PHYTASE PRODUCTION BY SOLID-STATE FERMENTATION AND BIOREACTOR DESIGN

Ph.D. THESIS

by

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JUNE, 2017

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A THESIS

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

of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"PHYTASE PRODUCTION BY SOLID-STATE FERMENTATION AND BIOREACTOR DESIGN"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2011 to June, 2017 under the supervision of Dr. Sanjoy Ghosh, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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

(SIDHARTH ARORA)

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

(Sanjoy Ghosh)
Supervisor

Date:

ABSTRACT

Solid-State Fermentation (SSF) is the process of cultivation of microorganisms on moist solid substrates or inert carriers that take place in the absence or near absence of a free aqueous phase. Use of solid organic wastes for the production of large quantity of biologically active secondary metabolites, single cell proteins, enzymes, industrial chemicals, biofuel, food, phenolics, feed, and pharmaceutical products has made SSF technology as an attractive alternative to submerged fermentation (SMF). Over the last few decades, the technique with its broad application and operational advantage over SMF has led to improvement in bioreactor design, operation, and scale-up strategies. However, the true industrial potential of SSF technology has not been fully realized which is due to the lack of suitable bioreactors designs which can operate aseptically at high substrate bed loading, facilitate efficient heat transfer and mitigate heterogeneity of heat and mass.

In this thesis, design and the operation of a novel, intermittently mixed, modular in nature, SSF bioreactor system is proposed. The bioreactor performance was experimentally validated with a heat transfer design equation during the production of phytase, by *Rhizopus oryzae* MTCC 1987, using wheat bran and linseed oil cake (1:1) as the main substrate. Phytase is an important enzyme in the animal feed industry. It catalyzes the hydrolysis of phytate; an anti-nutrient compound present in cereal and grains, that forms complexes with phosphorus and other nutrients, thereby, making them unavailable for absorption. Phytase produced by *R. oryzae* in the present study is thermo-tolerant and acid stable. This is highly preferred for the enzyme to withstand the temperature of the pelleting process and to retain activity in the acidic environment of organism's digestive tract.

The Bioreactor system is modular in nature wherein multiple modules may be stacked vertically around a central pipe. The central pipe not only facilitates the supply of conditioned air into the module but also acts as the shaft of the mixing apparatus. The bioreactor operates at high substrate bed loading (59.2%, v/v) while providing strict aseptic condition; such that all the fermentation operations can be performed in a single module in a highly contained fashion.

Intense heat buildup and heterogeneity of heat and mass, across the substrate bed, constitute major challenges for scale-up of SSF process. To address these issues, a design equation describing heat transfer in the axial and radial direction was experimentally validated during phytase production in the proposed bioreactor at different operating conditions of inlet air flow rate and relative humidity (RH). The experimentally obtained bed temperature profiles were well predicted by the design equation (error < 5%). An inlet air flow rate of 4 L min^{-1} at 80% RH was optimum for phytase production. Sensitivity analysis of the system's transport and kinetic parameters showed that *R. oryzae* specific growth rate constant, maximum biomass concentration, and metabolic heat yield, and substrate density and thermal conductivity had a significant effect on critical bed height. Among the parameters, the effect of specific growth rate constant was most profound. Phytase production was examined in a 0.5 L mini-bioreactor system using optimized synthetic media, in submerged fermentation (SMF) conditions. Phytase yield in SMF was 15.9 times less than SSF. Water activity in the substrate bed was optimized by varying RH of the inlet air stream. Desorption Isotherm of the substrate was used to correlate water activity and bed moisture content. A bed moisture content of 0.55 mL g-dry-$solid^{-1}$ corresponding to bed water activity ≥0.95 was optimum for phytase production.

Mixing constitutes a critical design parameter in SSF bioreactor and its effect on heat and water transport, and microbial growth in substrate bed can significantly influence overall productivity. Effects of mixing events were studied on phytase production in the proposed SSF bioreactor. A critical mixing phase was identified, in the absence of which fungal growth led to the onset of heat accumulation and subsequent bed drying. At this critical phase, the tensile strength required to disengage *R. oryzae* hyphal bonds was experimentally estimated (600 N m^{-2}) and related to the mixing intensity in the bioreactor that gave an optimum working value of 15 rpm. Effect of mixing time on bioreactor performance was also investigated where a 3 min mixing duration, at every six hours, increased the biomass and phytase productivity to 2.2- and 4.5-fold, respectively than packed bed bioreactor (PBR) operation. The proposed bioreactor system with intermittent mixing gave better performance than PBR and tray bioreactor, concerning maximum bed temperature, axial bed temperature and biomass gradient, average bed moisture content, biomass, and phytase productivity.

Direct measurement of cell biomass is difficult in an SSF process involving filamentous fungi since the mycelium and the solid substrate are often inseparable. However, respiratory data is rich in information for real-time monitoring of microbial biomass production. In this regard, a correlation was obtained between oxygen uptake rate (OUR) and biomass concentration (X) of *Rhizopus oryzae* during phytase production in the proposed SSF bioreactor. To obtain the correlation, various models describing sigmoidal growth were tested, namely the logistic, Gompertz, Stannard, and Schnute models. Regression analysis of experimental results, at different operating conditions of inlet air flow rate and relative humidity, suggested that OUR and X were correlated well by the logistic model ($R^2 > 0.90$). To corroborate the use of respiratory data for on-line measurement of metabolic activity, OUR was related to metabolic heat generation rate (R_q), and the logistic model was found to satisfactorily correlate R_q and X as well. The model parameter, $Y_{Q/X}$, when substituted into the heat transfer design equation, along with the values of other parameters and operating variables, gave reliable estimates of bed temperature. The correlations developed in the present study, between respiratory activity and biomass concentration may be extended on to other SSF processes for further validation.

Effect of periodic air pressure pulsation (APP) was studied on bed temperature and moisture content, and on biomass and phytase production in a different fermentation setup for SSF. Air pressure amplitude of 60 mBar for 1 min duration, at a frequency of 10 min, during the log phase of *R. oryzae* increased the cell biomass and phytase productivity by 1.43 and 1.90 times than packed bed operation. Scale-up of phytase production, using design equations, in the proposed SSF bioreactor with APP shall be taken up in future studies.

LIST OF PUBLICATIONS

PATENT:

1. "A novel bioreactor system for solid state fermentation and a process of operation thereof" Patent application number: 201611020038, dated 11/06/2016.

PUBLICATION IN REFEREED JOURNALS

1. **Arora S,** Dubey M, Singh P, Rani R and Ghosh S "Effect of mixing events on the production of a thermo-tolerant and acid stable phytase in a novel solid-state fermentation bioreactor". *Process Biochem.* DOI: **10.1016/j.procbio.2017.06.009**

2. Rani R, **Arora S,** Kumar S and Ghosh S (2013). "Optimization of Medium Components for the Production of Phytase by R. Oryzae using Statistical Approaches". *J. Bioremed. Biodeg.* S18: 003. DOI: **10.4172/2155-6199.S18-003**

PAPERS SUBMITTED IN REFEREED JOURNALS

1. **Arora S,** Singh P, Rani R, and Ghosh S "Heat transfer validation and optimization of bed water activity in a novel solid-state fermentation bioreactor".

2. **Arora S,** Rani R, Singh P, and Ghosh S "Oxygen uptake rate as a tool for on-line estimation of cell biomass and bed temperature in a novel solid-state fermentation bioreactor".

3. **Arora S,** Rani R, Singh P, and Ghosh S "Bioreactors in solid-state fermentation technology: design, applications and engineering aspects".

ACKNOWLEDGEMENT

Completing a Ph. D. is truly a great marathon event, and I would not have been able to complete this long journey without the cooperation and guidance of a number of people to whom individually I owe a great sense of gratitude for their valuable contributions.

I have always believed, hard work in the right direction is the key to success. Parents give that direction to their children so that they live a life, one which is of value! Likewise, my supervisor Dr. Sanjoy Ghosh, Assoc. Professor, Department of Biotechnology, IIT Roorkee, gave an excellent direction, to make my career, of value! Dr. Ghosh was always with me from the formative stages of this thesis to the final draft, for his ingenious advice, deep concerns, constitutive criticism and able guidance throughout the tenure of this study. Dr. Ghosh was instrumental in encouraging and inspiring me to cultivate critical approach to planning experiments, analyzing and presenting the data. I owe him high gratitude for the help extended to me and for showing me the path of leadership, shaping my carrier to the place where I stand confidently. I shall forever treasure the enriching experience of working with Dr. Ghosh as my guide.

I extend my profound gratefulness towards Prof. and Head, Dr. Partha Roy, Department of Biotechnology, IIT Roorkee, for providing all the facilities to accomplish my task smoothly and efficiently.

I would like to extend my sincere regards to my seniors Dr. Richa, Dr. Rashmi, Dr. Gaurav, Dr. Pawan, Dr. Lalit, Dr. Rohit, Dr. Shilpi and Dr. Meenu for their help and encouragement. I would cherish the memorable moments, with my lab mates Shailendra, Archana, Akash, Kartik, Mayank, Afifa, Komal and Raikamal. I express my deep appreciation to all of them.

Special thanks to Dr. Richa Rani and Mr. Pritam Singh for extending their unconditional support and prolific help, whenever required.

My acknowledgement is due to the all the technical and non-technical staff of the Biotechnology department, IIT Roorkee for their untiring support.

I wish to extend my warmest thanks to all those who have joined me while travelling along this exciting road. In this regard, I would like to convey heartfelt thanks to Alok, Atin, Madhusudan, Narender sir, Rajat, Shailendra,

Snehashish and Tamoghna for making my stay comfortable and joyful at IIT Roorkee. Special thanks to Mr. Manish Jangid for being there at the Tables.

My heartfelt appreciation goes to my M. Tech. colleagues for their kind support and immense motivation. Heart goes out to my B.E. batchmates Gautam, Hemang, Kamal, Rohan and Manjeet with whom I learned various facets of biotechnology and shared one of the best days of my life.

My gratitude is due to my teachers Prof. N.G. Karanth, Prof. Surender Kumar and Prof. M. B. Saidutta, who inculcated in me the concepts of Biochemical engineering. Special thanks to my M. Tech. supervisors Dr. B.D. Prasanna and Dr. Lisette D'Souza. The encouragement and blessings I got from you were priceless.

Words are not enough to express my indebted appreciation for my parents. My father and mother have always been very proud parents. It was their dream and ambition that made me strong enough to pursue higher studies. My father's confidence in me and my mother's indefinable contributions in my life shaped my dreams and aspirations into reality.

I cannot express my feelings for the love and blessings showered on me by my Grandfathers Late Sri Bishambar Lal Arora and Late Sri Bana Ram that always boosted my spirits throughout this academic pursuit. My heart goes out to my maternal grandmother Late Shanti Devi ji for her endless love and care. I would also like to affectionately acknowledge my grandmother Pushpa Rani ji for her endless love, and inculcating in me discipline and forbearance.

In the reminiscence, I might forget a few names. Many years from now those faces will flash back to my fading memories to me a feeling that I was lucky enough to spend some time with them.

Finally, yet importantly, I am immensely thankful to Ministry of Human Resource and Development (MHRD) for providing financial assistance. Also sincere thanks to Department of Biotechnology, Ministry of Science and Technology, for funding the filing of patent for SSF Bioreactor.

