	Variation in lactic acid production between different concentrations of magnesium sulphate	4	3070.31	767.57	504.98**
	Variation in lactic acid production within the different concentration of magnesium sulphate	2	0.30	0.15	0.09
	Error	8	12.239	1.52	
Zinc Sulphate	Variation in lactic acid production between different concentrations of zinc sulphate	4	7543.904	1885.975	1907.9**
	Variation in lactic acid production within the different concentration of zinc sulphate	2	3.892	1.946	1.9889
	Error	8	7.908	0.9885	

**, Significant at 1% level; *, Significant at 5% level

Table 9. Defined set of conditions and factors favourable for lactic acid production in submerged fermentation condition with glucose as a substrate

	Parameters		Conditions/Factors derived
(A)	Physical parameters		
	(i)	pH	5.5
	(ii)	Temperature	30 °C
	(iii)	Agitation speed	150 rpm
(B)	Biological parameters		
	(i)	Spore concentration	10^6 spores / ml
	(ii)	Inoculum level	1 %
(C)	Chemical parameters		
	(i)	Carbon source	12% (glucose)
	(ii)	Nitrogen source	Ammonium sulphate (0.2 % w/v)
	(iii)	Phosphate source	Potassium dihydrogen phosphate (0.015% w/v)
	(iv)	Magnesium source	Magnesium sulphate heptahydrate (0.025% w/v)
	(v)	Zinc source	Zinc sulphate heptahydrate (0.005% w/v)
	(vi)	Buffering agent	Calcium carbonate (4% w/v)

Table 10. **Evaluation of kinetic parameters for lactic acid production using glucose as a substrate under derived conditions of fermentation**

Kinetic parameters*	Wild type strain *R. oryzae* RB-5	Mutant strain *R. oryzae* RBU2-10
Lactic acid yield[a] (%) ($Y_{P/S}$)	49.25	70.22
Degree of conversion[b] (g/g)	0.476	0.585
Lactic acid production rate (g/l/h)	0.793	1.154
Specific glucose uptake rate (g/l/h)	1.611	1.644
Glucose utilization (%)	96.68	98.66
Biomass yield[c] (g/g) ($Y_{X/S}$)	0.057	0.070
Lactic acid from cells[d] (g/g) ($Y_{P/X}$)	8.52	9.89
Specific biomass growth rate (g/l/h)	0.093	0.116

* Analysed at 72 h under derived conditions of fermentation

[a] $Y_{P/S}$, Values calculated as per utilized glucose

[b] Degree of conversion (g/g), (η= p/s) p, product ; s, initial glucose concentration

[c] $Y_{X/S}$, values calculated on the basis of biomass obtained and the glucose utilized.

[d] $Y_{P/X}$, values calculated on the basis of lactic acid produced and the biomass obtained.

Fig. 10. LACTIC ACID PRODUCTION UNDER DIFFERENT FERMENTATION CONDITIONS

Chopped sugarcane bagasse, coconut coir and groundnut shell as solid supports were added with glucose (12%) in the defined fermentation medium for solid state fermentation. The fermentation was performed for 96 hours at 30°C (pH, 5.5). Submerged fermentation was carried out in the conditions as derived in Table 9.

▨, ▥ ; ▤, ▦ ; represent lactic acid production and lactic acid yield by mutant *R. oryzae* RBU2-10 and wild type *R. oryzae* RB-5 respectively.

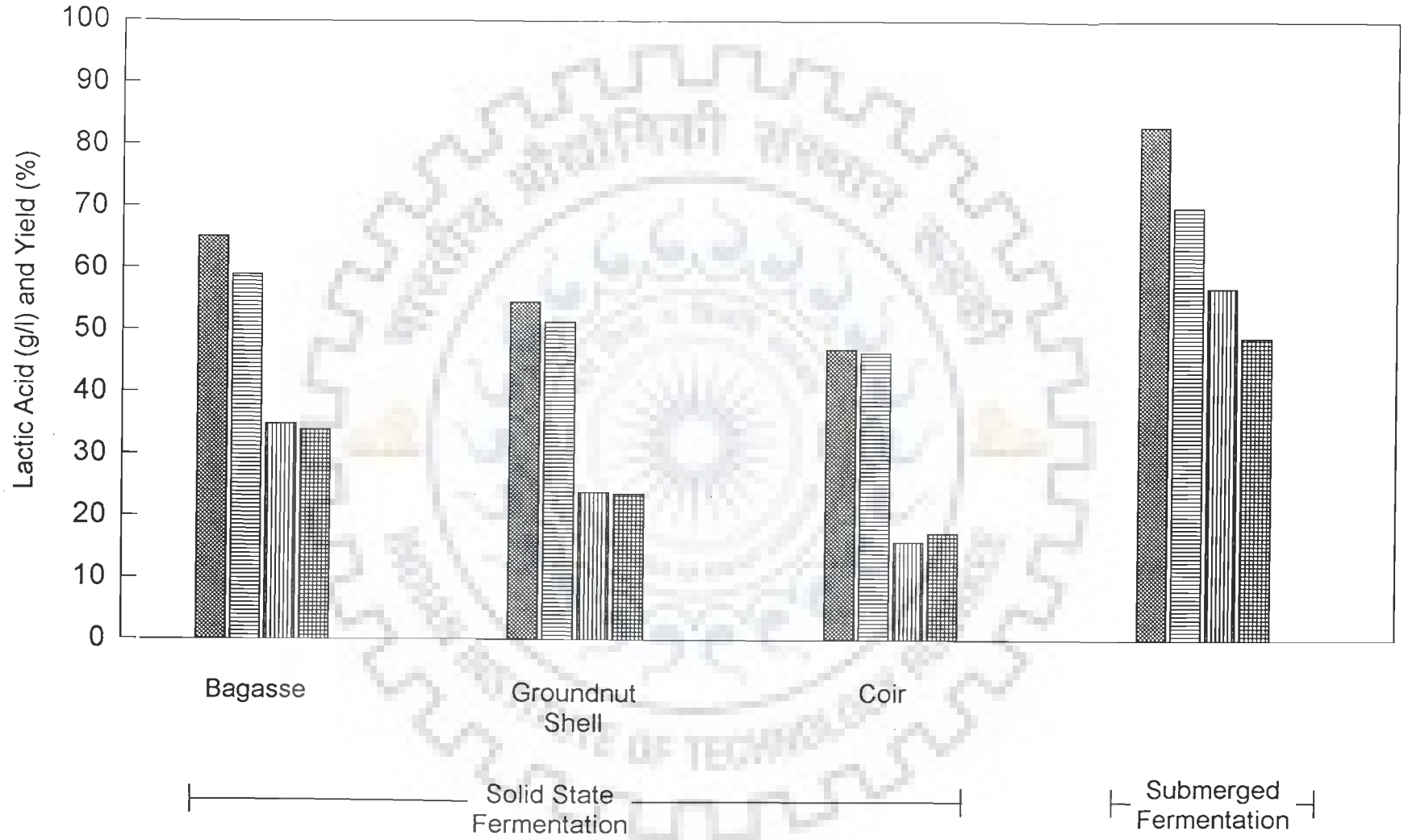

Lactic Acid (g/l) and Yield (%)
100
90
80
70
60
50
40
30
20
10
0
Bagasse
Groundnut Shell
Coir
Solid State Fermentation
Submerged Fermentation

Fig. 10

Fig. 11. LACTIC ACID PRODUCTION AT DIFFERENT TIME PERIODS DURING SOLID-STATE AND SUBMERGED FERMENTATION.

Solid-state (using bagasse as solid matrix) and submerged fermentation were carried out in conditions as defined in the legends for Fig. 10.

□ , ▨ ; represent the lactic acid production and yield by mutant *R. oryzae* RBU2-10 respectively.

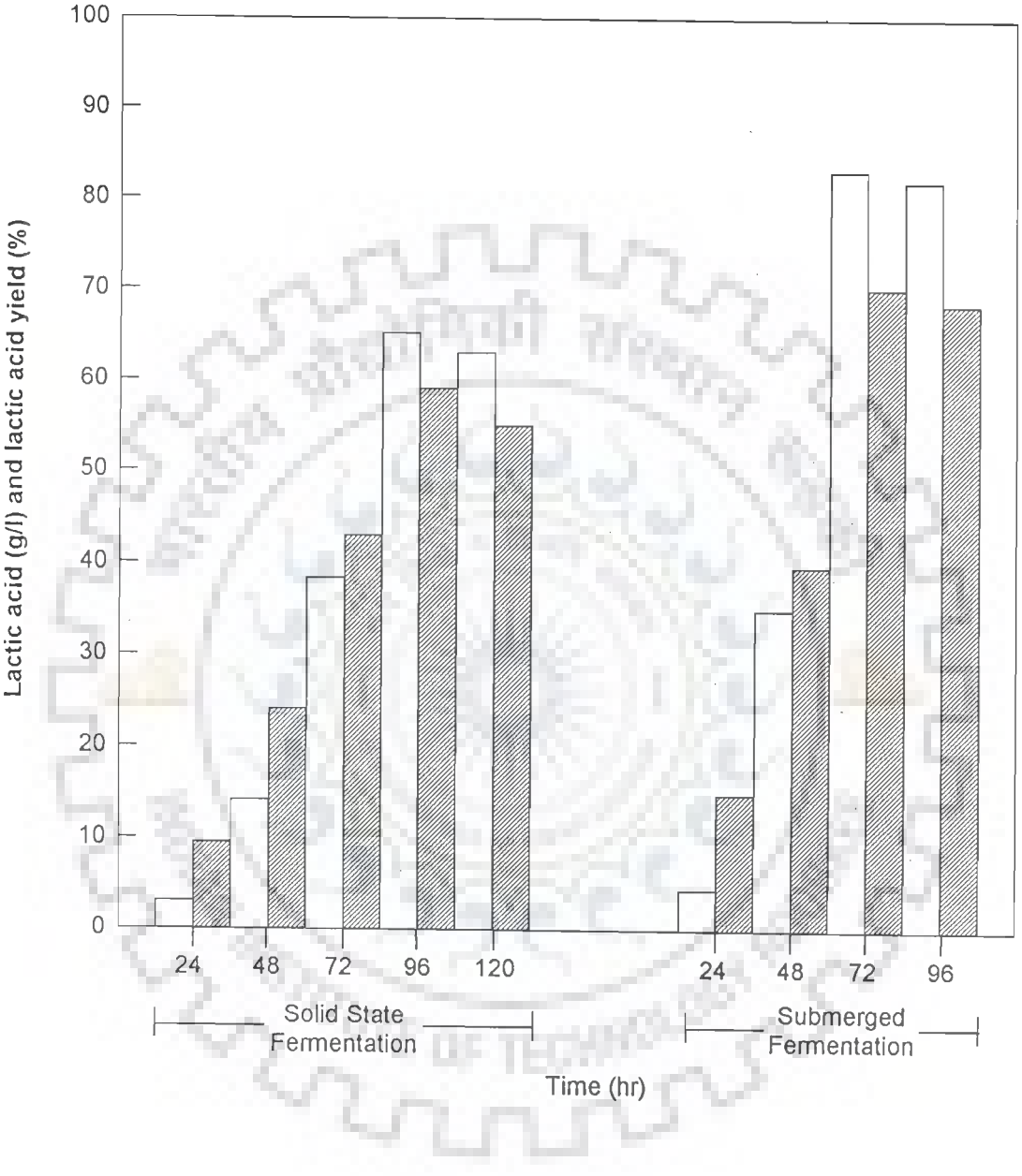

Lactic acid (g/l) and lactic acid yield (%)
100
90
80
70
60
50
40
30
20
10
0
24
48
72
96
120
24
48
72
96
Solid State Fermentation
Submerged Fermentation
Time (hr)

Fig. 11

of production was observed after 96 hrs (65.27 g/l), whereas the maximum levels of production under submerged condition (83.15 g/l) was observed after 72 hr of fermentation (Fig. 11).

4.5 LACTIC ACID PRODUCTION WITH CHEAPER CARBOHYDRATE SOURCES

Among the carbohydrate substrates, molasses and starch based substrates which are low priced and are available in plenty particularly in this part and in other parts of this country, were evaluated for lactic acid production. In the starchy substrates, broken and pest infested rice, market refuge potatoes, sago and arrowroot starch (acid and enzyme hydrolysed) were used.

Sugarcane molasses in the crude form has higher heavy metal content which are inhibitory to the lactic acid production. The crude molasses when used as a substrate had led into 17.14 g/l of lactic acid production with 18.98 % yield. However, rectification of molasses using potassium hexacyanoferrate had resulted into a increase in production level (31.46 g/l) as well as the yield of 28.80% by mutant strain RBU2-10 (Fig. 12).

Broken and pest infested rice, market refuge potatoes, arrowroot and sago starch were enzyme (α amylase, glucoamylase) or acid hydrolysed, since untreated starch following autoclaving may get contaminated due to incomplete sterilization inside the gelatinized starch particles. These substrates yielded relatively higher levels of production but maximum production (81.20 g/l) was obtained by the enzyme hydrolysed rice starch (Fig. 12) followed by the potato (74.04 g/l), arrowroot (69.72 g/l) and sago starch (68.36 g/l). Since among the cheap carbohydrate sources,

Fig. 12. LACTIC ACID PRODUCTION WITH CHEAPER CARBOHYDRATE SOURCES

Lactic acid production by mutant *Rhizopus oryzae* RBU2-10 was determined after 72 hours under submerged fermentation. Conditions of fermentation were as derived in the Table 9.

□, ▨ ; ▧ , ▩ ; ▤ , ▥ ; represent lactic acid production , lactic acid yield and biomass obtained by using acid and enzyme hydrolyzed starch substrates (12% sugar) respectively and crude and rectified molasses (12% sugar) respectively.