My thesis is a culmination of my parent's dream and their aspirations.

(SIDHARTH ARORA)

DEDICATION

THIS THESIS IS DEDICATED TO MY PARENTS,

WHO SACRIFICED THEIR TODAY

FOR MY BETTER TOMORROW

Acronym Abbreviation and Symbols

AFB	Anaerobic fluidized bed bioreactor
AFR	Air flow rate
APP	Air pressure pulsation
APP-SSF	Air pressure pulsation solid-state fermentation
ASFB	Air-solid fluidized bed bioreactor
Bi	Dimensionless Biot number
Bt	*Bacillus thuringiensis*
$H_{critical}$	Critical bed height
CER	Carbon dioxide evolution rate
CFU	Colony forming unit
CMC	Carboxy methyl cellulose
DDF	Dimensionless design factor
DPS	Discrete particle simulation
EA	Ethyl acetoacetate
EC	Expanded clay
GDD-SSF	Gas double dynamic solid-state fermentation
g-ds	Gram-dry-solid
gfs	Gram-fermented-solid

HLD	Honeycomb loading device
HRT	Hydraulic retention time
IM	Intermittent mixing
IMC	Initial moisture content
IM-SSF	Intermittently mixed solid-state fermentation
IU	International unit
kg-ds	Kilogram-dry-solid
L/D	Length to diameter ratio
L min^{-1}	Litre per minute
NO	Nitric oxide
OUR	Oxygen uptake rate
PA	Pressure amplitude
PBR	Packed bed bioreactor
PC	Data logger
PG	Pectinase
PSM	Phytase screening media
Pt	Platinum
RDB	Rotating drum bioreactor
RH	Relative humidity
rpm	Rotations per minute

SB	Sugarcane bagasse
SBL	Substrate bed loading
SCP	Single cell protein
SMF	Submerged fermentation
SS	Stainless steel
SSF	Solid-state fermentation
TB	Tray bioreactor
TPH	Total petroleum hydrocarbons
VAC	Volatile aroma compounds
WB	Wheat bran
~	Nearly to
ΔE	Energy accumulation
Δl/l	Elastic recovery
Δr	Difference between two radial distances
Δt	Time interval
Δz	Difference between to axial distances
°C	Degree celcius
μ	Specific growth rate constant
μm	Micrometer
μM	Micromolar

a_w	Water activity
C_{pa}	Heat capacity of the air
C_{pb}	Heat capacity of the bed
C_{ps}	Heat capacity of substrate particles
D	Axial dispersion coefficient
d	Day
D	Reactor diameter
D_e	Effective diameter of bed particle
f	Change in water carrying capacity with temperature
g	Gram
g	Gravitational force
h	Hour
H	Reactor bed height
h_a	Convective heat transfer coefficient of air
H_{sat}	Moisture content in saturated air
J	Joule
K	Kelvin
k_a	Thermal conductivity of the air
k_b	Thermal conductivity of the bed
KJ	Kilojoule

k_La	Mass transfer coefficient
k_s	Thermal conductivity of the substrate
L	Litre
M	Meter
M	Molarity
M_a	Mass of the air
M_b	Mass of the bed
mBar	Millibar
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
m_o	Maintenance coefficient for O_2
MPa	Megapascal
m_q	Maintenance coefficient for heat
N	Rotational speed
N	Normality
nm	Nanometer
nmol	Nanomole
P_e	Peclet number

$Q_{a,cond}$	Axial conduction
$Q_{a,conv}$	Axial convection
$Q_{r,cond}$	Radial conduction
r	Radial distance
R	Bioreactor radius
r_e	Effective radius of bed particle
R_q	Metabolic heat generation rate
s	Second
T	Temperature
t	Time
T_b	Temperature of bed
$T_{experimental}$	Experimentally obtained bed temperature
T_o or T_{in}	Initial bed temperature
T_{opt}	Optimum temperature
$T_{predicted}$	Bed temperature predicted by design equation
T_s	Temperature of surrounding
u_s or V_z	Superficial air velocity
Λ	Latent heat of vaporization
v/v	Volume by volume
vvm	(Air volume)(reactor volume)$^{-1}$min^{-1}

W	Watt
w/v	Weight by volume
w/w	Weight by weight
X	Biomass concentration
X_m	Maximum biomass concentration
X_o	Initial biomass concentration
$Y_{q/x}$	Yield of metabolic heat from growth
$Y_{x/o}$	Yield of biomass from O_2
z	Axial distance
ϵ	Void fraction in the bed
λ	Enthalpy of vaporization of water
π	3.142
ρ_a	Density of air
ρ_b	Density of the bed
ρ_s	Density of substrate

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1 Chapter 1

Introduction

1.1 Solid-state fermentation

Solid-state fermentation (SSF) may be defined as the growth of microorganisms on solid particles in the absence or near absence of free water. The solid particle may act as the sole source of nutrients, or an inert material may support organism's growth, and the nutrients may be supplied externally. The space between the solid particles is occupied by a continuous gas phase; however, droplets of water or film of water on particle surface may also be present [1]. A majority of the SSF process involve filamentous fungi [2], although some include bacteria and yeasts as well.

SSF has been practiced for many centuries especially in some Asian countries [3] for the production of traditional foods such as tempeh, miso, koji, red rice and soy sauce [4, 5]. However, biochemical mechanisms and socio-economic potential of the process have been understood only during the 20th century. SSF was considered for the production of enzymes in the early 1900s, and for penicillin production in the 1940s, but the interest diminished due to advances in the submerged fermentation (SMF) technology [1]. The resurgence of interest began in the mid-1970s with the prospect of utilization of agriculture waste as the solid substrate for fermentation. Use of solid agro-wastes as the energy or nutrient source, for microbial growth, is advantageous not only because of the low raw material cost but also for the higher yield of some fungal products (enzymes, spores, etc.) compared to SMF [6, 7]. Use of SSF technology also circumvents the discard and burning of agro and food processing wastes. Indeed, a significant number of agro-wastes (wheat bran, rice bran, rice husks, oil cakes, apple pomace, citrus pulp, coffee pulp, banana peel, sugar beet molasses, etc.) have shown potential utility as the solid substrate in various research reports.

1.2 Current and prospective applications of SSF

Over the last three decades, significant research has been undertaken for the development of SSF bioprocesses, which can be attributed to the advantages that SSF offers over SMF. In some cases, SSF operation has proven to be highly advantageous than SMF for a multitude of reasons [8-10]. Firstly, because of the absence of free water less reactor volume is required; there are fewer chances of contamination, there is no foam formation, and downstream processing is simpler. Besides, the raw material (solid substrate) is cheaper, simple and is available throughout the year. Moreover, the solid nature of substrate mimics the natural habitat of filamentous fungi which more often than not results in high volumetric productivity of the desired metabolite. Also, SSF is a natural process which helps in biomass energy conservation, waste management, and pollution control.

The current and potential application of SSF includes the production of a myriad of different products [11-13]:

- Enzymes such as amylases, pectinases, phytases, cellulases, lipases, tannases, proteases, especially in those processes where the fermented product may be used directly as enzyme source;
- Antibiotics, such as penicillin and meroparamycin;
- Biological control agents, including bioinsecticides and bioherbicides;
- Pigments, aroma and flavor compounds;
- Industrial chemicals, such as citric acid, lactic acid, ethanol, and polyhydroxyalkanoates;
- Protein-enriched agricultural residues, single cell protein.

There has been an increasing interest in the scientific community regarding the use of microorganisms in SSF conditions for bioconversion of industrial waste. Some of the processes include [1]:

- Decolorization of dyes;
- Bioremediation;

- Biopulping;
- Bioleaching.

Treatment of industrial wastes with SSF technology would not only help in curbing pollution but also aid in solid waste management and reduce the operating cost of industries [14].

1.3 The Need: SSF Bioreactor design and design equation

Solid-state fermentation with its broad application and operational advantage over SMF has led to significant research input over the last three decades which has resulted in improvement in reactor design, operation and scale-up strategies [1]. In spite of these developments, currently used bioreactors do not fully meet all the criteria required for commercial utilization of a broad spectrum of bioprocesses, in particular, scalability and sterility issues [2, 15]. This is probably due to a combination of three factors, i.e., lack of efficient bioreactor design, very limited use of design equations describing the transport and kinetic phenomenon at macro and microscopic levels, and the absence of online process monitoring and control strategies (Fig. 1.1). The majority of SSF research is limited to flasks, columns, and trays where the quantitative characterization of relevant phenomenon is most often ignored. In fact, SSF in trays constitutes a major proportion of commercial processes in fermentation industry [16, 17], where, scale-up is usually achieved by increasing the surface area of the tray and/ or increasing the number of trays [18]. However, this not only results in low productivity; it makes the process more labor intensive, requires large aseptic rooms, sophisticated procedures, and equipment for workers which often prove prohibitive and highly costly.

To address the above-mentioned issues, there is a need for a biochemical engineering approach which would involve quantitative characterization and experimental validation of the key phenomenon occurring during an SSF process. Mathematical description of macro and microscopic phenomenon needs to be incorporated into design equations. Design equations for packed bed and rotating drum bioreactors have been shown to be of potential utility in guiding bioreactor design, mode of operation and control strategies [19-23]. However, many of these utilities are restricted to theoretical predictions; without being validated by experimental

results. One possible reason for this may be the lack of technical know-how and dependency on trays.

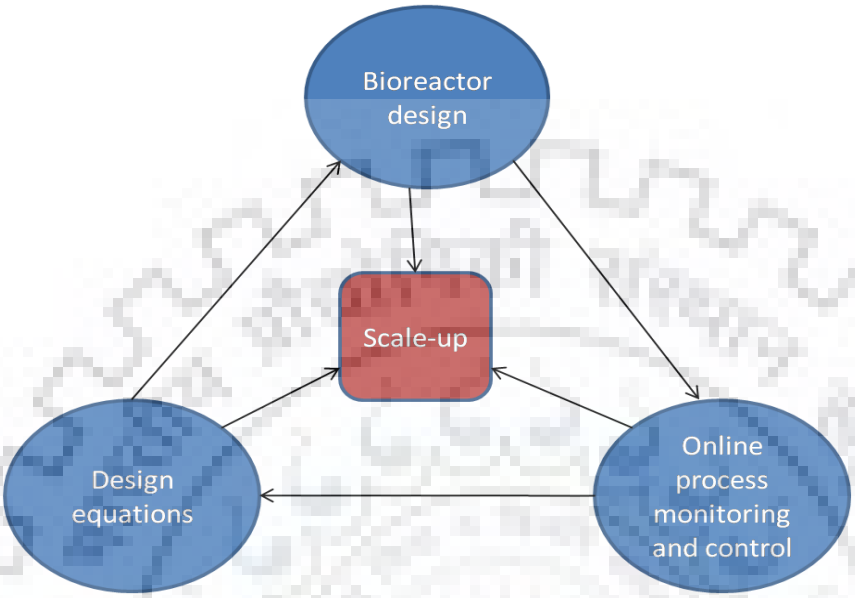

Figure 1.1 Factors affecting the scale-up of SSF bioreactor

1.4 Scope and organization of the thesis

In this thesis, design, and the mode of operation of a novel, intermittently mixed, modular, solid-state fermentation bioreactor system is proposed and experimentally validated for the production of a thermo-tolerant and acid stable phytase, by *Rhizopus oryzae*, utilizing wheat bran and linseed oil cake as the main substrate. The bioreactor claims to operate at high substrate bed loading and under strict aseptic conditions; such that all the fermentation operations can be performed in a single module, in a highly contained fashion. The quantitative characterization of key phenomenon has been presented in a heat transfer design equation. The latter has been experimentally validated in the proposed bioreactor. Phytase production through SSF has been compared with that carried out under SMF. A novel approach, based on the tensile strength of hyphal bonds and respiratory activity of the organism, has been developed for the optimization of mixing events in the bioreactor. Significant impetus has also been given to facilitate real-time monitoring of cell biomass and bed temperature during fermentation. The

thesis has been divided into seven chapters. Fig. 1.2 summarizes the rationale and major highlights of each chapter.