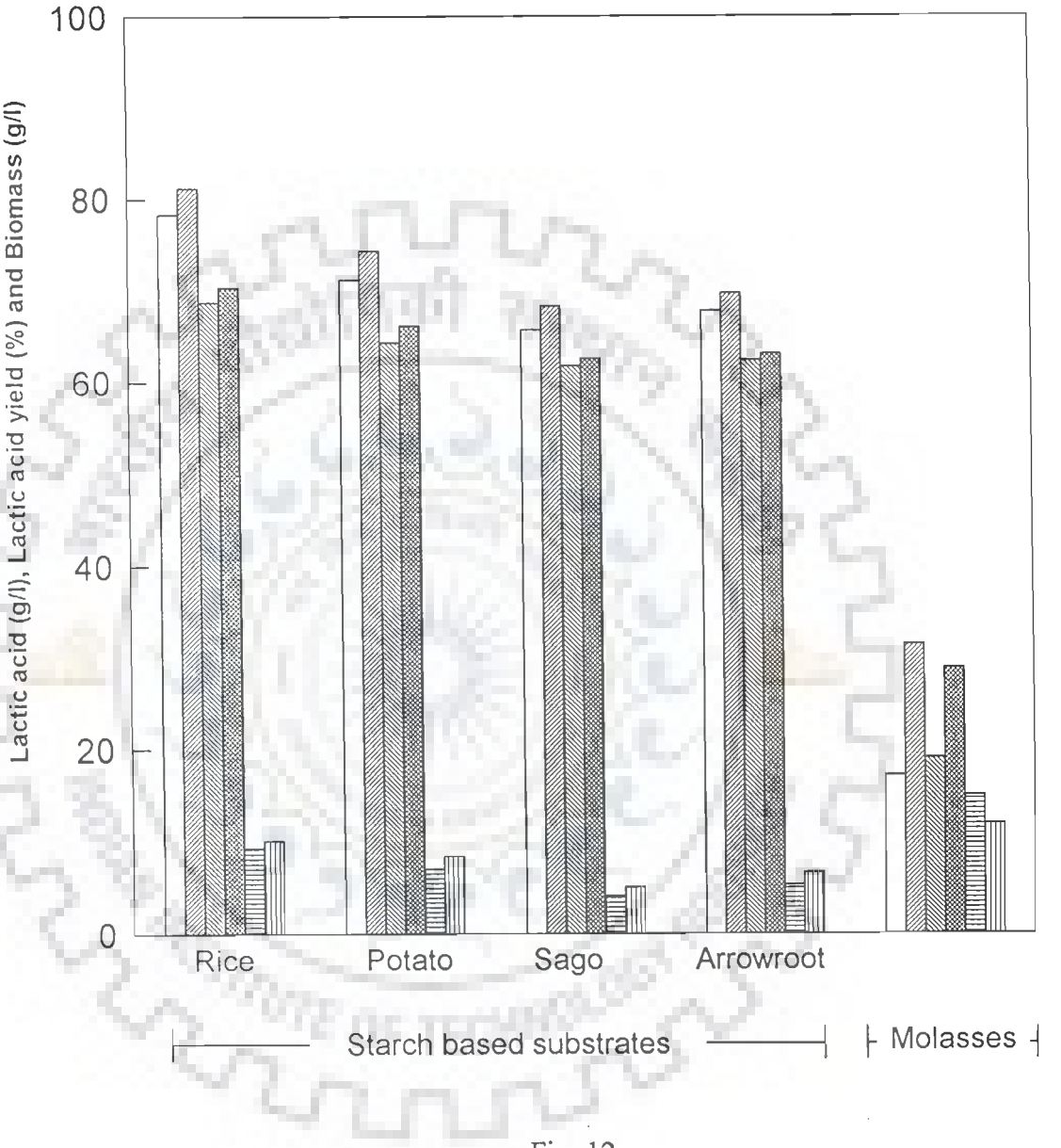

100
80
60
40
20
0
Lactic acid (g/l), Lactic acid yield (%) and Biomass (g/l)
Rice
Potato
Sago
Arrowroot
Starch based substrates
Molasses

Fig. 12

Fig. 13. EFFECT OF VARYING CONCENTRATIONS OF NUTRIENTS ON LACTIC ACID PRODUCTION USING HYDROLYZED RICE STARCH

Production of lactic acid was measured by mutant *Rhizopus oryzae* RBU2-10 under submerged fermentation in the conditions as defined in the legends for Fig. 12. The optimal level of nutrient derived in each stage was used for detecting the optimal level of nutrient in the subsequent stages. □, ▩; represent lactic acid level and yield respectively obtained by mutant *R. oryzae* RBU2-10.

rice starch yielded the highest concentration of lactic acid (81.20 g/l), therefore, evaluation of the various critical parameters for maximizing the lactic acid production by mutant *R. oryzae* RBU2-10 was carried out using this substrate.

4.5.1 Lactic Acid Production as a Function of Medium Composition in Rice Starch based Substrate

Some of the major nutrients affecting lactic acid production were added in varying concentrations. It was observed that $(NH_4)^{2+}$, PO_4^{3-}, Mg^{2+} and Zn^{2+} were essentially required respectively at (w/v) 0.1 %, 0.015 %, 0.025 %, 0.005 % concentrations for achieving the maximum production (86.13 g/l) of lactic acid (Fig. 13). A significant variation in lactic acid production ($P < 0.01$) was observed between various salt concentrations (Table 11).

4.5.2 Lactic Acid Production in Rice Starch Medium as a Function of Time

Minimal level of lactic acid production was detected after 24 hrs of fermentation. Rapid increase, in lactic acid production thereafter was observed leading to the maximum levels (86.13 g/l) obtained after 72 hrs of incubation (Fig. 14). An yield of 73.48% was obtained under these conditions.

Analysis of various kinetic parameters using glucose and enzyme hydrolysed rice starch had shown that higher degree of conversion (0.717 g/g) and the production rate (1.196 g/l/h) were observed with the enzyme hydrolysed starch substrate denoting therefore that enzyme hydrolysed rice starch was a potential substrate over glucose for lactic acid production by the mutant *Rhizopus oryzae* RBU2-10 (Table 12).

Fig. 13. EFFECT OF VARYING CONCENTRATIONS OF NUTRIENTS ON LACTIC ACID PRODUCTION USING HYDROLYZED RICE STARCH

Production of lactic acid was measured by mutant *Rhizopus oryzae* RBU2-10 under submerged fermentation in the conditions as defined in the legends for Fig. 12. The optimal level of nutrient derived in each stage was used for detecting the optimal level of nutrient in the subsequent stages. □, ▩; represent lactic acid level and yield respectively obtained by mutant *R. oryzae* RBU2-10.

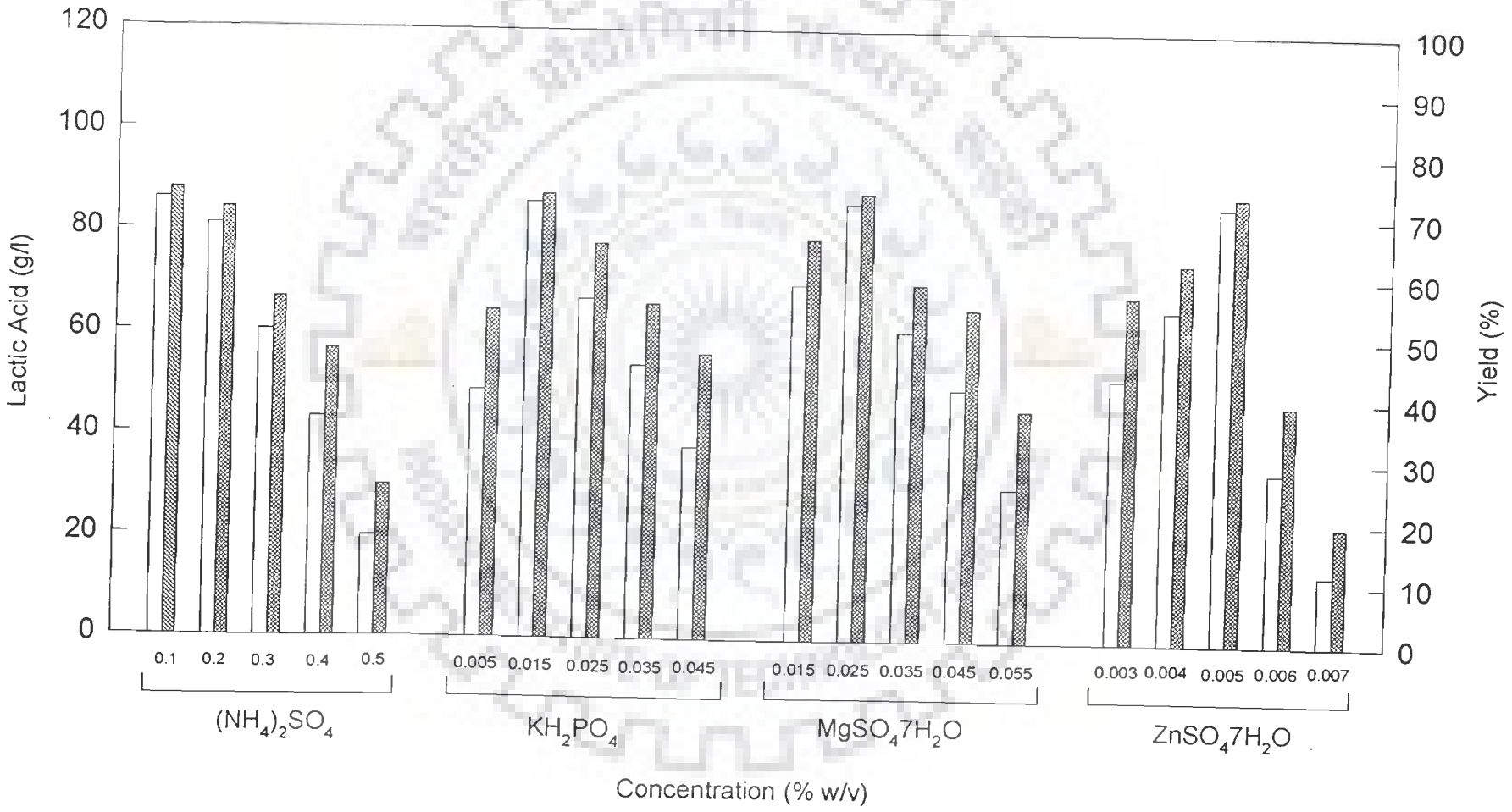

120
100
80
60
40
20
0
Lactic Acid (g/l)
100
90
80
70
60
50
40
30
20
10
0
Yield (%)
0.1 0.2 0.3 0.4 0.5
0.005 0.015 0.025 0.035 0.045
0.015 0.025 0.035 0.045 0.055
0.003 0.004 0.005 0.006 0.007
(NH4)2SO4
KH2PO4
MgSO47H2O
ZnSO47H2O
Concentration (% w/v)

Fig. 13

Table 11. **Analysis of variance for different nutrient concentrations on lactic acid production by *R. oryzae* RBU2-10 using enzyme hydrolyzed rice starch**

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Ammonium sulphate	Variation in lactic acid production between different concentrations of ammonium sulphate	4	8923.037	2230.7	1615.2**
	Variation in lactic acid production within the different concentration of ammonium sulphate	2	1.289	0.6445	0.4666
	Error	8	11.051	1.381	
Potassium dihydrogen phosphate	Variation in lactic acid production between different concentrations of potassium dihydrgen phosphate	4	4100.17	1025.04	1083.838**
	Variation in lactic acid production within the different concentration of potassium dihydrogen phosphate	2	1.861	0.9305	0.98392
	Error	8	7.566	0.9457	
Magnesium sulphate	Variation in lactic acid production between different concentrations of magnesium sulphate	4	5292.27	1323.06	603.03**
	Variation in lactic acid production within the different concentration of magnesium sulphate	2	42.865	21.432	9.768*
	Error	8	17.552	2.194	
Zinc sulphate	Variation in lactic acid between different concentration of zinc sulphate	4	9317.897	2329.474	347.68**
	Variation in lactic acid production within different concentration of zinc sulphate	2	30.1	15.05	2.24
	Error	8	53.667	6.70	

**, Significant at 1% level; *, Significant at 5% level

Table 12. **Evaluation of kinetic parameters for lactic acid production by *R. oryzae* RBU2-10 using glucose and enzyme hydrolyzed rice starch as the substrates under derived conditions of fermentation**

Kinetic parameters*	Substrate utilized	
	Glucose	Enzyme hydrolyzed rice starch
Lactic acid yield[a] (%) ($Y_{P/S}$)	70.22	73.48
Degree of conversion[b] (g/g)	0.585	0.717
Lactic acid production rate (g/l/h)	1.154	1.196
Specific substrate uptake rate (g/l/h)	1.644	1.629
Substrate utilization (%)	98.66	97.75
Biomass yield[c] (g/g) ($Y_{X/S}$)	0.070	0.069
Lactic acid from cells[d] (g/g) ($Y_{P/X}$)	9.89	10.633
Specific biomass growth rate (g/l/h)	0.116	0.112

* Analysed at 72 h under derived conditions of fermentation

[a] $Y_{P/S}$, Values calculated as per utilized substrate

[b] Degree of conversion (g/g), (η= p/s) p, product ; s, initial substrate concentration

[c] $Y_{X/S}$, values calculated on the basis of biomass obtained and the substrate utilized.

[d] $Y_{P/X}$, values calculated on the basis of lactic acid produced and the biomass obtained.

Fig. 14 LACTIC ACID PRODUCTION IN RICE STARCH MEDIUM AT DIFFERENT TIME PERIODS.

Production of lactic acid in submerged cultivation in the medium containing rice starch (12% sugar) supplemented with ammonium sulphate, potassium dihydrogen phosphate, magnesium sulphate and zinc sulphate (% w/v 0.1, 0.015, 0.025, 0.005 respectively) was carried out under conditions as defined in the legends of Fig. 12.