Phytase (*myo*–inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) is an essential enzyme in the food or feed industry [24, 25] because it catalyzes the hydrolysis of phytate. Phytate, an anti-nutritional compound present in cereal, grains, etc., chelates phosphorus and other nutrients thereby making them unavailable for absorption. Phytase hydrolysis phytate, rendering phosphorus and other vital supplements, present in the feed, available to monogastric animals and thereby obliterating the need for the additional supply of nutrients into the feed [26]. Phytases are produced by a large number of plants, animals, and microorganisms; however, the phytases from microbial sources especially of fungal origin have supremacy in the commercial industry [27]. Although compendia of phytases are reported, a thermo-tolerant and acid stable phytase is highly preferred for the enzyme to withstand the temperature of the pelleting process and to retain activity in the acidic environment of organism's digestive tract [14]. Moreover, phytase production by *Rhizopus oryzae* strain is advantageous since it falls in the category of generally regarded as safe (GRAS) organism. Also, crude enzymes produced by GRAS strain on various feed supplements (bran, straw, oil cakes, etc.) could serve as a value–added supplement by providing other fungal proteins, sugars, and some accessory enzymes along with the primary enzyme source.

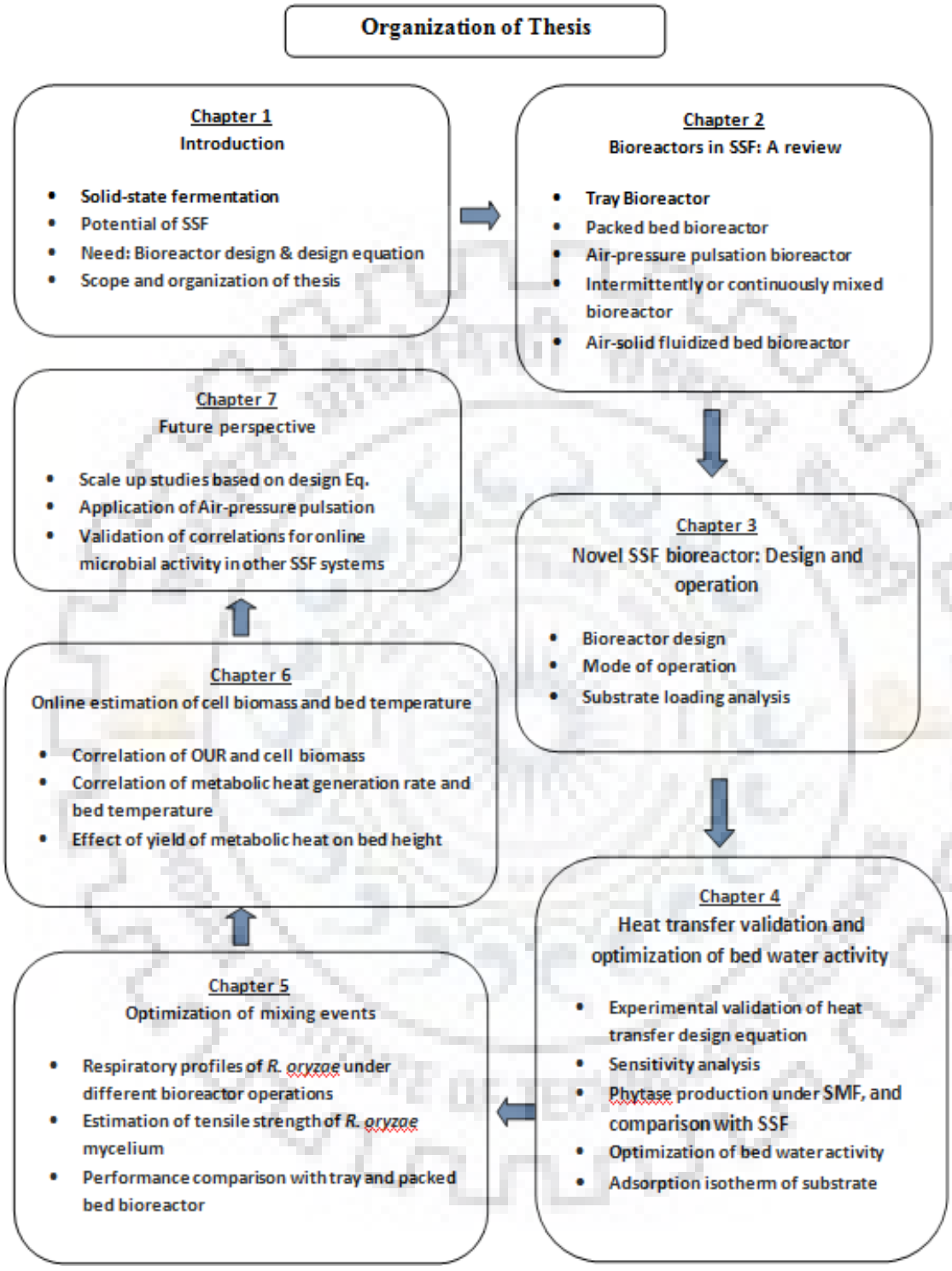

Figure 1.2 Organization of thesis

2 Chapter 2

Literature Review

Summary

In recent years, substantial credibility in employing solid-state fermentation technique has been witnessed owing to its numerous advantages over submerged fermentation. In spite of enormous advantages, the true potential of SSF technology has not been fully realized at industrial scale. The lack of rational and scalable bioreactor designs, backed by design equations and automated control system, that could facilitate maintenance of optimum bed temperature and water activity, successfully address the heterogeneity concerning heat and mass, and operate aseptically remains the prime reason for it. As a result, there still exists vast scope in SSF bioreactor research and development to cater broad spectrum of biotechnological applications. This chapter reviews the state-of-the-art in SSF technology with the focus on bioreactors that have been employed for bioprocess applications. Based on the mode of operation, bioreactors are classified into five categories with emphasis on design features, the effect of operating conditions on productivity, applications, and limitations. Selected modeling studies developed over the years have been revised and presented in problem specific manner to address limitations. Some interesting designs, including few recent ones that have been proposed and employed at the pilot and industrial level are discussed in more detail.

2.1 Introduction

The recent surge in demand for large quantity of biologically active secondary metabolites, single cell proteins, enzymes, industrial chemicals, biofuel, food, phenolics, feed, and pharmaceutical products [1] [2] has made SSF technology as an alternate production method to submerged fermentation (SMF), the need of the hour. In addition to the production of specialty products of commercial interest, there is also a growing popularity of SSF to be used as waste management technology; applications of which may include bioremediation, detoxification, bioleaching and biopulping [1, 3, 4]. The technique with its broad application and operational

advantage over SMF [5-8] (Table 2.1) has led to significant research input which has resulted in improvement in reactor design, operation and scale-up strategies [9]. In spite of developments, the major hindrance in the industrialization of SSF process remains the lack of simple, efficient and easily scalable bioreactors that could facilitate efficient heat transfer, address the heterogeneity, and at the same time operate with complete sterility [10]. Although in recent years there have been reports of few bioreactor systems that have partially overcome these challenges for a particular application, there still exists a vast scope for improvement to address a broad spectrum of biotechnological applications.

In this chapter, based on the mode of operation, SSF bioreactors have been classified into five categories. The description of each category begins with an introduction to the reactor type and highlights of recent case studies. The case studies present a holistic view of reactor configuration, the effect of operating conditions on the productivity of the desired metabolite, advantages and limitations. Design equations are powerful mathematical tools which can aid in bioprocess optimization, provide scale-up guidelines and facilitate control and automation. Selected studies involving mathematical models and design equations have been revisited and cited at the end of each category with an objective to address limitations, discuss scale-up strategies and promote greater interaction between biologists and engineers. However, model equations, assumptions, and solution techniques are not discussed, and readers are advised to refer to the original article for that matter. At the end of this chapter, Table 2.2 shows examples of SSF bioreactor systems used in recent bioprocesses.

2.2 SSF Bioreactor classification

The SSF bioreactors have been classified into the following five categories based on their mode of operation.

I. **Tray Bioreactor**
II. **Packed Bed Bioreactor**
III. **Air Pressure Pulsation Bioreactor**
IV. **Intermittent or Continuously Mixed Bioreactors**
V. **Air-Solid Fluidized Bed Bioreactor**

Table 2.1 Advantages of SSF over submerged fermentation

Absence of free water	Lower reactor volume required Negligible chances of bacterial contamination Lower cost of treatment of liquid effluents No foam formation
Fermentation medium	Simple and natural Low manufacturing costs due to utilization of agro-industrial wastes as substrate Throughout year availability of substrates Value-added utilization of cheap wastes Ample amount of substrates Minimum mineral supplementation is required
High interfacial area	Easier to meet aeration requirement Reduced or eliminated aeration machinery cost
Area to liquid volume ratio	Low processing energy requirement
Natural environment	Microorganisms easily grow in this condition up to sufficient extent
Volumetric productivity	High Yields are reproducible
Downstream process	Simple and easier Low cost of product purification and recovery
Environmental aspects	Biomass energy conservation Helps in waste management Pollution control
Product quality	Heat resistant Greater resistance to UV light and heat Long shelf life when used as a bio control agent

2.2.1 Tray Bioreactor

SSF in trays has traditionally been used for the production of fermented foods such as tempeh, miso, koji, and soy sauce [11, 12] in some Asian countries [13]. Trays are usually made of wood, metal or plastic, with or without perforations, packed with substrate-support and stacked one above the other in temperature and humidity controlled rooms (Fig. 2.1). Scale-up is achieved by increasing the surface area and the number of trays. The current section starts with recent reports describing the utility of different substrates for enzyme production in tray bioreactor (TB). Emphasis has been on the operating bed heights, chemical nature and initial moisture content (IMC) of the substrate and their effect on productivity of the desired

metabolite. Reports citing operational difficulties, issues related to poor O_2 and moisture levels, high bed temperatures and corresponding control strategies are discussed.