● , ■ , ▲; represent the lactic acid level, yield and biomass respectively.

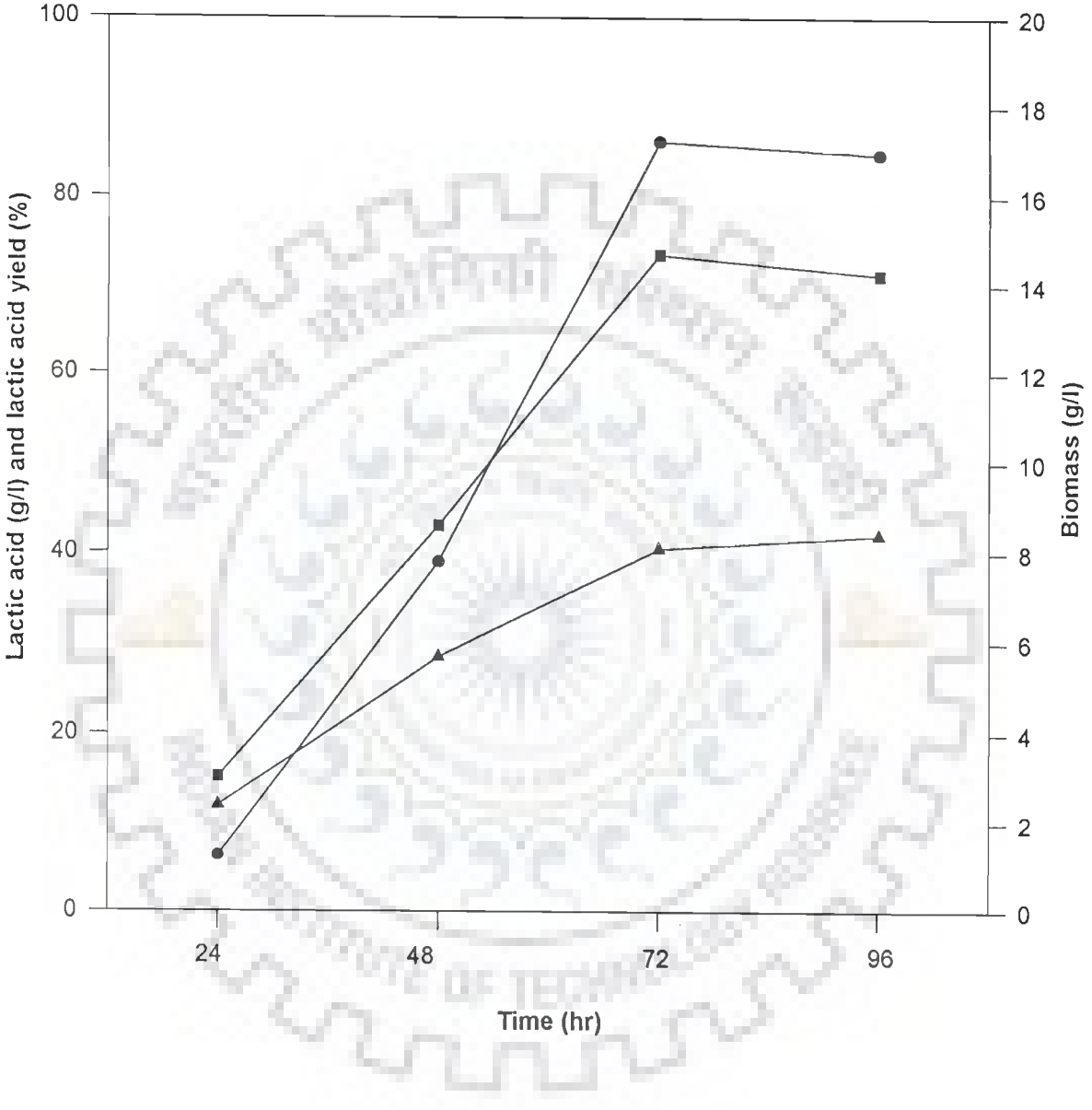

100
80
60
40
20
0
Lactic acid (g/l) and lactic acid yield (%)
20
18
16
14
12
10
8
6
4
2
0
Biomass (g/l)
24
48
72
96
Time (hr)

Fig. 14

4.6 LACTIC ACID PRODUCTION BY IMMOBILIZED CELLS OF *R. ORYZAE* RBU2-10 USING HYDROLYZED RICE STARCH CONTAINING MEDIUM

Immobilization of *R. oryzae* RBU2-10 spores in two matrices i.e. calcium alginate beads (0.3 cm diameter) and loofa sponge (1.6 x 1.8 x 0.35 cm size) was carried out and the defined rice starch containing fermentation medium was used for detecting the lactic acid production levels. Since loofa sponge (Plate 6) is a natural material hence its ability to withstand the wider variations in the pH and temperature was studied. The pieces of loofa sponge remained unchanged with respect to its shape and texture when they were treated with buffers of varying pH (1.1 – 14) for 15 days and also resisted any change after repeated autoclaving for different time periods (20 – 40 min at 121°C) and as much as even 4 times at 121°C for 20 minutes.

4.6.1 Evaluation and Derivation of Various Conditions for Immobilized *R. oryzae* RBU2-10 for Lactic Acid Production

4.6.1.1 Lactic Acid Production as a Function of Size of Immobilized Matrices

Use of different sized alginate beads for production had shown that beads of 0.5 cm diameter had resulted into maximum production (54.25 g/l) (Fig. 15a) ($P < 0.01$, Table 13). Loofa sponge on the other hand at the dimensions of 1.6 x 1.8 x 0.35 cm had resulted into comparatively higher levels (63.27 g/l) of production (Fig. 15b) ($P < 0.01$, Table 14). Thus loofa sponge had shown 16.62% higher levels of production than the calcium alginate beads.

PLATE 6. **LOOFA SPONGE**

A : SURFACE VIEW

B : VIEW OF HORIZONTAL SECTION

C – D : VIEW OF VERTICAL SECTION

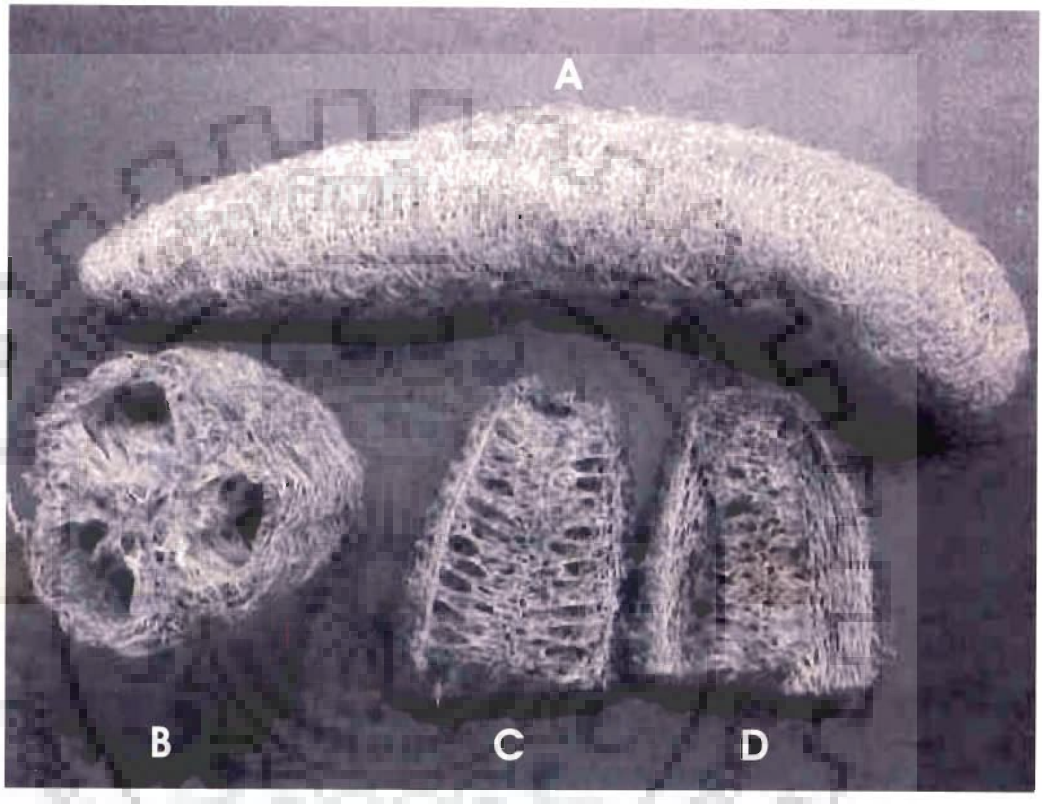


PLATE - 6

5 LACTIC ACID PRODUCTION AS A FUNCTION OF VARIATION OF SIZE OF IMMOBILIZED MATRICES

Rhizopus oryzae RBU2-10 immobilized in (a) calcium alginate beads and (b) loofa sponge of varying size were used for submerged fermentation in the rice starch (12% sugar) containing fermentation medium. Other conditions of fermentations were same as described in the legends of Fig. 14.

▨ , ● ; represent the lactic acid level and biomass respectively.

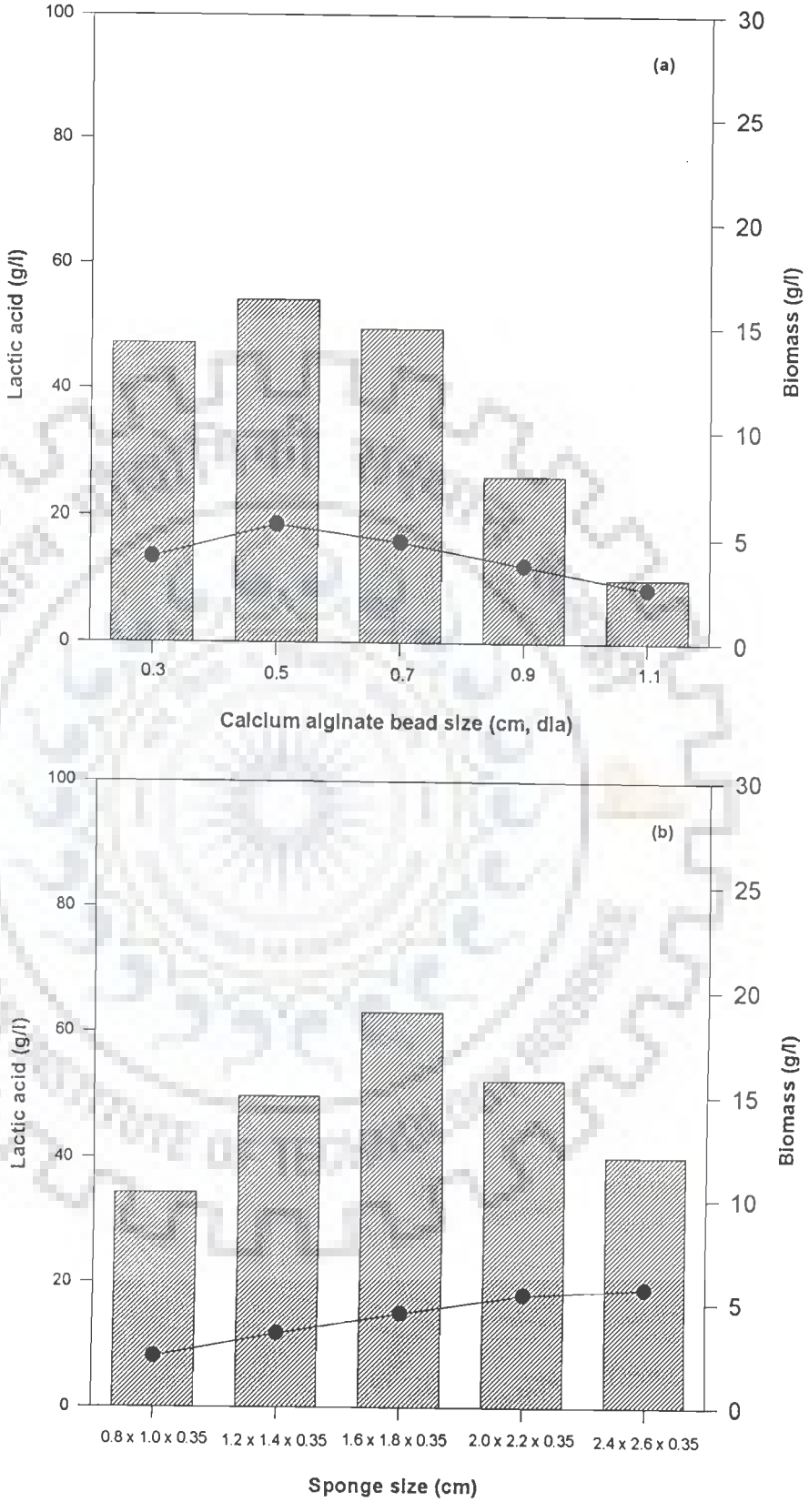

(a)
Lactic acid (g/l)
Biomass (g/l)
100
80
60
40
20
0
30
25
20
15
10
5
0
0.3
0.5
0.7
0.9
1.1
Calcium alginate bead size (cm, dia)
(b)
Lactic acid (g/l)
Biomass (g/l)
0.8 x 1.0 x 0.35
1.2 x 1.4 x 0.35
1.6 x 1.8 x 0.35
2.0 x 2.2 x 0.35
2.4 x 2.6 x 0.35
Sponge size (cm)

Fig. 15

4.6.1.2 Lactic Acid Production as Function of Spore Suspension in Various Matrices

An increase in spore suspension from 1% - 3% led to 28.62% increase in lactic acid production (69.78 g/l) (Fig. 16a) ($P < 0.01$, Table 13) by the calcium alginate immobilized spores of *R. oryzae* RBU2-10. However, a further increase in spore suspension to 4% led to an increase in the biomass but a reduced level of lactic acid production. The spore suspension at 3% was also favorable for lactic acid production (73.66 g/l) ($P < 0.01$, Table 14) by loofa sponge immobilized cells of *R. oryzae* RBU2-10. Higher levels of spore suspension decreased the lactic acid production ability of the loofa sponge immobilized *R. oryzae* RBU2-10 cells (Fig. 16b) (Plate 7).