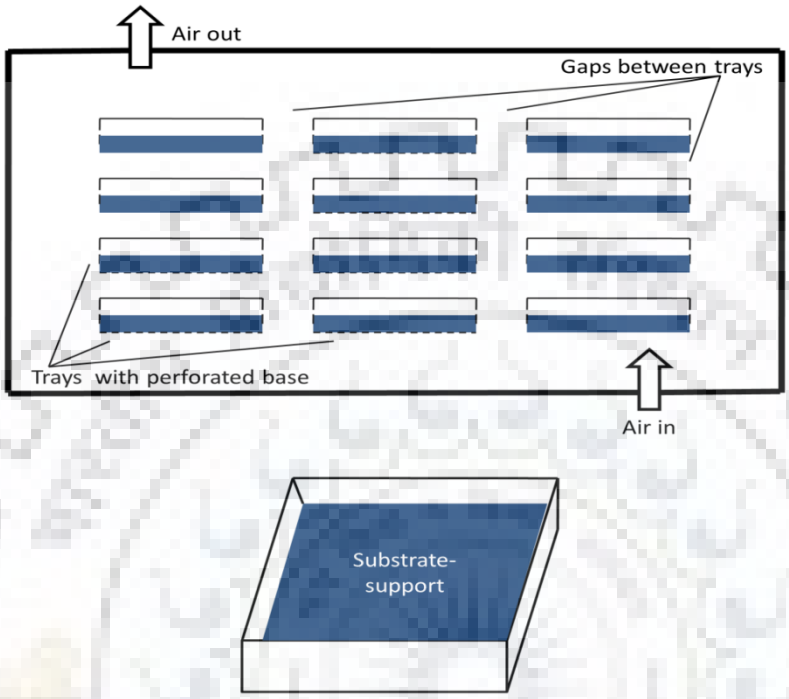

Figure 2.1 Schematic diagram of a tray bioreactor and for an individual tray.

Laccase production by *Trametes hirsute* was studied using grape seeds as substrate-support in TB [14]. Use of grape seeds as substrate-support over nylon cube sponge resulted in a threefold increase in laccase yield. Using the same organism, high laccase activity (12260 U/L) was achieved when orange peel was used as substrate in a TB of 1 cm bed height [15]. High enzyme activity was attributed to high pectin and cellulose content in orange peels and the absence of mechanical stress. Application of laccase production was examined for the removal and decolorization of synthetic dyes, in TB (0.5 cm thickness) under the semi-solid-state condition, using *Trametes pubescens* [16]. Initially, dyes were adsorbed onto dry sunflower shell seeds which were subsequently used as solid-support for fermentation. Remarkably high laccase activity (40172 U/L) was obtained when 0.5 mM Cu^{2+} and 50 µM tannic acid were added as supplements to growth media, on the 3rd day of cultivation. TB was used for

cellulolytic enzyme production using a co-fermentation technique involving *Trichoderma reesei* and *Aspergillus oryzae* [17]. Soybean meal and wheat bran (4:1) were used as the substrate (1 cm bed height), and optimum operating conditions of bed temperature (30ºC), moisture (70%) and pH (5) were obtained. These workers emphasized the importance of an appropriate C:N in the substrate and concluded that the process not only facilitated high cellulase yield but also resulted in a balanced production of glucanase (endo and exo) and β-glucosidase, which is generally recommended in biomass processing for biofuel production. Higher β-glucosidase levels were reported [18] during fermentation of apple pomace using *Aspergillus niger* and *Trichoderma reesei* in TB. Still higher β-glucosidase levels were obtained (91.8 IU/gfs) when *Aspergillus niger* was cultivated in plastic trays (40×25×12 cm) [19], and operating conditions were optimized using response surface methodology technique. High IMC (>70% w/w) resulted in lower enzyme activity due to low O_2 levels, decrease in bed porosity and substrate aggregation. This was an important observation which asserts IMC as a critical design parameter for TB.

Fath et al. [20] proposed a novel trickling TB which consisted of a medium reservoir, a bioreaction chamber, and a product storage tank. The bioreaction chamber housed perforated trays (17×11.5×4.2 cm) that acted as the site of fermentation and through which the crude enzymatic solution was made to trickle down from the medium reservoir in batch and semi-batch fashion. Rice bran (primary substrate) was supplemented with wheat bran (to increase porosity), soybean meal (as nitrogen source) and wheat flour (inducer for protease). The whole setup was kept in an insulated wood cabin at 30ºC and provision for exchange of gases was also provided. High protease activity (748 U/g-ds) was achieved on the 3rd day, which however was not exceedingly high to that obtained with flasks (530 U/g-ds). Operating bed heights were not discussed, but high substrate bed loading (SBL) may have been responsible for the moderate increase. Countercurrent movement of trickling liquid and fresh air might be an attractive prospect as this is expected to enhance retention time of fluid in bed and increase the interfacial area for heat and mass transfer. Ruiz et al. [21] proposed a column TB which consisted of 8 perforated base trays in a cylindrical column during fermentation of lemon peel pomace for pectinase (PG) production. The sterile moist air was circulated through perforations to increase O_2 availability and circumvent bed drying, which resulted in high PG activity (2181U/L).

These workers also emphasized the importance of suitable substrate particle size, bed porosity and heat and mass transfer characteristics of the bed on PG production. TB (320×400×50 mm) were used [22] for the production of *Phlebiopsis gigantia* spores, to be used as biofungicide. The 'root and butt rott' caused by *Heterobasidiun annosum*, a natural antagonist of *P. gigantia*, results in substantial capital loss to forest and wood industry of some European countries. Its production using SSF has significant commercial potential since yields are higher than with SMF [23]. Starch mash was used as the substrate, and supplemented with silica to retain bed moisture. Spore viability of ~5.4×10^{6} CFU/g-substrate was achieved, which was comparable to that obtained in packed bed bioreactor (PBR) and disposable plastic bags. These workers asserted that although TB was simple in design and prevented overheating, its operation was highly labor intensive since each tray had to be loaded, monitored, emptied and cleaned individually. Moreover, low SBL was prominent as dead space between the trays was necessary for uniform air circulation.

A decrease in lipase activity by *Rhizopus oryzae* was reported [24] when the substrate (sugarcane bagasse) bed height was increased from 0.5 to 3 cm, in a TB; consisting of three trays (35×25×5cm) mounted one above the other. The high bed temperature was responsible for the decrease in lipase production. Xie et al. [25] studied the effect of SBL on conidia production of *Beauveria bassiana* in a TB containing rice as substrate. Conidia yield of 2.70×10^{12} conidia kg^{-1} rice was reported in a 60×40×2 cm bed. With an increase in bed thickness to 8 cm, the yield reduced to 1.02×10^{12} conidia kg^{-1} which was due to the inefficient dissipation of metabolic heat. Maximum bed temperature exceeded 10ºC from optimum (25ºC), most likely impeding fungal growth. However, when the bed was cut into 100 pieces, each of size 6×4×2 cm and with a 0.5 cm gap between them, yield increased to 3.94×10^{12} conidia kg^{-1} and the maximum bed temperature did not exceed 31ºC. TB of different sizes were used for the production of a thermo-tolerant and acid stable phytase from *Rhizopus oryzae* [26]. Optimized growth media consisted of wheat bran and linseed oil cake (1:1) as the main substrate. Different SBL resulting in various bed heights (0.2-2 cm) were tried. Increasing the substrate bed height up to 2 cm did not lead to significant decrease in maximum phytase yield (149.2 U/g-ds). However, a 3.5 cm bed height halved the phytase yield which was probably due to inadequate moisture level and steep gas gradients (unpublished data). Similar observations were made

during [27] the production of thermo-tolerant phytase by *Sporotrichum thermophile*. The enzyme yield decreased significantly as the bed height increased beyond 1.5 cm.

The above studies revealed the drawbacks of employing high SBL rates in TB and necessitated the use of design equations, involving system's transport and kinetic parameters, and operating variables, to optimize bed heights and design suitable control strategy. Rao et al. emphasized on analyzing the effect of model parameters, such as bed diffusivity, maximum specific growth rate constant, maximum biomass concentration, on the critical bed height in a TB [28]. Rajagopalan and modak [29] worked on heat and O_2 transfer in TB and took into account the conduction and metabolic heat generation due to microbial activity. Simulation results suggested that for optimum temperature control, the best strategy was to employ thin bed heights (1-2 cm) and the headspace air temperature to be kept near to the optimum for microbial growth. An increase in bed thickness resulted in low O_2 concentration in bed, which may well be unavoidable in trays. The importance of optimum operating temperature was emphasized in a two-phase growth model [30] where the effect of temperature upshift was investigated on the growth kinetics of *Rhizopus oligosporus*. Modeling approach was used [31] to study the effect of bed O_2 concentration on hyphal elongation, branching frequency and radial elongation rate of *Aspergillus oryzae* for α-amylase production in TB. An improved energy balance was proposed [32] by incorporating the phenomenon of removal of heat by evaporation to the model of Rajgopalan and modak [29]. Montero et al. [33] developed heat and mass balances in a TB (80.6 L) where convective heat transfer was enhanced with the internal circulation of air. These workers emphasized on the effect of Reynolds number, a function of airflow in the bioreactor headspace, on heat removal, mass transfer and maximum possible bed heights. They also estimated coefficients of heat and mass transfer using exhaust CO_2 data and bioreaction stoichiometry. Reliable estimates of exhaust O_2 and CO_2 could provide online insights into the production of metabolic water, the rate of substrate consumption, product formation, etc., which are otherwise very tedious to determine in an SSF process. Correct estimation of process parameters is also of paramount importance for satisfactory validation of SSF bioreactor. Parameter values obtained from related processes may significantly vary from an actual fermentation process [34].

Although TB constitutes a major proportion of industrial SSF processes in fermentation industry [35] [36], this is not without disadvantages. Heat transfer is mainly through conduction and because of low thermal conductivity of substrate; heat dissipation is often not efficient. As a result, control of bed temperature and moisture within the reactor bed is very limited especially at large bed heights. Moreover, TB requires large operational area and the process is labor intensive. Most often the substrate requires separate sterilization, and the process is not contained. It is tedious to apply this technology to sterile operations, except only if large aseptic rooms are built, and sophisticated procedures and equipment are provided for workers, which may be prohibitive and highly costly.

2.2.2 Packed Bed Bioreactor

The characteristic feature of a PBR is forced aeration through static bed which aids in the replenishment of O_2 and moisture and mitigates high levels of temperature and CO_2. PBR are usually employed where mixing is undesirable or deleterious for microbial growth. They offer better control and facilitate high SBL than TB. Construction generally consists of a cylindrical glass or metal drum, which houses the substrate and the walls of the cylindrical glass or drum may be jacketed. Provision of cooling plates may also be provided within the bed to facilitate heat transfer. Fig. 2.2 represents the traditional design of a PBR. The section begins by reviewing recent reports studying the effect of high air flow rate (AFR) on reactor performance. This is followed by issues related to bed compaction, pressure drop, air channeling and strategies to overcome them in PBR. Finally, modeling studies and control strategies addressing the problems of heat accumulation and heterogeneity are reviewed.

Effect of forced aeration was examined [37] on the production of glucoamylase and protease during fermentation of waste bread pieces, by *Aspergillus awamori*, in a 0.5 and 1 L PBR. AFR of 1.5 vvm was found to be optimum, and a model describing its effect on enzyme production was proposed, which successfully predicted the adverse effect of low and high AFR.

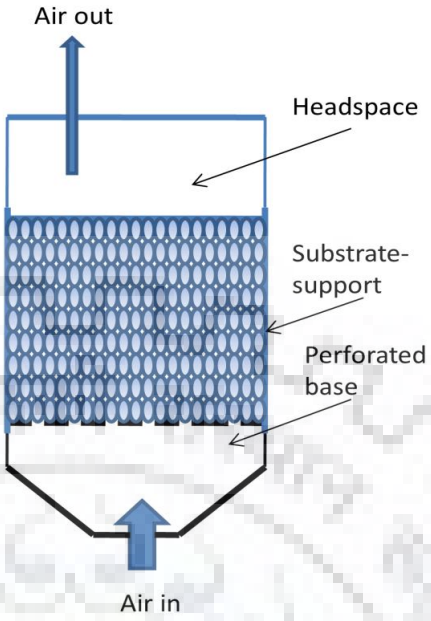

Figure 2.2 Schematic diagram of a traditional packed bed bioreactor.

Similar observations were made during the production of cellulases from olive oil and wine processing waste in a glass column. High AFR decreased cellulase production, which was due to low bed water activity (a_w) and high shear stress on *Aspergillus uvarum* [38]. Inulinase production was negatively affected at high AFR [39] that caused bed moisture to drop and increased the shear stress on fungus. These workers emphasized the importance of IMC of copra waste (substrate), where a higher than optimum level (60%, v/w) resulted in substrate agglomeration and depleted O_2 level, whereas, low levels were inhibitory for inulinase production. The choice for forced aeration may also be governed by the type of product in question. Medeiros et al. [40] used two different AFR to test the production of volatile aroma compounds (VAC) in a PBR during fermentation of cassava bagasse by *Kluyveromyces marxianus*. They observed that lower specific AFR (0.06 L h^{-1} g^{-1}) resulted in higher concentrations of total VAC than high AFR (0.12 L h^{-1} g^{-1}), although bed moisture remained near optimum in both conditions. Higher production was attributed to lower O_2 exposure to yeast, that resulted in higher concentrations of ethyl acetate, ethanol, and acetaldehyde.

A common phenomenon observed in a forcefully aerated PBR is high degree of substrate compaction resulting in air channeling, pressure drop across the bed and subsequent heterogeneity of heat and mass. These problems were observed during the production of Iturin A, an antifungal agent, by a strain of *Bacillus subtilis* [41]. High AFR stimulated the production of poly-γ-glutamic acid which increased bed viscosity, reduced free passage of air, caused a high-pressure drop and consequently decreased oxygen uptake rate (OUR). The issues related to bed compaction were pertinent during pectinase (PG) production, by *Aspergillus niger*, in a pilot scale PBR (200 L) [42]. 20 kg of wheat bran (WB) at 30°C with an IMC of 62% (w/w) resulted in maximum PG productivity of 1350 U kg^{-1} h^{-1}, which was; however, lower than that obtained with lab scale column bioreactor (1930 U kg^{-1} h^{-1}). This decrease was attributed to substrate compaction which resulted in bed shrinkage along the walls of bioreactor that caused air channeling and bed temperature to rise to 37°C. To address this problem, sugarcane bagasse (SB) was used to enhance bed porosity and allow uniform distribution of moist air. 10% of WB was substituted by SB that successfully prevented air channeling and maintained bed temperature to near optimum (30°C). However, this further decreased the productivity (810 U kg^{-1} h^{-1}) as now less WB was available for fermentation. The increase in substrate loading (30 kg) again led to bed compaction, increase in maximum bed temperature (43°C) and moisture gradient. Productivity increased to 1840 U kg^{-1} h^{-1} only when inlet air temperature was cooled down to 24°C during peak heat generation. However, issues of bed shrinkage, heat accumulation, and heterogeneity persisted which suggested that under the given operating conditions, high SBL was not suitable. Biz et al. [43] made further inroads for pilot scale production of PG in the bioreactor system used by Pitol et al. by using *Aspergillus oryzae* and enhancing the mechanical properties of substrate-support. This not only resulted in higher enzyme yield but also circumvented the problems encountered by Pitol et al. Use of citrus pulp (51.6% w/w) and SB (48.4%, w/w) as substrate (15.5 kg) increased pectinase yield to 37 U g^{-1}, from the 20 U g^{-1} reported by Pitol et al. This is a classic example which underscores the importance of suitable substrate parameters (e.g., thermal conductivity, density, specific heat, size, void fraction) and kinetic parameters (e.g., specific growth rate constant, metabolic heat yield, maximum cell biomass concentration) that may significantly affect metabolite production.

Production of hydrolases was investigated during the fermentation of babassu cake by *Aspergillus awamori* [44]. Babassu cake is rich in carbohydrate, known for induction and secretion of enzyme pool, and exhibits good mechanical properties. PBR was employed to overcome scale-up limitations encountered with TB in their previous study [45]. Except for xylanases, higher enzyme yields were reported for cellulases, proteases, endoamylases and exoamylases. Interestingly, isoamylase production was only observed with forced aeration. However, high bed temperature, and heterogeneous temperature and a_w profiles were prominent across the bed, and as a result, these workers called for further improvements. In PBR, bed temperatures can exceed to more than 20ºC than optimum [46] which could potentially render 20-30% biomass inactive particularly in the upper regions of bed [47]. Use of water jacketed system may not be preferred since radial heat loss is often not significant in reactors with a large diameter. Operation with high superficial air velocity (u_s) can be one strategy that may address high bed temperature [48]. Simulation results on the growth model of *Rhizopus oligosporus* showed that cell death could be minimized if u_s of 0.4 ms^{-1} or more were employed, in a 0.3 m bed height. However, this may cause bed compaction and heterogeneity, and also be energy intensive. Other alternatives were to cool the inlet air during peak heat generation and maintain the aspect ratio ≤ 1:1 [48]. Sangsurasak and Mitchell [49] worked on an improved model by including 'evaporation' term in the energy balance. Simulation results showed that convection and evaporation contributed significantly to heat loss. The model assumed that growth is limited by high temperatures and not by bed a_w. However, significant water loss can be expected where evaporation contributes greatly (78%) to heat loss. Using pseudo-steady-state approximation, Weber et al. [50] developed both enthalpy and water balance to study the effect of bed temperature and a_w on sporulation of *Coniothyrium minitans* in PBR. Simplification of enthalpy balance gave a temperature control strategy where AFR was defined as a function of OUR and the rate of metabolic heat generation. Further simulations gave a real-time estimation of bed water content. Ashley et al. [47] worked out a model describing heat control strategies in PBR. These workers argued that when the bed is bottom-aerated for the entire process, upward traversing air gets hotter with distance and loses its ability to cool the bed beyond a critical bed height, and which is why top regions in a PBR experience overheating problems. Using the values of process parameters from the work of Ghildyal et al. (1994), these workers concluded that overheating was inevitable with

unidirectional flow of air. Simulations with air reversal strategy at different time intervals also proved unsatisfactory with central regions of bed remaining above the critical temperature. However, frequent mixing was successful as this facilitated distribution of cooler media throughout the reactor, thereby enhancing the convective heat transfer. To prevent overheating in PBR, an advanced version of the packed bed was proposed by Roussos et al. [51], called 'Zymotis' (Fig. 2.3). Dynamic heat transfer model for Zymotis was also worked out [52, 53].

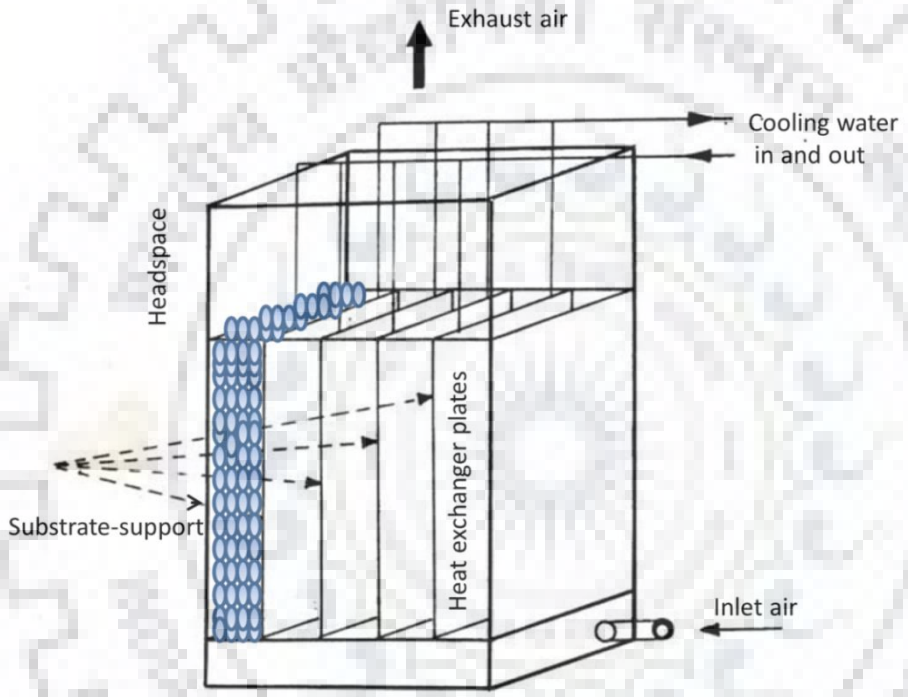

Figure 2.3 Schematic diagram of 'Zymotis' packed bed bioreactor.

SSF operation in packed beds is a significant improvement over TB where higher SBL is possible along with better process control. PBR constitutes a popular mode of operation when mixing is undesirable. However, PBRs even at laboratory scale are susceptible to problems of detrimental temperature rise, substrate compaction, bed drying and process heterogeneity, thereby imposing limitations on operating bed heights. Moreover, in situ and aseptic operation of fermentation processes such as sterilization, inoculation, product removal and post fermentation treatment of the bed is highly unlikely.

2.2.3 Air Pressure Pulsation SSF Bioreactor

Bioreactors under this category employ periodic pulsation of air pressure; which may be coupled with forced air circulation, to enhance the microbial activity and mitigate heterogeneity. A process comprising both the pulsation event and forced air circulation is referred to as gas double-dynamic solid-state fermentation (GDD-SSF). The high partial pressure of O_2 in the gas phase during the compression is intended to increase O_2 concentration in the bed while decompression phase causes bed to swell, thereby, facilitating removal of heat and CO_2. Repeated cycles of the two events simulate mixing action, with an advantage that forces acting are not shearing but normal. Since this bioreactor category has received very little attention in SSF reviews [54], early reports of APP-SSF bioreactors have also been reviewed. The section begins by discussing the effect of operating variables; such as pressure amplitude, pressure duration and frequency, exhaust time and velocity of air circulation, on bioreactor performance. Heat and mass transfer studies though very few available have been reviewed next. The section concludes with the description of honeycomb loading device (HLD) [55], a novel bioreactor design under this category, which works on the principle of air pressure pulsation and forced air circulation.