4.6.1.3 Lactic Acid Production as a Function of Immobilized Inoculum Level

Amongst the various levels of inoculum used, 15% w/v calcium alginate beads containing immobilized *R. oryzae* RBU2-10 had resulted into 75.09 g/l of lactic acid production (Fig. 17a). Level of significance are up to 1% between different levels of inoculum (Table 13). However, the mutant RBU2-10 immobilized in the loofa sponge appeared more productive. As a 4% inoculum (4 pieces of loofa sponge, of 1.008 cm^3 : 1.6 x 1.8 x 0.35 cm, per 100 ml media) had led to increased levels of lactic acid production (80.75 g/l) ($P < 0.01$, Table 14) (Fig. 18b).

4.6.1.4 Lactic Acid Production by *R. oryzae* RBU2-10 at Varying Time Intervals

Lactic acid production with calcium alginate immobilized *R. oryzae* RBU2-10 was detected after 24 hours of fermentation and reached to the maximum (75.09 g/l) after 72 hrs of incubation with a biomass generation of about 6.40 g/l (Fig. 18a) whereas higher levels of production (80.75 g/l) was obtained in relatively shorter

Fig. 16. LACTIC ACID PRODUCTION AS A FUNCTION OF SPORE SUSPENSION IN VARIOUS MATRICES

Varying levels of spores (1-5 x 10^6 spores/ml) of Rhizopus oryzae RBU2-10 were immobilized in (a) calcium alginate (0.5 cm dia) beads and (b) loofa sponge (1.008 cm^3 : 1.6 x 1.8 x 0.35 cm) using the derived rich starch (12% sugar) containing fermentation medium. The lactic acid production was estimated in calcium alginate and loofa sponge immobilized matrices after 72 hours and 48 hours of incubation respectively. Other conditions of fermentation we same as described in the legends of Fig. 14.

▨ , ● ; represent the lactic acid level and biomass obtained respectively.

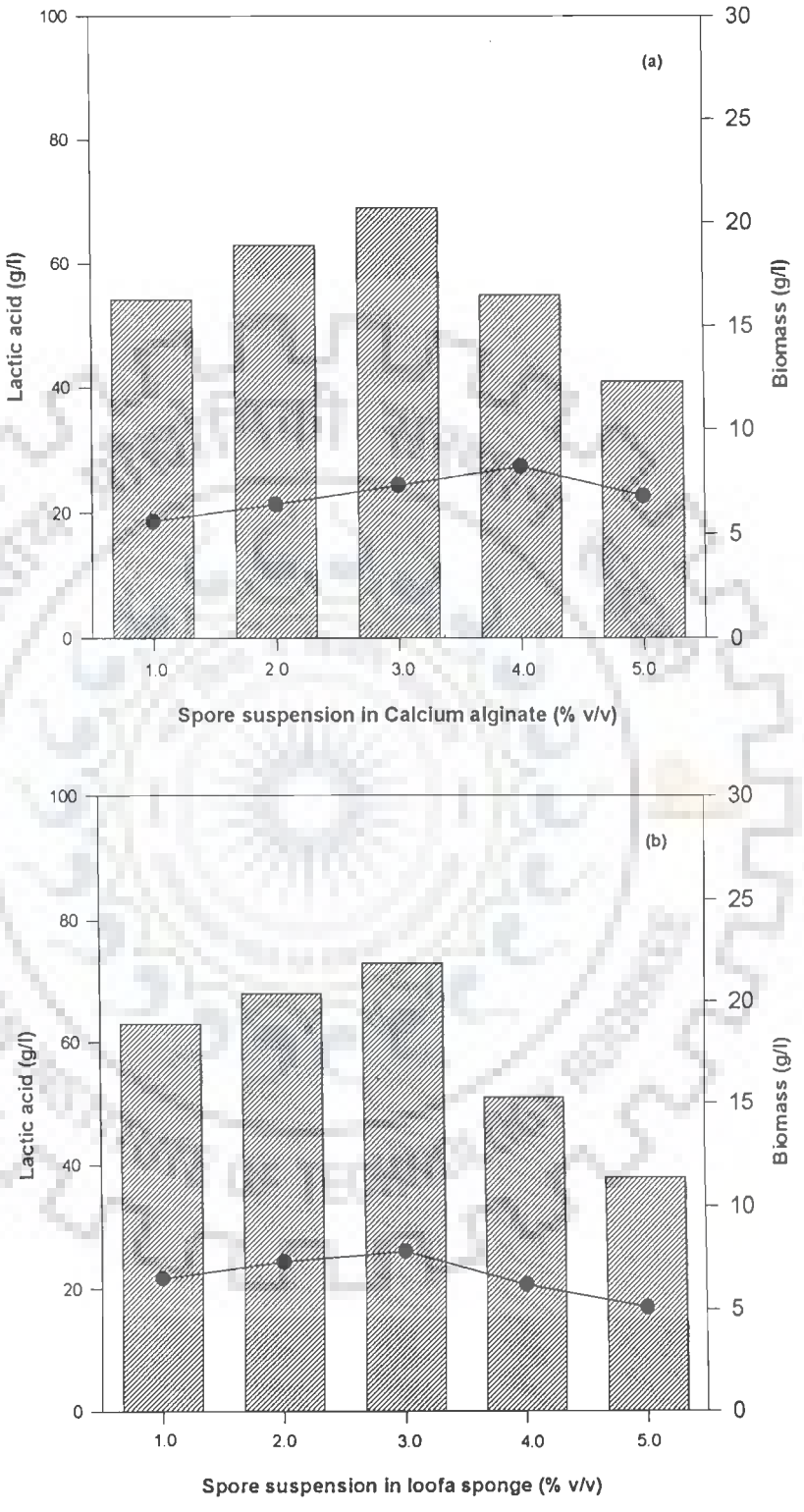

(a)
Lactic acid (g/l)
Biomass (g/l)
100
80
60
40
20
0
30
25
20
15
10
5
0
1.0
2.0
3.0
4.0
5.0
Spore suspension in Calcium alginate (% v/v)
(b)
Lactic acid (g/l)
Biomass (g/l)
Spore suspension in loofa sponge (% v/v)

Fig. 16

Fig. 17. LACTIC ACID PRODUCTION AS A FUNCTION OF IMMOBILIZED INOCULUM LEVEL

Lactic acid production with varying inoculum levels of (a) calcium alginate beads (0.5 cm dia) and (b) loofa sponge (1.008 cm^3 : 1.6 x 1.8 x 0.35 cm) immobilized spores (3 x 10^6 spores/ml) of mutant *R. oryzae* RBU2-10 was used. The fermentation was carried out using rice starch (12% sugar) containing medium in the conditions as described in the legends of Fig. 14.

▨ , ● ; represent the lactic acid levels and biomass obtained respectively.

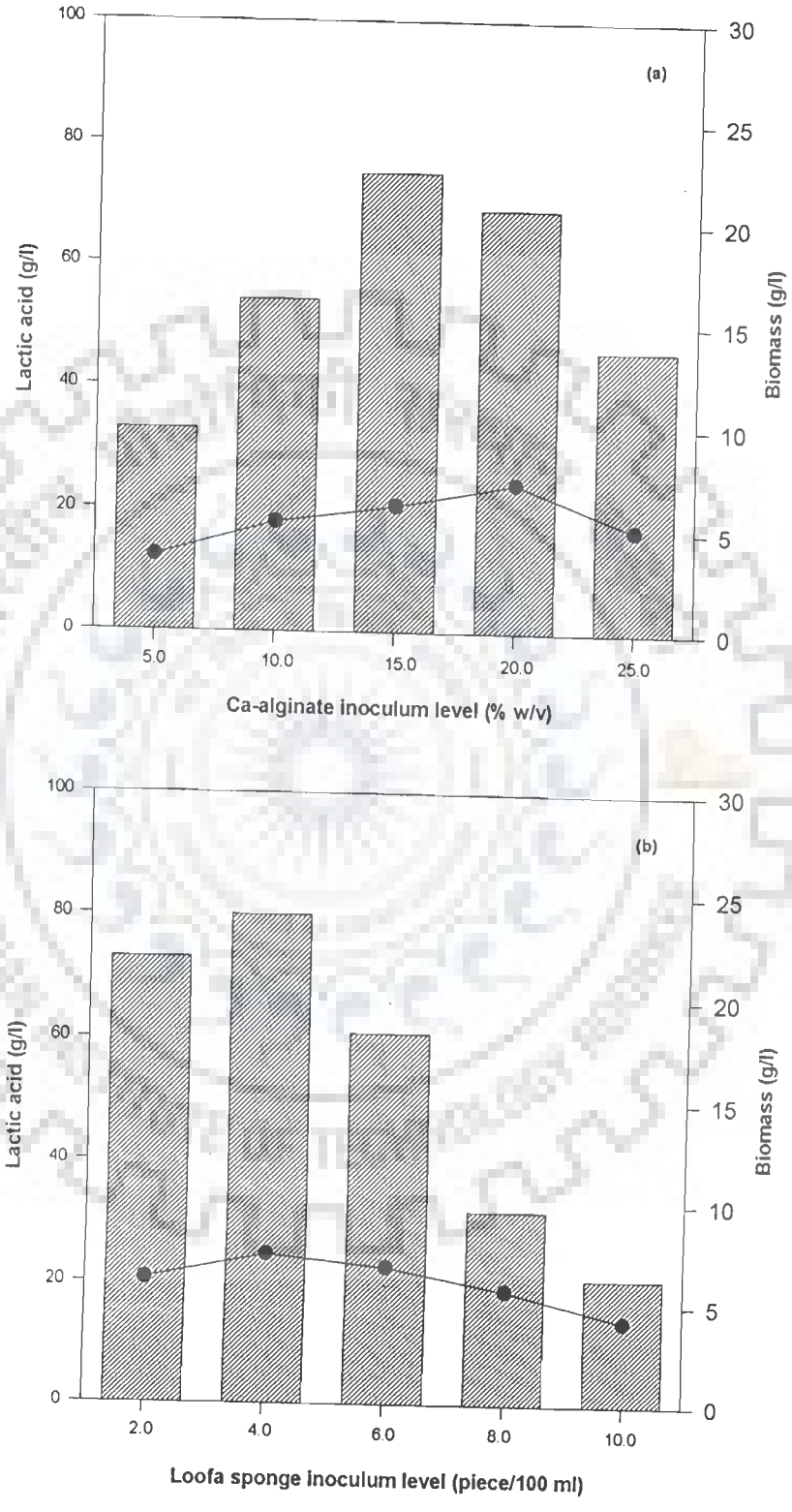

(a)
Lactic acid (g/l)
Biomass (g/l)
100
80
60
40
20
0
30
25
20
15
10
5
0
5.0
10.0
15.0
20.0
25.0
Ca-alginate inoculum level (% w/v)
(b)
Lactic acid (g/l)
Biomass (g/l)
2.0
4.0
6.0
8.0
10.0
Loofa sponge inoculum level (piece/100 ml)

Fig. 17

Table 13. Analysis of variance for multiple parameters for lactic acid fermentation by calcium alginate immobilized cells of *R. oryzae* RBU2-10

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Bead size	Variation in lactic acid production between different sizes of beads	4	4180.12	1045.03	262.834**
	Variation in lactic acid production within the bead size	2	2.212	1.106	0.278
	Error	8	31.808	3.976	
Spore suspension	Variation in lactic acid production between different spore concentrations in calcium alginate beads	4	1366.31	341.577	73.9664**
	Variation in lactic acid production within the spore concentration	2	3.884	1.942	0.4205
	Error	8	36.949	4.618	
Inoculum level	Variation in lactic acid production between different inoculum levels	4	3453.655	863.413	527.114**
	Variation in lactic acid production within the each inoculum level	2	0.449	0.2245	0.1370
	Error	8	13.104	1.638	

**, Significant at 1% level; *, Significant at 5% level

Table 14. **Analysis of variance for lactic acid production by *R. oryzae* RBU2-10 cells immobilized in loofa sponge**

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Sponge size	Variation in lactic acid production between different sponge size	4	1501.357	375.339	115.6715**
	Variation in lactic acid production within the sponge size	2	10.001	5.0005	1.541
	Error	8	25.959	3.2448	
Spore suspension	Variation in lactic acid production between different concentrations of spore in loofa sponge	4	2371.746	592.936	250.183**
	Variation in lactic acid production within the each spore concentration	2	2.604	1.302	0.549
	Error	8	18.96	2.37	
Inoculum level	Variation in lactic acid production between different inoculum levels	4	7940.86	1985.215	36094.81**
	Variation in lactic acid production within each inoculum level	2	28.756	14.378	261.418
	Error	8	0.444	0.055	

**, Significant at 1% level; *, Significant at 5% level

Fig. 18. LACTIC ACID PRODUCTION AS A FUNCTION OF FERMENTATION TIME

(a) Lactic acid production using 15% of calcium alginate beads and (b) loofa sponge (4 pieces/100 ml) immobilized *R. oryzae* RBU2-10 was carried out under conditions as described in the legends of Fig. 17.

△ , ● ; represent the lactic acid level and biomass obtained respectively.