A SSF bioreactor system was designed [56] where the surface area of the bed exposed to surrounding air was increased by periodic pulsation of air pressure. The pulsation action simulated periods of inhaling and exhaling which was referred to as 'fermenter breathing,' analogous to lungs action. To ensure O_2 was not limiting, the air pressure was varied inside the bioreactor [57]; which consisted of perforated trays supported in a stainless steel (SS) cylinder. The bioreactor was used to study the effect of APP on cellulase production by *Trichoderma viride*, and a threefold increase in enzyme production was observed, compared to TB (static bed operation). Effect of GDD-SSF was investigated on cellulase production by *Penicillium decumbens* [58], where, air pressure pulsation of 0.20 MPa and air circulation at 1.5 m s^{-1} resulted in high productivity. Electron microscopy revealed that substrate was held loose and this probably facilitated efficient heat and mass transfer. However, further increase in air pressure resulted in disruption of mycelia. APP-SSF was used for the production of alkaline protease using *Bacillus pumilus* [59]. Effect of pressure amplitude and duration was examined

on enzyme yield, productivity, and substrate bed loading. With the increase in air pressure to 0.05 MPa and 0.1MPa, protease activity increased by 63% and 95%, respectively, than static bed operation. Here again, APP-SSF simulated mixing action as the bed was held loose. Enzyme yield decreased significantly as the bed height was increased from 1.5 to 6 cm in TB, whereas only a marginal decrease was observed with APP-SSF. Feruloyl esterase production by *Aspergillus niger* was studied [60] during fermentation of rice straw and wheat bran (4:1). Conical flasks containing the substrate were kept in a 25 L cylinder, where pressure amplitude of 0.2 MPa, high and low pressure duration of 20 s and 30 min, respectively, increased the enzyme yield and productivity, resulted in better bed temperature control, circumvented steep gas gradients and enhanced the respiration intensity.

Hongzhang et al. [61] argued that frequency of APP should be a function of organism growth stage and carefully selected as high frequency may not only be deleterious to microbial growth but also be energy intensive. These workers used GDD-SSF for the production of *Bacillus thuringiensis* (Bt) in a 70 m^3 bioreactor; where numerous trays were stacked in an SS cylinder. APP with an upper and lower limit of 1.5 kg cm^{-2} and 0.05 kg cm^{-2}, respectively, along with internal air circulation was more effective and gave maximum Bt activity (18000 IU). Moreover, temperature gradients were minimized when trays were kept in a horizontal position (0.8ºC cm^{-1}) than vertical configuration (4.2ºC cm^{-1}). Yang et al. [62] emphasized the importance of optimum range and duration of air pressure amplitude during the production of a thermo-tolerant xylanase by *Thermomyces lanuginosus*. Exposure for prolonged periods of high pressure negatively affected fungal growth and enzyme yield. Hendges et al. [63] observed lower pectinase production by *Aspergillus niger* with APP, where sudden pressure shock proved detrimental to fungal mycelia. These reports suggested that operation with APP should be designed carefully after studying the morphology of the microorganism.

APP resulted in high xanthan production by *Xanthomonas campestris* using polyurethane foam as inert support [64]. Higher production than the TB, even at high initial glucose concentration (60 to 80 g L^{-1}) was attributed to better O_2 transfer. Both xanthan production and OUR were significantly enhanced after 50 h. Similar observations were made [65] during cellulase production by *Trichoderma reesei*. ATPase activity and cell permeability were used as the

measure of microbial metabolism, and the effect of pressure pulsation on microbial activity was investigated. With APP, the increase in cellulase activity corresponded with an increase in ATPase activity, which suggested that external stimulation might have enhanced metabolism. He and Chen [66] tested the performance of a pilot scale (800 L) GDD-SSF bioreactor for the production of proteases, pectinases, glucoamylases, and cellulases. The bioreactor consisted of two cylindrical tanks where pre-inoculated multiple trays could be stacked on a frame and rolled into tanks. In comparison to the static operation, glucoamylase and protease production were enhanced to ~three folds, and pectinase and cellulose production were enhanced to ~two folds, respectively. Moreover, fermentation times were shortened, and axial temperature gradients were reduced in all the four enzyme systems.

Chen et al. [67] discussed the importance of exhaust time in APP-SSF operation during cellulase production in a 2 L bioreactor. Exhaust time was defined as the time required for exhaust gases to completely discharge the vessel during decompression phase, and was a function of pressure pulsation and vent aperture area. As the air pressure increased up to 0.2 MPa, the cellulase activity enhanced; however, further increase of pressure to 0.3 MPa proved undesirable as this resulted in a large exhaust time resulting in lower enzyme yield. Vent aperture size was identified as an important design parameter that had a significant effect on cellulase production. Chen et al. [68] studied temperature control in GDD-SSF and found that air pressure amplitude of 0.20 MPa, for 5 min duration, coupled with an air circulation rate of 1.5 m s^{-1} brought down the maximum bed temperature from 53ºC (recorded during static bed operation) to 33ºC. At still higher APP (0.30 MPa) temperature control was satisfactory, but this proved detrimental to fungal growth. Axial temperature gradients were reduced to 0.12 cm^{-1}, which was a remarked improvement from trays (3 cm^{-1}) [69]. Chen et al. [70] extended the GDD-SSF for studying mass distribution inside the substrate bed. Bed moisture, cell biomass, and cellulase activity were determined using multivariate calibration models that involved the use of near infrared spectroscopy and chemometrics. Jian and Yang [71] estimated the contribution to heat loss by conduction, evaporation, and convection in static and APP-SSF for the production of cellulases by *Trichoderma koningii*. APP-SSF proved beneficial since heat loss through conduction was enhanced (evident from higher wall temperature) and total water loss doubled; as a result, 657 KJ more energy was dissipated than the static bed. Zhao et al.

[72] worked out a model to determine optimum pressure pulsation frequency and its effect on fermentation productivity.

Chen and He [55] designed HLD device (Fig. 2.4), which worked on the principle of GDD-SSF. The bioreactor consisted of nine stainless steel tubes, laid on a metal frame. The metal frame housed two steel discs as end shields and nine grids were located and welded in between the shields. These steel tubes had openings with a series of little holes on pipe walls. While in operation the inoculated media was transferred into HLD, and the device was pushed into a steam sterilized fermenter, and the cover was closed. Fig. 2.5 shows experimental setup for HLD. The performance was tested by investigating bed temperature variance, between GDD-SSF and a jacketed water system, and comparing the spore viability of *Bacillus cereus* DM423 in the absence of GDD-SSF (static bed). Maximum temperature variation with GDD-SSF was 7.7ºC, and that with circulating water in a jacketed system was 19.8ºC. Also, the spore count was better than what was achieved without GDD-SSF. The apparatus gave an impressing bed loading coefficient of 66.8% (v/v), which is almost two-fold higher than conventional SSF bioreactors. HLD was applied successfully in the industrialization of cellulase and biopesticide. Use of design equations involving heat and mass transfer studies may further help in designing optimum operating conditions and predict temperature variations during scale-up.

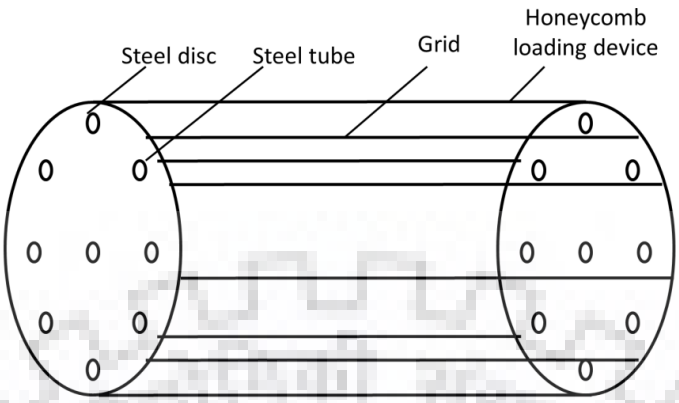

Figure 2.4 Schematic diagram of Honeycomb Loading Device (HLD).

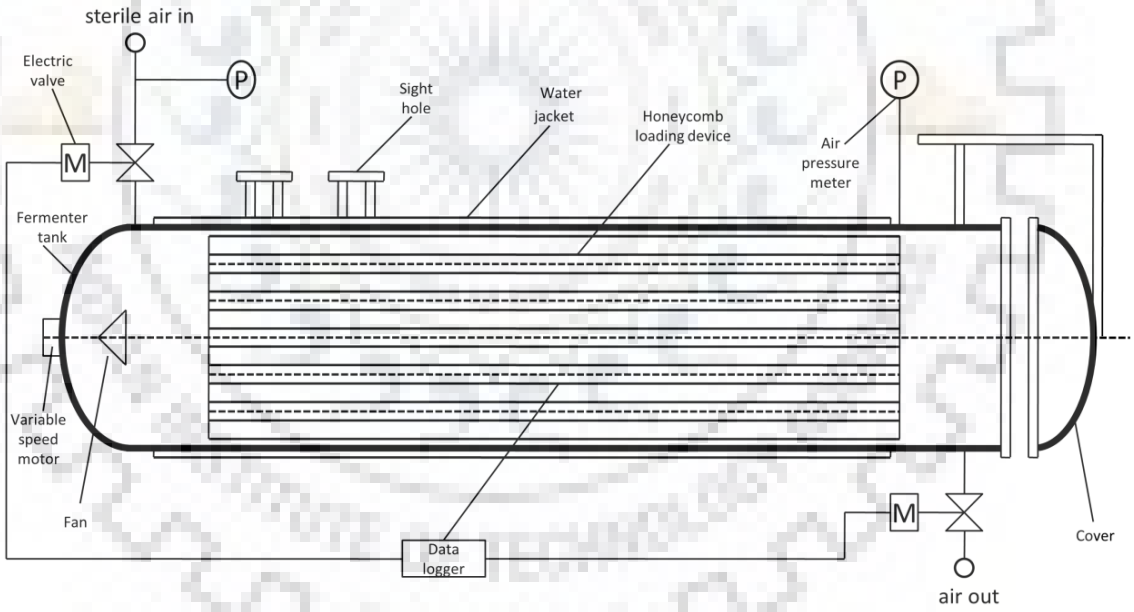

Figure 2.5 Schematic diagram for experimental set up for HLD.

Recent reports suggest that APP/GDD-SSF is a marked improvement over TB. However, there is a dearth of heat and mass transfer studies which could answer some important questions such as: what maximum bed heights are possible with APP for a given set of transport and kinetic

parameters? What would be the effect of operating variables on SBL? Will APP-SSF along with moderate intermittent mixing significantly enhance productivity without affecting fungal growth? Or will the addition of inert support, such sugarcane bagasse, perlite, vermiculite, polyurethane, glass fiber etc., [73] enhance transport properties and reduce process heterogeneity? All these questions only underscore the scope and tremendous research opportunities under this category. Fermentation operations such as substrate pretreatment, inoculation and product extraction are carried out outside the cultivation chamber which may lead to contamination, require large sterile rooms, health hazard, procedures, and protocols for workers. Development of modular bioreactors with APP/GDD-SSF where all the fermentation operation could be carried out in a highly contained fashion may be an interesting prospect.

2.2.4 Intermittently or Continuously Mixed SSF Bioreactor

To enhance heat and mass transfer and stimulate microbial growth, bioreactors under this category involve mixing of substrate bed which is most often coupled with forced aeration. Mixing augments convective transport since it increases the surface area of the substrate exposed to moist air or cooling fluid. However, the challenge is to maximize productivity with fewer mixing events since mixing, even at low intensity, could potentially damage fungal mycelia and also be energy intensive. The current section begins with reports investigating the effect of mixing on biological activity, heat and mass transfer and process productivity in rotating drum bioreactor (RDB) and other intermittently or continuously agitated, forcefully aerated systems. Few recent studies where traditional RDB design has been modified for use in waste treatment have been discussed. Pilot and industrial scale bioreactors that have come up over the years, under this category, have been discussed next. The section ends with a discussion on the utility of mathematical modeling and design equations in guiding appropriate bioreactor configuration and operating conditions.

RDB comprises a drum-shaped container that may be mounted on a roller (rotating device) and consists of three subsystems i.e. the wall of the drum, the headspace and the substrate. Air is typically blown through the headspace above a tumbling bed of substrate particles, and the bioreactor may be intermittently or continuously rotated. Mixing of substrate bed is facilitated by rotating action of the drum around its central axis (Fig. 2.6 a), however, stirred drum

bioreactors may also be used, where paddles mounted on a central shaft facilitate mixing, while the drum remains stationary (Fig. 2.6 b). The drum may also be fitted with internal baffles or lifters (Fig. 2.6 c), of different sizes and shapes, to facilitate mixing.

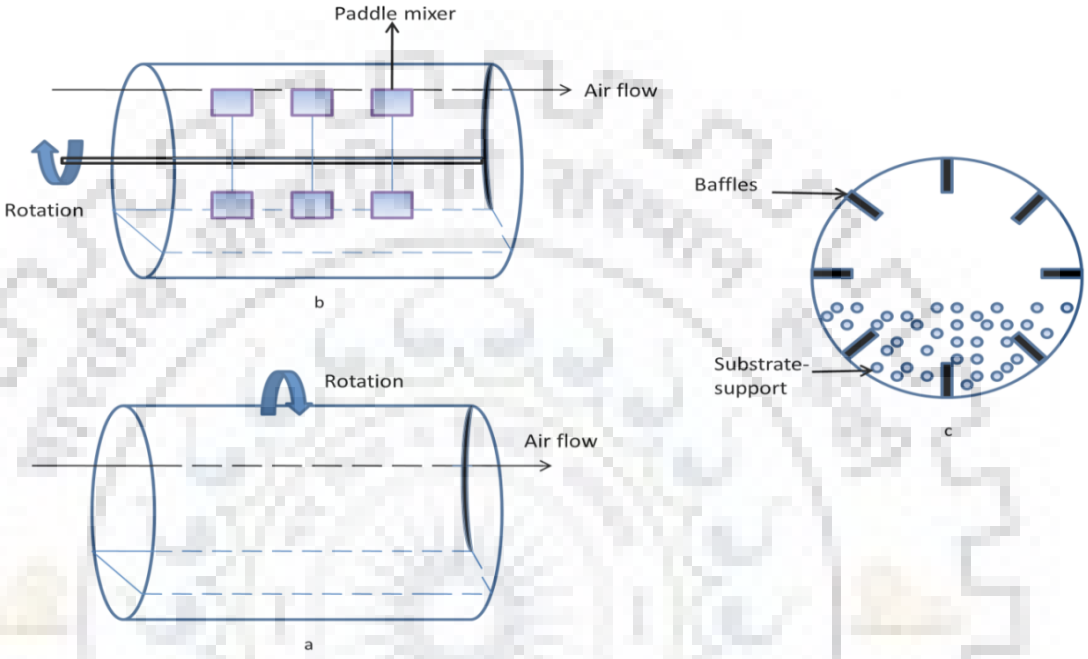

Figure 2.6 a. Schematic diagram of rotating drum bioreactor. b. Stirred drum bioreactor. c. Cross section of RDB showing arrangement of baffles.

Using 4 kg of empty fruit bunch as substrate, a two-fold increase in cellulase production was reported in a 50 L RDB than flasks [74]. The increase was attributed to better aeration and mixing in RDB. Simultaneous saccharification and fermentation of alkali pretreated sugarcane bagasse were performed in a 100 L RDB with internal baffles [75]. To achieve high ethanol productivity, a thermo-tolerant yeast *Kluveromyces marxianus* was used which could grow optimally at temperatures required (40-50ºC) for prolific saccharification. Gentle mixing at low intensity and frequency of rotation resulted in high ethanol yield (79%).

Effect of agitation on the production of hydrolases was studied [76] in a lab scale RDB (0.25 L) where *Aspergillus awamori* was grown on grape pomace and orange peels (1:1). Maximum

activities for xylanases, exo-polygalactouronase and CMC were obtained at high AFR (120 mL min^{-1} and 200 mL min^{-1}) and very low agitation rate (1 min d^{-1}). Continuous mixing without aeration caused substrate agglomeration which resulted in reduced growth. The intermittent rotation was found favorable for citric acid production during fermentation of apple pomace by *Aspergillus niger* [77]; however, continuous mixing resulted in 34% reduction in citric acid production. Mixing resulted in significant reduction in endoglucanase, glucosidase, and xylanase activity during fermentation of wheat bran by *Aspergillus niger* [78]. Cellulase production by *Aspergillus niger* was decreased by 17% with an increase in mixing frequency, in a novel bioreactor [79]. The above reports suggested that continuous mixing had a negative impact on process productivity. However, even if the organism is unable to tolerate mixing it may still be necessary to aseptically mix the bed during inoculation [80] and sampling [79] to ensure uniform distribution.

On the contrary, several reports have corroborated improvement in heat and mass transfer, and overall productivity, as a result of mixing action. Nagel et al. [81] observed that continuous mixing facilitated heat and mass transfer and there was negligible damage to microbe as the fungus mainly grew inside the wheat grain. Also, respiratory profiles of continuously mixed cultures were similar to those of unmixed ones. Effect of intermittent mixing (IM) was evaluated on sophorolipids production by *Starmerella bombicola* in 0.5 L flasks, using a mixture of winterization oil cake and sugar beet molasses (4:1) as substrate [82]. In contrast to the static operation, mixing action enhanced sophoroilipds yield and OUR by 31% and 15%, respectively. Effect of IM was studied on cellulase production [83] by *Trichoderma reesei* using wet corn distilled grains in flasks and glass columns. Mixing had an overall positive effect on microbial activity, however marginal (5-10%) decrease in cellulase activity was observed. Compared to static bed, a 23% increase in lovastatin production by *Aspergillus flavipes* was observed when IM was coupled with forced aeration [84]. Various other reports corroborate that IM did not negatively affect product yield [85-87]. No substantial decrease was observed in methylesterase production, CER and OUR of *Aspergillus tamari* during IM of the substrate bed [88]. Schutyzer et al. [89] argued that it may be impractical to derive a general mixing strategy for a broad spectrum of bioprocess and that the choice for mixing shall be

governed by fungal morphology, chemical and physical nature of the substrate, bioreactor configuration and mixing regime employed.

Few interesting configuration of RDBs reported for biological treatment of pollutants are discussed here. Nitric oxide (NO) removal efficiency was evaluated in a rotating drum biofilter [90]. The bioreactor comprised of a covered aluminum chamber which housed spongy media on aluminium drum frame with impermeable plates at both ends. Inoculum consisted of a concentrated sludge, derived from a wastewater treatment plant, and was supported on the sponge material. The inside of the drum contained the nutrient media, which was distributed onto the growing cultures through the drum rotating action. However, this also resulted in the formation of liquid biofilm across the sponge which offered resistance to the incoming NO stream. High rotation speed increased the thickness of the liquid film that resulted in low NO bioconversion. Rodriguez-Meza et al. [91] used a bench-scale RDB to study bioremediation of soil contaminated with total petroleum hydrocarbons (TPH). The removal efficiency of TPH was analyzed as a function of length to diameter ratio (L/D), rotation speed (*N*) and aeration in the bioreactor. Slurry, filled to 30% of reactor capacity, consisted of mineral medium and contaminated soil. 59.6% (w/v) of TPH removal was achieved at L/D of 1.5, and with helical fillers the TPH removal was enhanced to 62%. Treatment efficiency strongly depended upon *N* and the aeration rate.

An industrial scale IM-SSF bioreactor (15-ton capacity) was successfully employed for koji production by Nagata Brewing Industry Co. Ltd, Japan [92]. The detailed working operation was however not made available. Another bioreactor (1-ton capacity) in this category was developed for single-cell protein (SCP) production [93]. Mixing action was provided with screws that lifted the substrate with their rotating action, and was coupled with intermittent water addition. The bioreactor was successfully extended for the production of enzymes and biopesticides [94]. A similar bioreactor configuration (10-ton capacity) was employed [95] for the production of microbial protein using *Aspergillus tamari*. Perez-correa and Agosis [96] used a 200 kg capacity bioreactor for gibberlic acid production by *Gibberella fujikori*. Unlike the one developed by Durand and Chereau [93], agitator assembly was fixed, whereas the solid material was moved on a plate. However, only 40-60 cm bed heights were used. Fig. 2.7

represents schematic diagram for intermittently mixed and forcefully aerated bioreactors. Most of the bioreactors cited in this paragraph have similar designs, and are limited in their capacity to provide sterile operation, simple product extraction, and post extraction residue treatment. Although several reports are available in this category [97-101] where bioreactors have been operated under sterile conditions, however, substrate bed loading was limited (50 kg or less), and therefore, sterility and scalability appear to be inversely proportional for the existing SSF bioreactor designs.

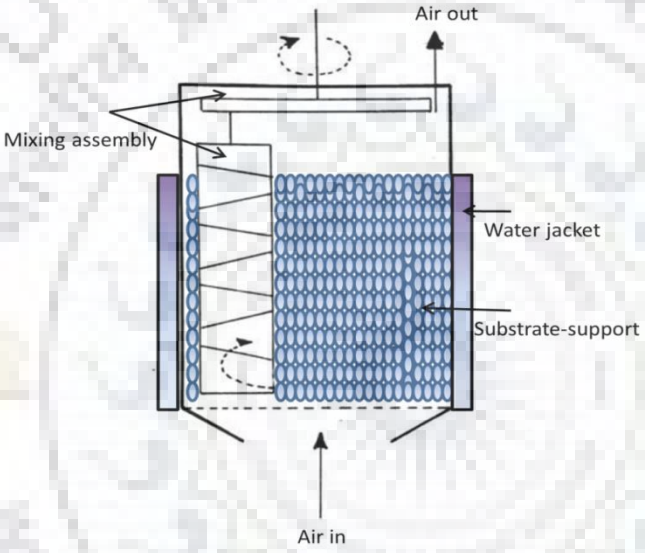

Figure 2.7 Schematic diagram of a forcefully aerated and intermittently mixed bioreactor.

Modular bioreactor configurations are promising prospects for next generation SSF bioreactor system, as they could potentially circumvent process heterogeneity [1] and operate aseptically. Bohmer et al. [102] worked on a mixed modular bioreactor for laccase production by *Trametes hirsutae* using pine wood chips and orange peels as substrate. PLAFRACTOR**,** a modular design patented by Biocon Ltd. [103] claimed for a self-contained SSF device that combined all the fermentation operations, i.e., sterilization, inoculation, cultivation, extraction, and post-extraction treatment, in a single unit.

The bioreactor was tested for sterile production of proteases, cyclosporine, amylases, and lovastatin. Multiple modules were stacked vertically (Fig. 2.8 a), where each module consisted of a base plate and a frame which formed the sides of a structure that served as a container for holding media (Fig. 2.8 c). The base plate consisted of two separate set of channels, i.e., communicating (carrying nutritive, extractive and sterile fluids) and non-communicating channels (carrying heating and cooling fluids) (Fig. 2.8 b). Mixing in each module was achieved with the help of two concentric shafts at the center of mixing arrangement. A working bed height of 4-8 cm was assumed in each module. Temperature control was claimed by conduction, which however is the least contributor for heat removal [104].