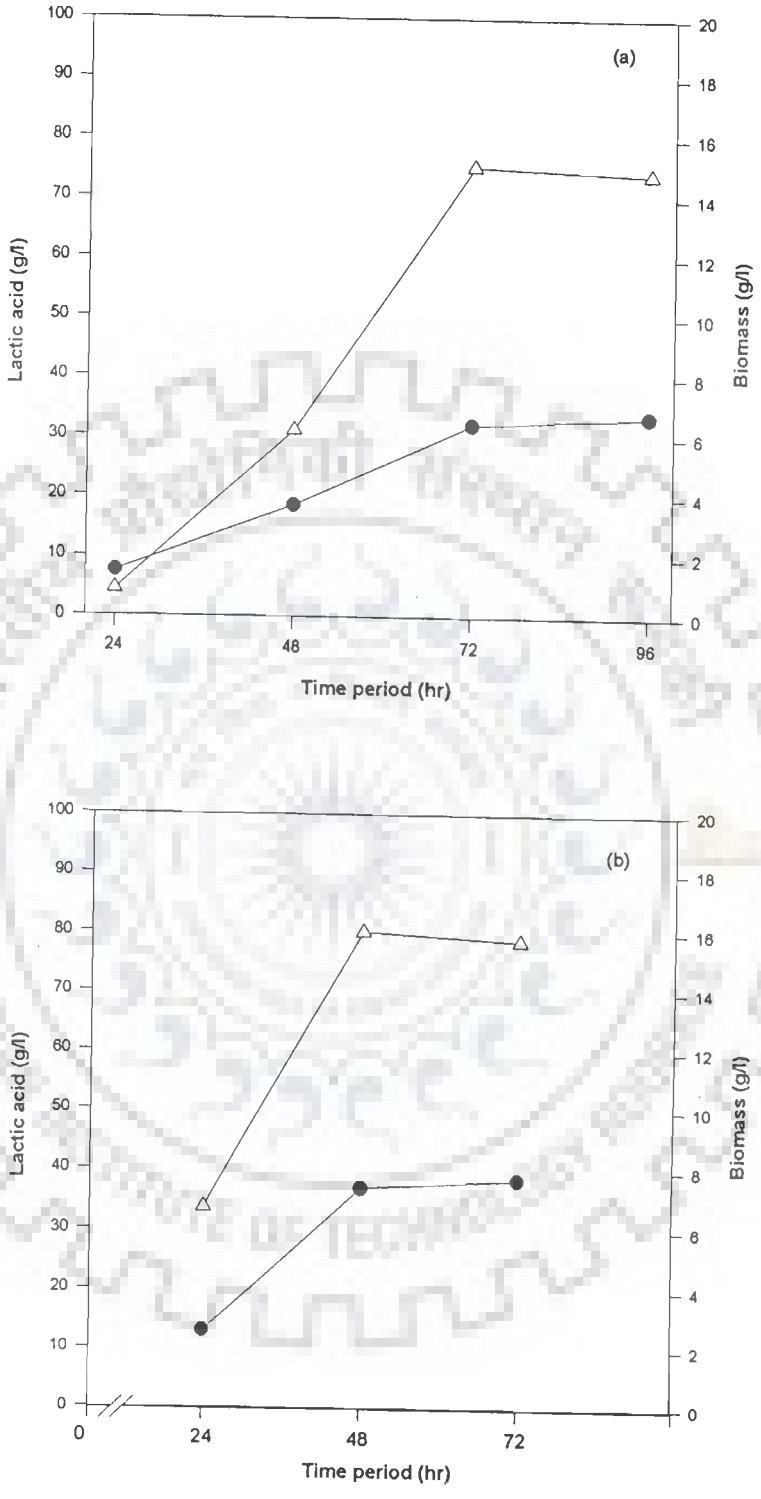

(a)
Lactic acid (g/l)
Biomass (g/l)
Time period (hr)
24
48
72
96
(b)
Lactic acid (g/l)
Biomass (g/l)
Time period (hr)
0
24
48
72

Fig. 18

period i.e. 48 hrs by *R. oryzae* RBU2-10 immobilized in loofa sponge (Fig. 18b) with comparatively higher biomass formation (7.61 g/l) under these conditions. The most favourable conditions for lactic acid production by *R. oryzae* RBU2-10 immobilized in loofa sponge have been defined in Table 15.

4.6.2 Semi-continuous Production of Lactic Acid by Calcium Alginate and Loofa Sponge Immobilized *R. oryzae* RBU2-10

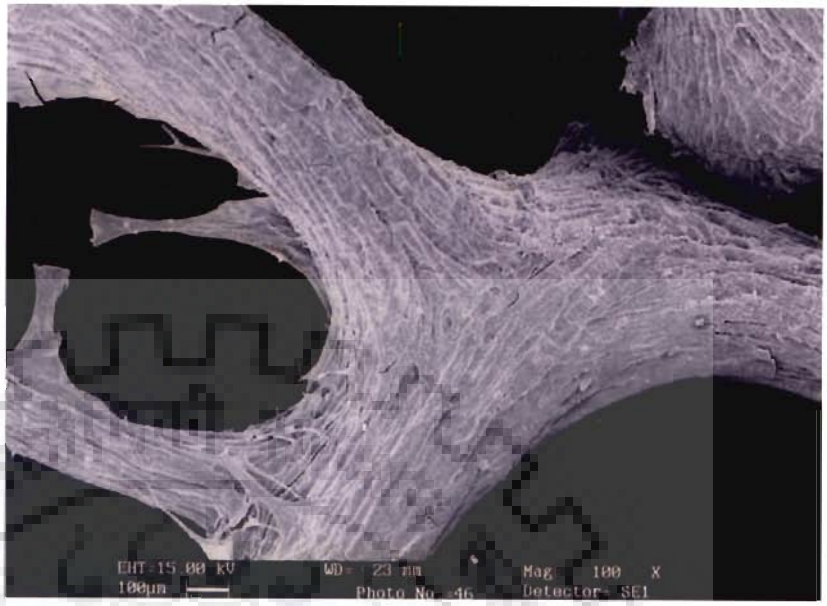
Lactic acid production with calcium alginate immobilized RBU2-10 cells reached to its maximum after 72 hrs as detected earlier. So fermentation medium was replaced after every 72 hrs so as to assess the ability of the immobilized cells to produce lactic acid during multiple fermentation cycles. The fermentation reaction was carried for a total of 8 cycles. The productivity obtained ranged between of 1.05-1.19 g/l/h during the first four cycles, thereafter, it decreased rapidly (Fig. 19).

R. oryzae RBU2-10 immobilized in loofa sponge were more productive and more than 60 g/l of lactic acid was produced during I – VII cycles of fermentation with a productivity varying between 1.27 – 1.84 g/l/h during these cycles (Fig. 20). Decrease in lactic acid productivity occurred thereafter. The third cycle of fermentation resulted into 88.68 g/l of lactic acid which was higher than the production obtained from free cells of *R. oryzae* RBU2-10 (86.13 g/l) under desired conditions. In semicontinuous production with free cells, the productivity ranging between 0.86 – 1.19 g/l/h was obtained during the I – IV cycle and decreased thereafter. The fermentation reaction can be performed till VI cycle as the pellets after VI^{th} cycle changed to mycelial flocs and chips (Fig. 21). Kinetic analysis of the lactic acid production in various cycles of production by loofa sponge indicated that higher

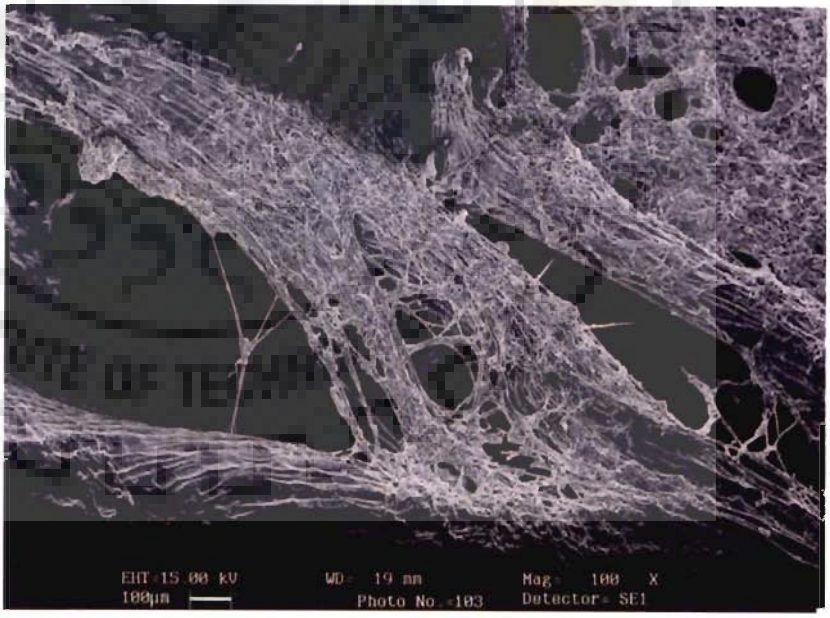
PLATE 7. **SCANNING ELECTRON MICROGRAPH OF *RHIZOPUS ORYZAE* RBU2-10 IMMOBILIZED ON LOOFA SPONGE**

A : UNINOCULATED LOOFA SPONGE

B : LOOFA SPONGE IMMOBILIZED WITH *RHIZOPUS ORYZAE* RBU2-10 MYCELIA


EHT=15.00 kV
WD= 23 mm
Mag= 100 X
100µm
Photo No.=46
Detector= SE1

A


EHT=15.00 kV
WD= 19 mm
Mag= 100 X
100µm
Photo No.=103
Detector= SE1

B

PLATE - 7

Table 15. Defined set of conditions and factors favourable for lactic acid production b *Rhizopus oryzae* RBU2-10 immobilized in loofa sponge with enzyn hydrolyzed rice starch as a substrate

	Parameters		Conditions/Factors derived
(A)	Physical parameters		
	(i)	pH	5.5
	(ii)	Temperature	30 °C
	(iii)	Agitation speed	150 rpm
	(iv)	Dimensions of loofa sponge	1.6 x 1.8 x 0.35 cm
(B)	Biological parameters		
	(i)	Spore concentration	3 x 10^6 spores / ml
	(ii)	Immobilized inoculum level	4 %
(D)	Chemical parameters		
	(i)	Carbon source	12% (reducing sugar)
	(ii)	Nitrogen source	Ammonium sulphate (0.1 % w/v)
	(iii)	Phosphate source	Potassium dihydrogen phosphate (0.015% w/v)
	(iv)	Magnesium source	Magnesium sulphate heptahydrate (0.025% w/v)
	(v)	Zinc source	Zinc sulphate heptahydrate (0.005% w/v)
	(vi)	Buffering agent	Calcium carbonate (4% w/v)

Fig. 19. SEMICONTINUOUS PRODUCTION OF LACTIC ACID BY CALCIUM ALGINATE IMMOBILIZED *RHIZOPUS ORYZAE* RBU2-10

Rhizopus oryzae RBU2-10 immobilized in calcium alginate beads were innoculated in defined rice starch fermentation medium containing 12% sugar for lactic acid production under the conditions as described in the legends for Fig. 17. The lactic acid was estimated at an interval of 24 hr till 72 hr of fermentation.

▨, ⊠, ▤ ; represent the lactic acid levels at 24, 48 and 72 hours respectively whereas ●, represent the productivity.

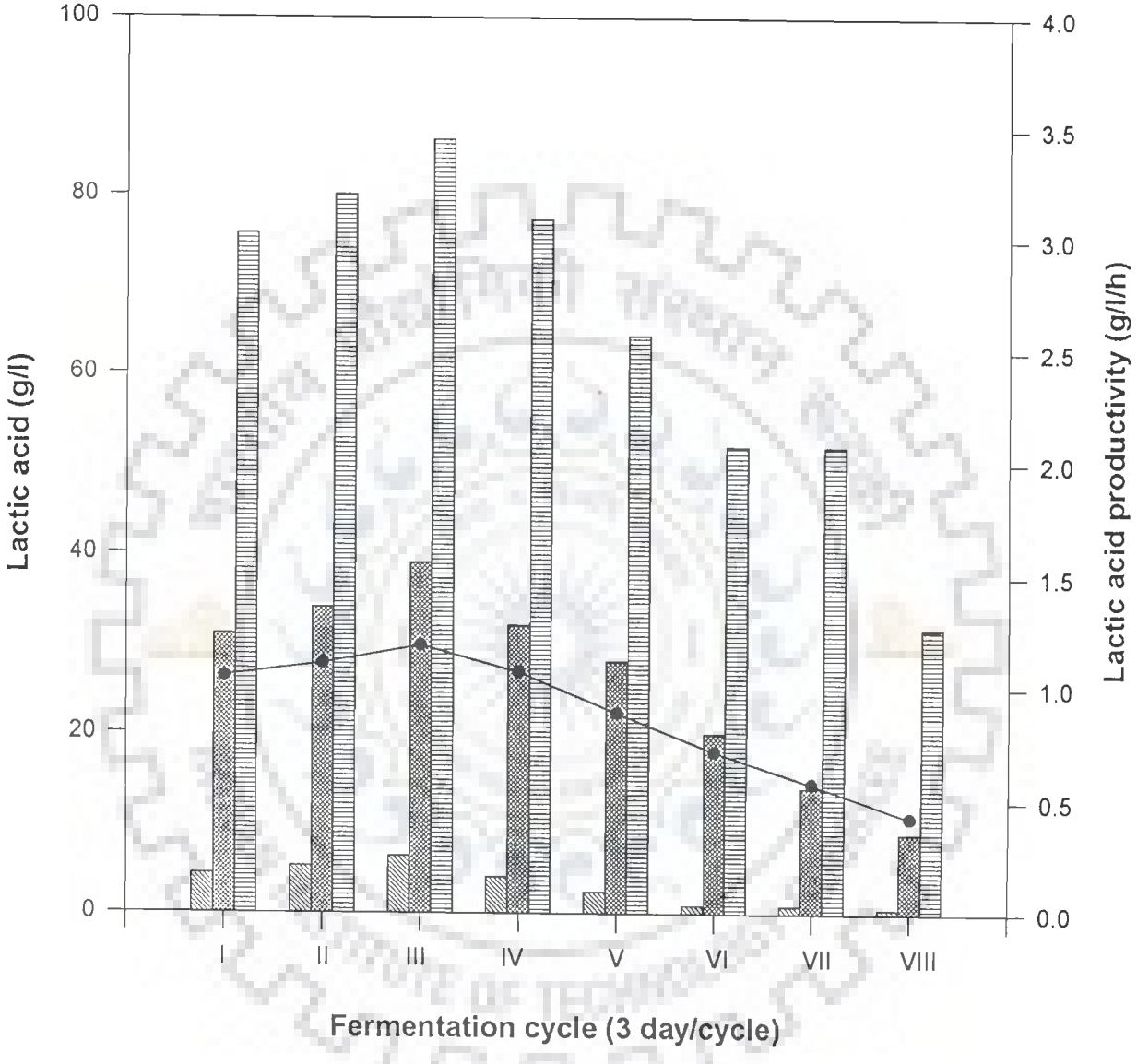

100
80
60
40
20
0
Lactic acid (g/l)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Lactic acid productivity (g/l/h)
I
II
III
IV
V
VI
VII
VIII
Fermentation cycle (3 day/cycle)

Fig. 19

Fig. 20. SEMICONTINUOUS PRODUCTION OF LACTIC ACID BY LOOFA SPONGE IMMOBILIZED *RHIZOPUS ORYZAE* RBU2-10

Rhizopus oryzae RBU2-10 immobilized in loofa sponge were inoculated in the derived rice starch fermentation medium containing 12% sugar for lactic acid production under conditions as described in the legends of Fig. 17. The lactic acid was estimated at an interval of 24 hr till 48 hr. ⊠ , ⊟ ; represent the lactic acid levels at 24 and 48 hours respectively whereas ● , represents productivity.

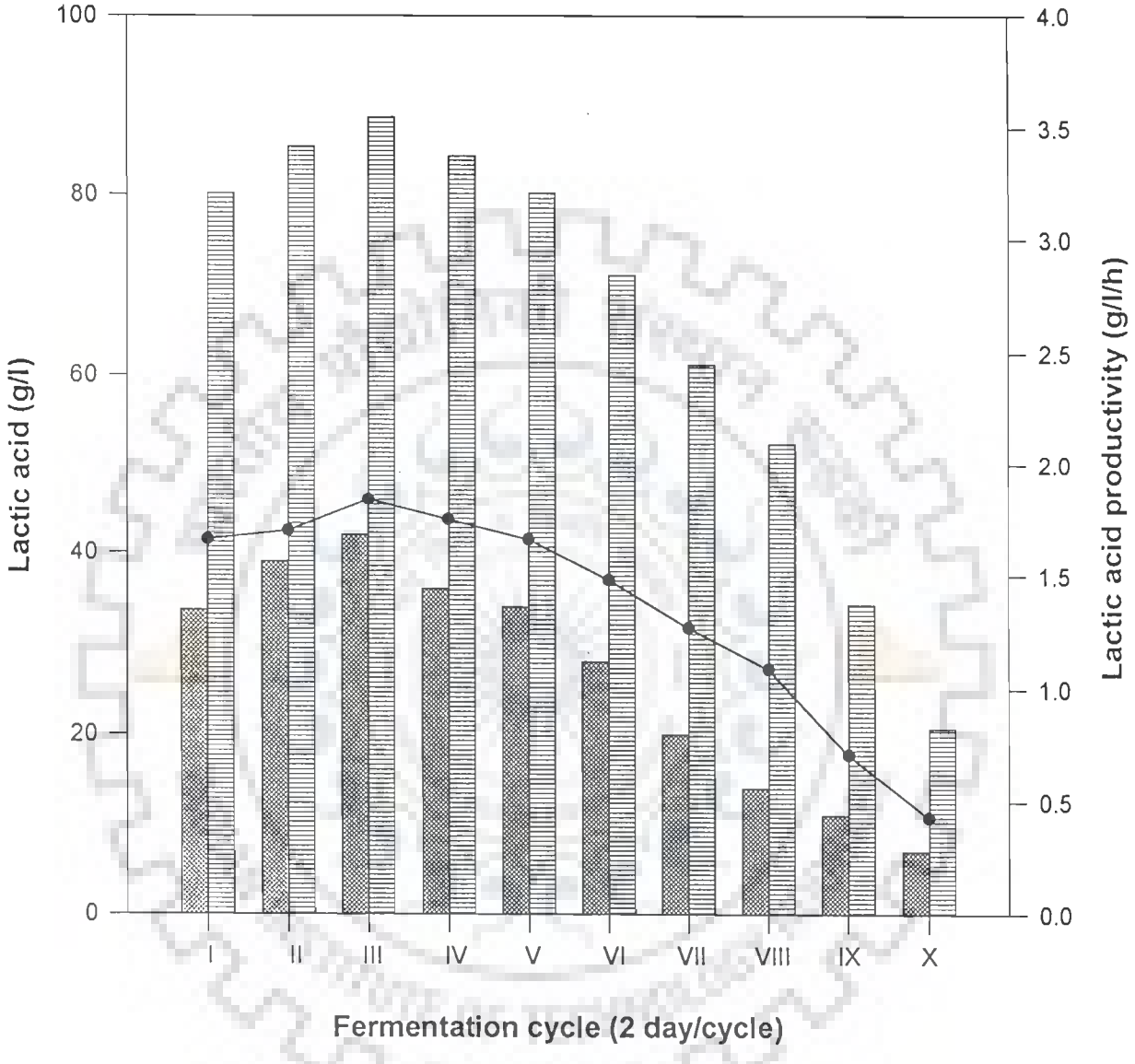

100
80
60
40
20
0
Lactic acid (g/l)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Lactic acid productivity (g/l/h)
I
II
III
IV
V
VI
VII
VIII
IX
X
Fermentation cycle (2 day/cycle)

Fig. 20

Fig. 21. SEMICONTINUOUS PRODUCTION OF LACTIC ACID BY FREE CELLS OF *RHIZOPUS ORYZAE* RBU2-10

Lactic acid production in the derived rice starch fermentation medium containing 12% sugar was carried out under conditions of Fig. 14. The lactic acid was estimated at an interval of 24 hr till 72 hr.

▨, ▩, ▤ ; represent the lactic acid levels at 24, 48 and 72 hours respectively whereas ●, represent the productivity.

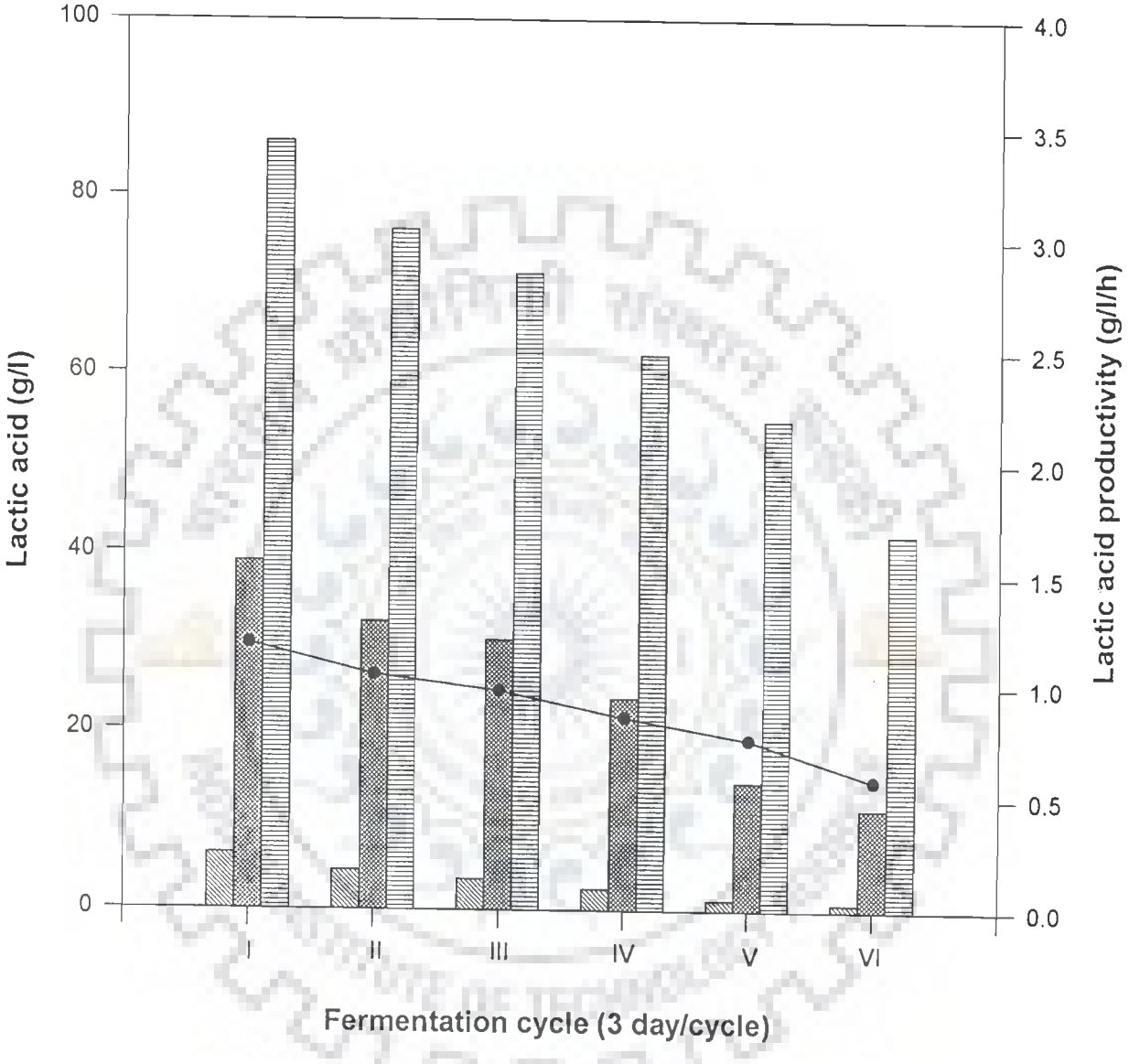

100
80
60
40
20
0
Lactic acid (g/l)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Lactic acid productivity (g/l/h)
I
II
III
IV
V
VI
Fermentation cycle (3 day/cycle)

Fig. 21

Table 16. **Evaluation of kinetic parameters for lactic acid production by *R. oryzae* RBU2-10 immobilized in loofa sponge cultivated in repeated batch process under derived conditions of fermentation.**

Batch/Cycle	Lactic acid (g/l)	Lactic acid yield* (%)	Substrate utilization (%)	Lactic acid productivity (g/l/h)
I batch	80.12	70.03	95.33	1.66
II batch	85.40	73.11	97.33	1.70
III batch	88.68	74.77	98.83	1.84
IV batch	84.31	73.89	95.08	1.75
V batch	80.14	71.48	93.41	1.66
VI batch	71.08	67.63	87.58	1.48
VII batch	61.19	63.60	80.16	1.27
VIII batch	52.35	59.42	73.41	1.09
IX batch	34.26	48.66	58.66	0.71
X batch	20.11	33.24	51.91	0.43
Process mean value	65.84	63.58	83.70	1.36

* Values calculated as per utilized substrate

utilization of sugars (80-99%) led to increased level of lactic acid production (61-89 g/l) obtained during I – VII cycles of fermentation (Table 17).

4.7 EXTRACTION OF LACTIC ACID

Out of the different concentrations (50% and 25%, v/v) of tri-n-octylamine (TOA) and tri-n-pentylamine (TPeA) in methyl isobutyl ketone (MIBK) and 1-octanol used in a phase ratio (solvent phase : aqueous phase) of 1 : 1, it was observed that 50% of TOA in MIBK and 1-octanol resulted in 77.5% and 50.1% extraction of lactic acid respectively. On the other hand 50% of TPeA in MIBK and 1-octanol had resulted into 60.2% and 28.5% extraction of lactic acid respectively (Fig. 22). TOA and TPeA when used at lower concentrations were less effective.

The improvement of the extraction efficiency by variations of phase ratio (solvent phase : aqueous phase) was attempted with 50% TOA in MIBK and 1-octanol. The increase in the solvent volume to two fold led to an increased extraction (85.2%) of lactic acid (Table 17). The lactic acid from the extractant (50% TOA in MIBK) was recovered by using 4 N NaOH. A recovery of 90% of lactic acid was observed when 4 N NaOH was added to extractant in a ratio of 1 : 1. 4 N NaOH when added to extractant in a ratio of 1 : 4 led to relatively lower levels (72%) of recovery.

Fig. 22. EXTRACTION OF LACTIC ACID WITH VARIOUS CARRIERS AND DILUENTS

Lactic acid extraction was carried out as described in methods with tri-n-octylamine and tri-n-pentylamine mixed with methyl iso-butyl ketone and 1-octanol as the diluents.

□, ▨ ; represent respectively 50% and 25% of carrier in diluent. A, Tri-n-octylamine in methyliso-butyl ketone; B, Tri-n-octylamine in 1-octanol ; C, Tri-n-pentylamine in methyliso-butyl ketone; D, Tri-n-pentylamine in 1-octanol.

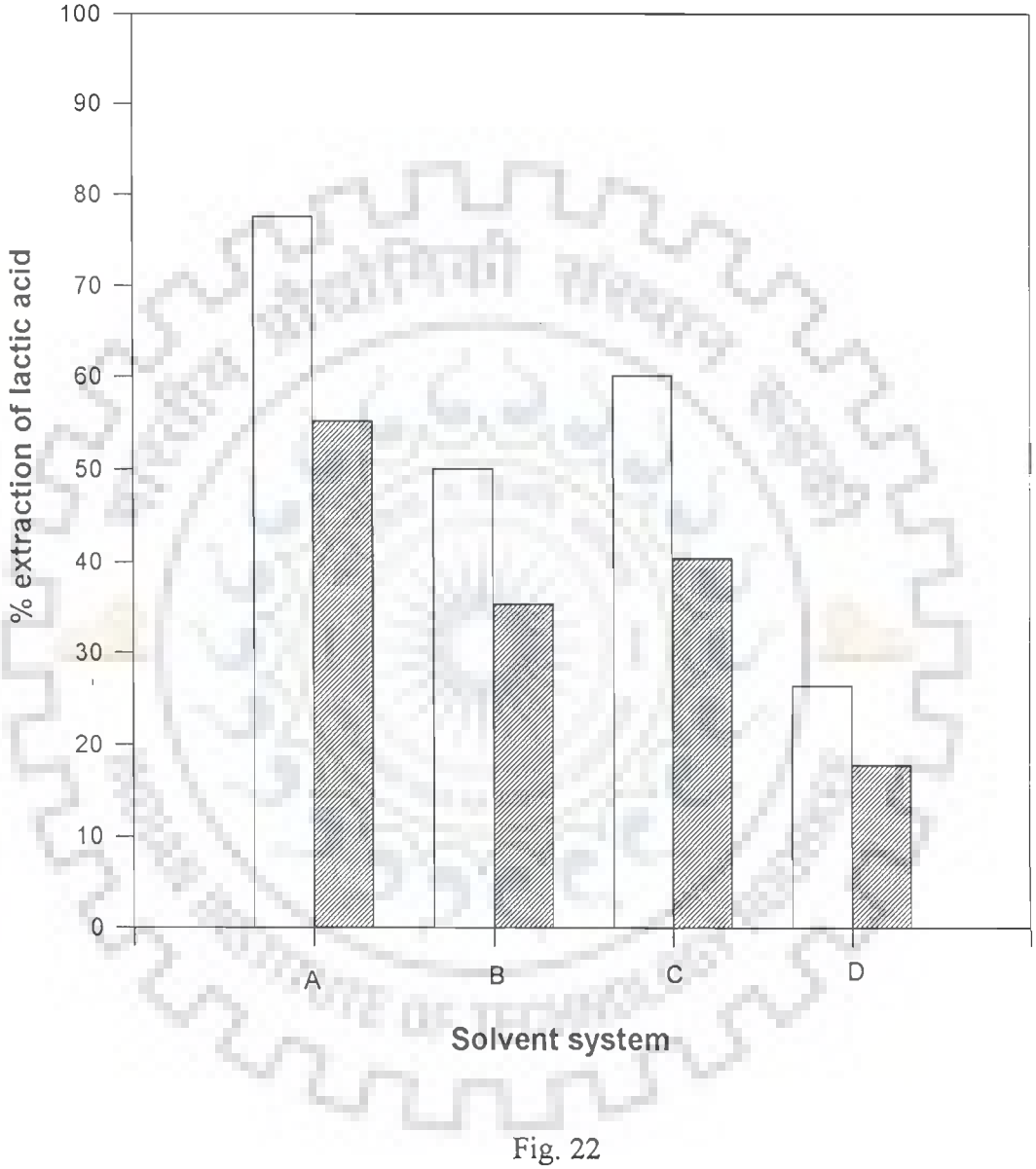

100
90
80
70
60
50
40
30
20
10
0
% extraction of lactic acid
A
B
C
D
Solvent system

Fig. 22

Table 17. Effect of the phase ratio on the extraction of lactic acid

Phase ratio (Volume of solvent phase : Volume of aqueous phase)	% TOA in MIBK (v/v)	Extraction (%)
1 : 1	50	77.5
2 : 1	50	85.2

Chapter 5

Discussion

5.1 ISOLATION AND SCREENING OF THE LACTIC ACID PRODUCING STRAIN

Nature abounds in microorganisms of diverse functions, so screening and selection of a potent microbial strain producing the compound of interest is a critical and exhaustive step for accomplishing the production of the desired compound. A thorough investigation into the spectrum of microbes for the isolation of potent lactic acid producing organism had been carried out by many investigators. The production of lactic acid by microorganisms was thought to be carried out mainly by the bacterial strains but several investigators have reported lactic acid production by fungi, mainly *Rhizopus* and *Mucor*.

Kristofikova *et al*, (1991) had isolated 36 *Rhizopus* strains from natural sources like soil, air and food. Out of these, four strains, had notable ability for lactic acid production and one strain having the highest production ability was chosen for further studies. Bai *et al*, (2003) had isolated a L(+) lactic acid producing strain of *R. oryzae* R 3017 from soil. Similarly, Miura *et al*, (2003) had isolated a strain of *Rhizopus* sp from soil for lactic acid production. In the present study different types of natural resources like soil samples (garden and forest) from local and adjoining areas, partially decomposed horticultural materials (fruits, vegetables) and decomposing food material (bakery products) were collected for isolation of a lactic acid producing strain. About 63 fungal strains were isolated from the various natural sources out of which 22 were from garden soil, 13 from forest soil, 11 from decomposing food and 17 were from the horticultural materials. Of these 63 isolates, 17 strains with higher

AU value were selected for further study. Following quantitative analysis for lactic acid production, strains RB-5, RB-4, RB-3 were found to produce higher levels of lactic acid, the maximum level resulted from strain RB-5 (36.25 g/l). This strain was identified to be *Rhizopus oryzae* from Indian Agricultural Research Institute, New Delhi and was denoted as *Rhizopus oryzae* strain RB-5.

The development of an economical fermentation process largely depends on the efficiency of the production strain involved in the process. The use of a high-yielding strain is a critical factor for the success of the process. The strains isolated from natural sources, usually are not very high yielding. Therefore, the improvement of such strains for the synthesis of a particular compound can be brought about by mutagenesis (Patel, 1997). The successful improvement in the productivity of various fungal metabolites and enzymes by mutagenesis with physical and chemical agents had been carried out (Tani *et al*, 1988; Jiang *et al*, 1991; Yang *et al*, 1994; Longacre *et al*, 1997 and Yu *et al*, 2000).

Attempts for improvement of *Rhizopus* for increasing its lactic acid production ability had been attempted earlier (Kristofikova *et al*, 1991; Suntornsuk *et al*, 1994; Longacre *et al*, 1997; Bai *et al*, 2004 and Miura *et al*, 2004). Improvement of lactic acid production ability of the isolated strain *R. oryzae* RB-5 was attempted by mutagenesis with UV, NTG and colchicine. Acid unitage value was determined to screen the mutants. This method is rapid, convenient and reliable for rapid screening of the mutants. Mutant RBU2-10 thus generated had 63.69% higher levels of lactic acid production (59.34 g/l) than the wild type strain *R. oryzae* RB-5. Mutants with improved production ability had been reported earlier (Suntornsuk *et al*, 1994;

Longacre *et al,* 1997; Bai *et al,* 2004 and Miura *et al,* 2004). Repeated subculturing of the mutant had shown similar levels of production for several generations. Wild type strain following mutagenesis had characteristic morphological variations.

The mycelia of the wild type strain *R. oryzae* RB-5 were white with black spores while mutant exhibited dirty white mycelia with greyish spores. In addition, higher rate of aerial growth was observed for the mutant than the wild type strain and during the submerged cultivation, RBU2-10 resulted into multiple, small sized, pellets compared to larger but relatively lesser number of pellets obtained from cultivation of wild type strain under similar conditions. Such distinguishing variations have also been observed earlier (Longacre *et al*, 1997 and Bai *et al*, 2004).

5.2 DERIVATION OF CRITICAL PARAMETERS FOR LACTIC ACID PRODUCTION

Among one of the major factors, needed for the success of a fermentation process, is the choice of the various components of a production medium. Various groups (Kristofikova *et al*, 1991; Soccol *et al*, 1994; Park *et al*, 1998; Dominguez *et al*, 1999 and Bai *et al*, 2003) have studied and identified the various parameters that are critical in regulating lactic acid production by the fungus *Rhizopus*. The strain RBU2-10 developed in our laboratory was evaluated for some major factors to define the desired conditions for achieving maximum levels of production. Among the sugar substrates, glucose followed by fructose were the preferred substrates. Glucose was also found to be the better substrate for lactic acid production by other groups. (Hamamci *et al*, 1994; Socool *et al*, 1994; Kristofikova *et al*, 1995 and Park *et al*, 1998). Concentration of the substrate not only regulates the level of product formation

but also plays a major role in the economy of the process. The sugar concentration when raised to 12%, led into maximum accumulation of lactic acid. Glucose at higher levels caused the increased endogenous concentration of glucose that favoured the synthesis of lactate dehydrogenase (Pirtchard, 1973). Further higher concentrations of glucose may result into an osmotic imbalance and may cause a declined rate of bioconversion of the substrate to lactic acid. These observations are in agreement with that of earlier workers (Mirdamadi *et al*, 2002).

Lactic acid generated acts as a inhibitor for cell growth, enzymatic hydrolysis and the microbial activity (Iyer *et al*, 1999), therefore monitoring the pH is a necessity to prevent the self inhibition. Lactic acid production brings down the pH to 3.8 and causes inhibition in production as reported earlier (Soccol *et al*, 1994 and Kristofikova *et al*, 1995). The stabilization of the pH by a buffering agent is an important step in lactic acid fermentation. Calcium carbonate is the most commonly used buffering agent for fermentation broth (Yu & Hang, 1989; Kristofikova *et al*, 1991; Soccol *et al*, 1994; Yin *et al*, 1997; Park *et al*, 1998 and Ruengruglikit *et al*, 2003). Variable amounts of calcium carbonate (1- 5%) were added in the fermentation broth and observed that addition of $CaCO_3$ (4%) helped in maintaining the pH required for bioconversion. Many investigators had observed that in the absence of $CaCO_3$ considerably lower levels of lactic acid formation had taken place (Soccol *et al*, 1994). However, excess addition of $CaCO_3$ did not result in increased fermentation efficiency of the strain. Calcium carbonate besides regulating the pH may also act as an inert support for the biomass. Thus, the biomass surface area increases which effects into

more nutrients utilization by the cells as the contact area between the two surfaces increases (Martak *et al*, 2003).

Diverse types of morphologies are exhibited by *Rhizopus* species in submerged fermentation, which is an important factor regulating lactic acid production by the mold. This morphological variation depends largely on inoculated spore concentration. The effects of variations in inoculated spore concentration (10^4–10^8 spores / ml) on lactic acid production by the fungal strain *R. oryzae* RBU2-10 was investigated in the present study. At a concentration of 10^6 spores / ml, the fungus exhibited pellet form of morphology, while at 10^7 and 10^8 spores/ ml clump like masses (small and large), were detected. Pellet shaped mycelial network favoured lactic acid production (Yin *et al*, 1998 and Miura *et al*, 2003). Trager *et al*, (1989) had observed that small pellets led to better yield compared to loosely packed mycelium, similar observations were noted in the present study. Decreased biomass at higher spore (10^8 spores / ml) concentration may be due to the nutrient and oxygen limitation in the center of a large clump of fungal mycelium which can result in autolytic process in the center of the clump. Pellet morphology obtained was also dependent on inoculum level. An inoculum level of 1% had generated the smaller pellets and favoured lactic acid production.

Among the nutrients, the nitrogen sources, ammonium sulphate followed by ammonium chloride led to maximum levels of lactic acid production, while sodium nitrate was the least preferred nitrogenous source not only in terms of lactic acid production but also in terms of fungal growth. Similar results have been observed by various investigators (Waksman *et al*, 1937 and Kristofikova *et al*, 1995). Ammonium

salts that may result into ammonia (Waksman *et al*, 1937) are preferable to nitrates and can be predominantly utilized by *Rhizopus* species Phosphates are the other essential component required for fungal growth and metabolism. KH_2PO_4 at a concentration of 0.15 g/l was most favourable leading to the increased metabolism of the fungus towards lactic acid production Similar requirement limits for phosphates were demonstrated by other research workers (Soccol *et al*, 1994 and Park *et al*, 1998). Requirement of magnesium sulphate in the present study (0.25 g/l) was similar as detected by earlier investigators (Lockwood *et al*, 1936 and Park *et al*, 1998). However, other groups have observed varying levels of Mg^{2+} either for spore formation or mycelial metabolism (Kristofikova *et al*, 1995 and Yin *et al*, 1998). Besides, zinc, due to its regulatory effect on lactate dehydrogenase (Pritchard, 1973) had affected the lactic acid production. Zinc more than its optimal concentration of 0.17 mM reduced the production levels, probably due to its inhibitory action for lactate dehydrogenase as observed earlier (Pritchard, 1973).

Lactic acid production using *Rhizopus* species has been largely carried out under submerged fermentation by various groups. (Yin *et al*, 1997; Park *et al*, 1998; Dominguez *et al*, 1999; Bai *et al*, 2003 and Huang *et al*, 2003). However, Soccol *et al* (1994) had mainly evaluated the solid-state fermentation (SSF) for lactic acid production by *Rhizopus* species. Lactic acid production ability of the mutant RBU2-10 strain was therefore mainly evaluated under solid-state and submerged cultivation condition. For solid state fermentation in the present study, three solid supports, i.e. sugarcane bagasse, groundnut shell and coconut coir impregnated with medium as derived in this study, were used. Air supply was maintained by periodically aerating

the fermentation medium. Maximum production of lactic acid was obtained with sugarcane bagasse (65.05 g/l) followed by groundnut shell (54.71 g/l) and coconut coir (47.16 g/l) in identical fermentation conditions. The difference in production levels is probably due to the structure of the three matrices which may differ in their ability of nutrient absorption, thus resulting into differential level of growth of *Rhizopus oryzae* RBU2-10 and hence affecting the product formation. Compared to SSF, levels of production was remarkably higher in submerged fermentation (83.15 g/l). Moreover, the total time taken for maximal production under submerged cultivation was lesser, i.e., 72 hrs, while during SSF, maximal production required at 96 hrs with an additional of 24 hrs required for the development of inoculum. Further, mycelium obtained during submerged fermentation can be utilized as biocatalyst by immobilizing these in a suitable matrix which cannot be accomplished during solid state fermentation. Therefore, submerged fermentation was chosen for further studies.

5.3 BIOCONVERSION OF CHEAPER CARBOHYDRATE SOURCES FOR LACTIC AID PRODUCTION

The success of a fermentation process largely depends on the selection of the initial raw material that are cheaper, easily available and do not add to the cost of the production process. This had led to search for cheaper carbohydrate sources which can be bioconverted into lactic acid. The sugarcane molasses and starch based substrates which are considerably low priced and abundantly available were evaluated for lactic acid production.

Sugarcane molasses is the byproduct of sugarcane processing industry and is rich in sugars and other minerals. The crude sugarcane molasses contain various

metal ions like Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} that are inhibitory (Clark *et al*, 1965, 1966) for the production (17.14 g/l). The potassium hexacyanoferrate based removal of these metal ions led to a 83.54% increase in lactic acid production (31.46 g/l) by *R. oryzae* RBU2-10. Among the other carbohydrate rich and agriculture based substrates, the starch based substrates like broken and pest infested rice, partially rotten potatoes, arrowroot and sago starch are considerably cheaper and are easily available in this and other parts of India. This region being the sugarcane and rice belt of the country has a number of rice processing mills and during processing, about 10-15% of the rice is generated as the broken rice and with no commercial value. This rice normally is unattended, becomes occasionally pest infested and is either rejected or used as the animal feed. In addition, 10% of rice during storage in the rice godowns gets pest infested and are wasted. Similarly, the market refuge rotten potatoes and sago and arrowroot which are considerably low priced and are easily available were also evaluated as substrate for lactic acid production. Starch gelatinizes when these substrates were heat sterilized, causing contamination because of unsatisfactory sterilization inside the starch gel. Such a problem was also encountered earlier (Yin *et al*, 1997). Starch was thus subjected to acid and enzymatic hydrolysis before use. It was observed that hydrolysed starch substrates led into significant levels of lactic acid production, however, the enzyme hydrolysed substrate was preferable over the acid hydrolysed substrate. Yin *et al*, (1997) had also observed the higher lactic acid production with α-amylase hydrolysed corn starch than the acid hydrolysed substrate. As proposed by Nigam and Singh (1995), the enzyme being specific in nature leads to the production of sugar syrups with well defined physical and chemical properties, in

addition the enzymatic hydrolysis being milder results in fewer side products and less browning. Amongst the substrates used, the maximum production was obtained with enzyme hydrolysed rice starch substrate, and this probably may be because of the difference in the intrinsic composition of the four substrates used. Xiadong *et al,* (1997) had observed marked variation in lactic acid yields from different starch based substrates. Levels of lactic acid production by hydrolysed rice starch further increased by analysing and enriching the medium with desired nutrients. The production levels thus obtained were even marginally higher (3.5%) than that observed with glucose as the substrate. Similar observations (Rosenberg *et al*, 1994 and Yin *et al*, 1997) relating to superiority of starchy substrates over glucose have also been reported by other investigators.

5.4 LACTIC ACID PRODUCTION WITH IMMOBILIZED CELLS

Immobilization of cells offers several advantages like higher productivity, operational stability and decreasing contamination of the product by free cells and thus has been extensively investigated for the improvement of fermentation processes (Tamada *et al*, 1992; Angelova *et al*, 1997; Slokoska *et al*, 1998; Sun *et al*, 1999; Yan *et al*, 2001 and Roble *et al*, 2003). The filamentous microorganisms when immobilized lead to decreased medium viscosity, enhancing the nutrient and oxygen transfer (Angelova *et al*, 1997) and can be effected into developing the repeated batch and continuous processes for economising the production process (Webb *et al*, 1986; Webb, 1989; Vassilev *et al*, 1992 and Federici, 1993).

Immobilization of *R. oryzae* RBU2-10 for lactic acid production was attempted using two different matrices, calcium alginate and loofa sponge. The fermentation

medium containing hydrolysed rice starch equivalent to 12% sugar was used as the substrate. The major physicochemical and biological parameters were analysed for increasing the production levels using immobilized cells. A 15% inoculum of calcium alginate beads immobilized with 3 x 10^6 spores/ml was more supportive for production. The calcium alginate trapped cells of *R. oryzae* RBU2-10 were repeatedly used for 8 cycles and maximum production was observed in the third cycle. However, significant levels of lactic acid production occurred during I - IV cycle having a productivity varied between 1.05–1.19 g/l/hr, productivity decreased thereafter.

Loofa sponge obtained from *Luffa cylindrica* was used for immobilization. These materials were porous, well aerated and observed to be stable over a wide range of pH and temperature. Analysis of essential parameters had indicated that 1.008 cm^3 (1.6 x 1.8 x 0.35 cm) sized sponge pieces added with 10^6 spores/ml had greatly favoured the production. Maximum accumulation of lactic acid was observed following 48 hours of incubation. Loofa sponge has earlier been shown to be suitable for production of ethanol and polymethylgalacturonase (Ogbonna *et al*, 1996; Ogbonna *et al*, 1997 and Slokoska *et al*, 1998). During semicontinuous production, more than 60 g/l lactic acid was produced during I – VII cycles with a productivity that varied in between 1.27 – 1.84 g/l/hr. Maximum level of lactic acid production occurred during III cycle which was higher (2.96%) than that produced by free cells.

A comparative study of lactic acid production indicated that loofa sponge is the preferable matrix than the calcium alginate beads as it not only favoured the lactic acid production but also the growth of the fungus. Higher levels of substrate utilization (80-98%) was observed with the loofa sponge immobilized mycelia.

Moreover, the duration of fermentation cycle was shorter in loofa sponge over calcium alginate beads. Further, the loofa immobilized fungal mass can be subjected to 10 cycles of fermentation where higher levels of lactic acid (61.19 – 88.68 g/l) and productivity (1.27 – 1.84 g/l/h) can be obtained during multiple cycles (I – VII) of fermentation. The alginate cells on the other hand had yielded 42.36 – 86.30 g/l of lactic acid having 0.58 – 1.19 g/l/h of productivity during I – VII cycles of fermentation. Thus loofa sponge immobilized mycelia of RBU2-10 appears to be a potential and promising system for obtaining significantly higher levels of lactic acid production. The free cells when used for repetitive batch fermentation had lower levels of production and had shown variations in the mycelial morphology during fermentation.

5.5 COMPARATIVE ANALYSIS OF EXTRACTION WITH CARRIERS AND DILUENTS COMBINATION

Solvent extraction of lactic acid has been a widely studied process (Baniel *et al*, 1982; Kertes and King, 1986; Yabannavar and Wang, 1991; King, 1992; Tik *et al*, 2001; Wasewar *et al*, 2002 and Matsumoto *et al*, 2003). In the present study, two carriers tri-n-octylamine and tri-n-pentylamine dissolved in two diluents methylisobutyl ketone and octanol were used. These two amines were selected since tertiary amines appear to be suitable for extraction of carboxylic acid from aqueous solution (Ricker *et al,* 1979). Among the two tertiary amies, tri-n-octylamine was more suitable for extraction. Similarly, Hong & Hong (1999) had observed that increase in chain length of polar diluents lead to better extraction of lactic acid. Methyl isobutyl ketone was observed to be a preferred diluent than the 1-octanol.

Choudhury *et al,* (1998) had also observed methylisobutyl ketone to be a highly suitable diluent. Following extraction, lactic acid was recovered using 4 N NaOH. A phase (extractant : aqueous) ratio of 1 : 1 led to 90% recovery whereas a ratio of 4 : 1 resulted in 72% recovery of lactic acid, indicating therefore the earlier combination to be significantly suitable for recovery.

Chapter 6

Summary & Conclusions

Among 63 fungal strains isolated from natural resources mainly forest, garden soils, decomposing food materials and from horticulture industry wastes, 17 strains denoting notable levels of lactic acid production were selected for further analysis. Of these strains, three strains that produced higher levels were selected, the strain RB-5 with maximal levels of production was identified to be *Rhizopus oryzae* from Indian Agricultural Research Institute, New Delhi and was selected for further study. Attempts to improve the production ability of the selected strain was undertaken by UV and chemical mutagenesis of the strain. The survival rate and distribution profile i.e. the positive, negative and corresponding mutants were determined after every stage of mutagenic treatment.

Among the various mutants obtained following UV and chemical mutagenesis, mutant *R. oryzae* RBU1-3 had increased levels of lactic acid production and was subjected to a second round of UV irradiation. This had led to the selection of a strain *R. oryzae* RBU2-10 which had shown 63.69% increase in lactic acid production levels than the wild type strain *R. oryzae* RB-5. The mutant *R. oryzae* RBU2-10 was found to produce stable levels of lactic acid after periodic subculturing for over a period of 10 months. The variations between wild type *R. oryzae* RB-5 and mutant strain *R. oryzae* RBU2-10 were not only limited to their lactic acid production ability but also to the morphological features. The wild type *R. oryzae* RB-5 had white mycelia with black spores, whereas mycelia of mutant *R. oryzae* RBU2-10 were dirty white with greyish spores, further, mutant had developed predominantly aerially directed

mycelia. Moreover in identical submerged cultivation, the wild type *R. oryzae* RB-5 had grown into large sized pellets whereas the pellets of mutant *R. oryzae* RBU2-10 were smaller and comparatively higher in number. The various physico-chemical and biological parameters for achieving increased levels of lactic acid production by the mutant *R. oryzae* RBU2-10 were evaluated in submerged cultivation conditions. Among various carbon sources, glucose at a concentration of 12% was found to be the most suitable for production. The medium supplemented with ammonium, phosphate, magnesium and zinc at 0.2, 0.015, 0.025 and 0.005% (w/v) concentrations respectively with an inoculum of 1% having 10^6 spores/ml and a fermentation period of 72 hrs were suitable for maximum levels of lactic acid (83.15 g/l) production. Production was also evaluated under solid state fermentation condition using three different supports i.e. bagasse, groundnut shells and coconut coir. The bagasse was found to be the preferred support and had led to 65.27 g/l of production by the mutant *R. oryzae* RBU2-10.

Further, the cheaper carbohydrate sources that are readily available and are low priced, were evaluated as substrates. These included sugarcane molasses and various starch based substrates, mainly, broken and pest infested rice, partially rotten potatoes, arrowroot and sago starch. Sugarcane molasses were subjected to rectification for removal of heavy metal content by hexacyanoferrate treatment before use of the substrate. The starch based substrates mainly broken and pest infested rice grains, partially rotten potatoes, sago and arrowroot were acid or enzyme hydrolyzed before use. Production of lactic acid by rectified molasses and the enzyme hydrolysed rice, potato, arrowroot and sago starch had resulted into 31.46 g/l, 81.20 g/l, 74.04 g/l,

69.72 g/l and 68.36 g/l of lactic acid production respectively. Production by the rice starch as substrate was further increased to 86.13 g/l by supplementing the medium with 0.1, 0.015, 0.025, 0.005% (w/v) of ammonium, phosphate, magnesium and zinc respectively. Higher degree of conversion (0.717 g/g), yield (73.48%) and production rate (1.196 g/l/h) were obtained under these conditions, indicating the suitability of rich starch substrate for lactic acid production. To economise the production further, two matrices, i.e., calcium alginate and loofa sponge were used for evaluating their suitability for semicontinuous production by immobilized *R. oryzae* RBU2-10. The various parameters for maximizing the levels of production were also analysed. Alginate beads at 15% (w/v) immobilized with 3 x 10^6 spores/ml were most suitable for production, whereas, loofa sponge (1.008 cm^3) having dimensions of 1.6 x 1.8 x 0.35 cm and immobilized with 3 x 10^6 spore/ml had yielded the maximum production by *R. oryzae* RBU2-10. Thus, 75.09 g/l of lactic acid was produced during one cycle of 72 hrs by calcium alginate whereas 80.75 g/l of the product was obtained following one cycle of 48 hrs duration by loofa sponge. The immobilized matrices were repeatedly used for multiple cycles of fermentation and kinetic parameters of lactic acid producion were studied. The RBU2-10 immobilized in the calcium alginate beads had yielded the higher levels of production during I – IV cycles of fermentation with a productivity that varied between 1.05 – 1.19 g/l/hr whereas the loofa sponge immobilized mycelia had resulted into higher levels of production during I – VII cycles of fermentation with a productivity that varied between 1.27–1.84 g/l/hr. Higher levels of substrate utilization (80-98%) were also observed under these conditions. Extraction of lactic acid with different combinations of carriers and

diluents were attempted. Tri-n-octylamine (TOA) at 50% (v/v) in methyl isobutyl ketone (MIBK) led to 77.5% extraction of lactic acid whereas TOA mixed with 1-octanol as diluent was less effective. Similarly, Tri-n-pentylamine at 50% (v/v) in methyl isobutyl ketone had resulted into 60.2% extraction, and lower levels of extraction were observed with 1-octanol as diluent. The solvent containing TOA in MIBK when added to the fermentation broth in a ratio of 2 : 1 led to 85.2% extraction of lactic acid. Lower levels of extraction (77.5 %) were observed when the similar solvent was mixed with the broth containing lactic acid in a ratio of 1 : 1. The lactic acid was finally recovered from the extractant using 4 N NaOH when added in the combination of 1 : 1 and 4 : 1. A ratio of 1 : 1 (extractant : 4 N NaOH) was found to be most suitable and resulted into higher recovery (90%) of lactic acid from the fermentation broth.

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