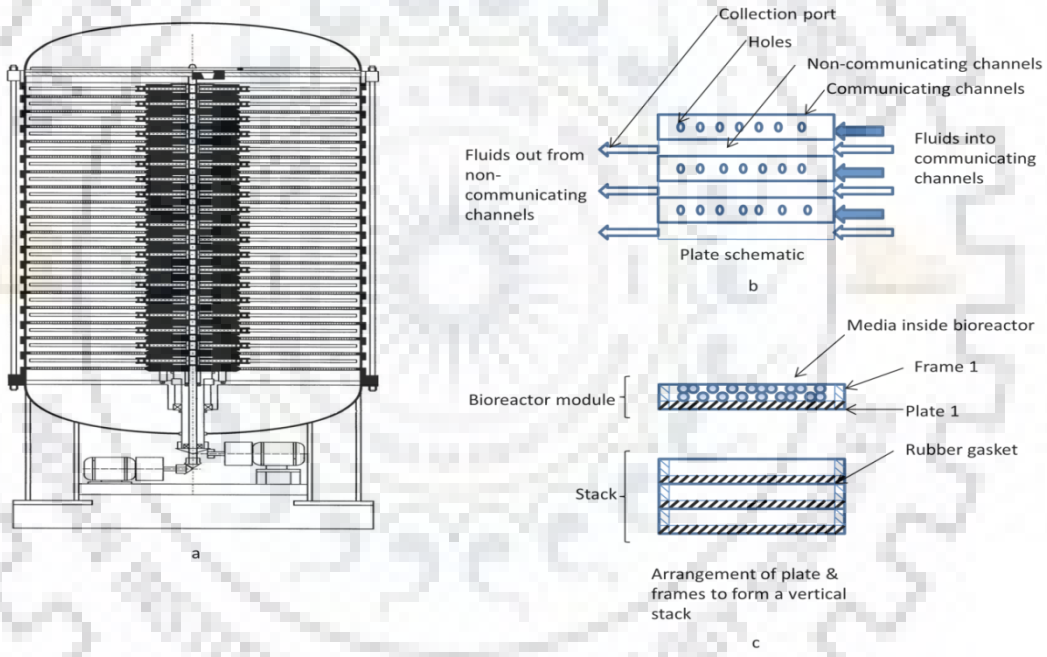

Figure 2.8 a. Schematic representation of PLAFRACTOR bioreactor showing multiple modules stacked vertically. b. Plate schematic. c. Arrangement of plate and frames to form vertical stack.

Use of mathematical design equations could be of great utility here, as they would help optimize working bed heights. Random and un-optimized bed heights could result in detrimental temperature rise, especially at top regions of the bed.

Novozymes Bio A/G patented [105] a novel bioreactor design, with maximum automation in operation. The bioreactor comprised of an upper and lower compartment separated by a perforated plate (Fig. 2.9). The upper compartment housed the substrate and was the site of fermentation, whereas the lower chamber facilitated the transfer of sterile air to the upper chamber. Each compartment housed one or more apertures, of which at least one was used for air supply, and could be controlled by an electric or pneumatic valve, thereby allowing the change of direction of air flow across the perforated plate. Access ports were provided to enable collection of solid samples via utensils or vacuum. The upper compartment also housed one or more nozzles for the supply of water, inoculum, extracting fluid, etc. Provision for mixing was also made to reduce or eliminate differential environment across the bed. Bioreactor units were connected to fermentation stations for supply/exhaust of liquid/gases, extraction and washing purposes. The transport and handling of bioreactor units was assigned to automated guide vehicles and industrial robots. Multiple such units (10-2000) could be employed depending upon the scale of operation.

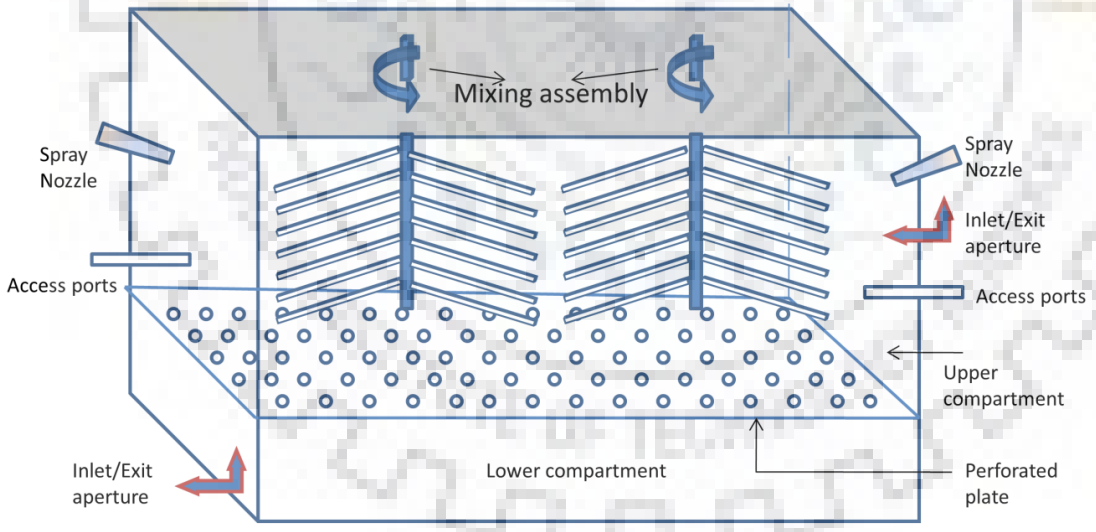

Figure 2.9 Schematic representation of modular bioreactor adapted for automation (Novozymes Bio A/G).

As with PLAFRACTOR, all the fermentation operations may be carried out, aseptically, in a single unit. The horizontal expansion of the bioreactor (100-400 cm or 150-300 cm) was larger than the vertical height (1 m, 2 m or 2.5 m) and was estimated to replace 50-100, 2 kg plastic SSF bags. Bed heights of 10-50 cm were claimed, which may well be possible since similar bed heights have been employed successfully in PBR [43]. As modeling studies and control strategies [106, 107] have shown, it is prudent to optimize SBL based on system parameters and operating variables. Here, inlet AFR, inlet air temperature and humidity, mixing regime, the direction of air flow and the system's transport and kinetic parameters can be used to design operating bed heights, where even larger bed heights may be possible than presently claimed by the inventors.

In RDB, useful space for fermentation is usually 30% of total drum volume [55]. As corroborated by modeling studies [108], high SBL may lead to process failure unless reactor design and operation are backed by a sound understanding of heat and mass characteristics, mixing behavior and control strategies. Jin et al. [109] investigated the effect of air spargers and lifters on mass transfer coefficient (k_La) during bioleaching process in RDB. Zhang et al. [110] extended mass transfer studies in an 18 L bioreactor [109] and developed a correlation between Sherwood number, reactor configuration and operating parameters for scale-up. Wang et al. [111] studied the effect of operating conditions and reactor configuration on power consumption, in an RDB. Liu et al. [112] did extensive work on power consumption in a 703 L RDB [109] and concluded that specific power requirement was significantly less than stirred tank reactor of similar size.

AFR, drum rotational speed, and SBL were accounted in Peclet number (P_e), and the latter was used for estimation of k_La [113]. Using this approach, k_La for different RDBs could be calculated at different P_e values. Hardin et al. [114] improvised on the modeling work of Saucedo-castaneda et al. [115] and developed a design tool, which was called as dimensionless design factor (DDF). DDF was defined as the ratio of kinetic (rate of metabolic heat generation) and transport (rate of heat removal) process in the reactor and was used to select operating variables such as AFR, inlet air temperature, and humidity to control bed temperature and guide scale-up. Nikakhtari et al. [116] studied the effect of SBL and *N* on k_La in a bead mill

and RDB with baffles. These workers provided scale-up strategies by correlating k_La with Froude, Reynolds and Schmidt number. Marsh et al. [117] studied mixing behavior in RDB and determined axial dispersion coefficient (*D*). Correct estimates of *D* could predict mixing speed and mixing time required for complete distribution of inoculum, water, and nutrient within substrate bed, as well as the degree of substrate agglomeration. However, estimates were made in the absence of microorganism and mixing characteristics may considerably differ in an actual fermentation. Discrete particle simulation (DPS) was used [89, 118] to characterize mixing in two and three dimensions in RDB with *Aspergillus niger*. The tensile strength of *A. niger* mycelium was obtained at different fermentation times, and used in DPS, for optimization of mixing events. Using modeling studies, Nagel et al. discussed strategies for temperature [81] and moisture [119] control in RDB. Wang et al. [120] worked on the modeling of RDB for ethanol production under anaerobic conditions.

Although IM makes it possible to replenish and homogenously distribute water in the bed, it is not a simple matter to aseptically determine on-line bed a_w during fermentation. Khanahmadi et al. [34] estimated the moisture content of bed by determining inlet and outlet gas temperature. A model for water balance was used to derive the relationship between the rate of change of water and dry matter, with the rate of metabolic heat generation. However, it was assumed that air is saturated and remains in thermal equilibrium with solid, which may not hold true where dry air is used to cool the bed. For the case where dry air is used to cool the bed, air and solid were considered two different phases, not at equilibrium, and energy and water balances were developed for them separately [106]. A control strategy based on model simulations suggested satisfactory control of moisture with fewer mixing events. However, at low mixing frequency, the bed temperature quickly returned to a high value, and a better control strategy was necessitated. Meien et al. [107] tested proportional integral derivative and dynamic matrix control strategy to the model of von meien and Mitchell [106] for IM and forcefully aerated PBR. Simulations showed that varying inlet air temperature and keeping it saturated, along with intermittent water supply was a good strategy. However, irrespective of the control strategy employed, temperature gradients could not be avoided in a 2 m bed height which underscored the utility of modeling studies for designing bioreactor operation.

Intermittently mixed SSF bioreactors offer better control options than TB and PBR and also augur high SBL rates. Limitations with some of the earlier reported designs are nonsterile operation, cumbersome product extraction and post fermentation residue treatment. Modular design, such as those patented by Biocon Ltd [103] and Novozymes Bio A/G [105] are promising in their ability to aseptically perform all the fermentation operations in a single module. However, such systems should be validated for various bioprocesses and backed by suitable design equations and control strategies to ensure satisfactory scale-up. At present, very few performance reports are available for the automated modular system, and vast scope for reactor development and validation exist in this category.

2.2.5 Air-solid fluidized bed bioreactor

Air-solid fluidized bed bioreactor (ASFB) is another category of SSF bioreactor where substrate and microorganism are constantly kept in a fluidized state by the action of upward flow of air. The fluidized state effectively increases the surface area of substrate available for microbial growth [121]. Efficient heat transfer is also achieved due to a high degree of turbulence arising from high superficial air velocities. The section starts by describing the basic design features of AFSB which is followed by few case studies where it has been mainly used for the production of volatile products. Only a few reports are available where ASFB have been applied to SSF bioprocess and whatever is available has been mostly limited to lab scale operation. On the other hand, Anaerobic Fluidized Bed bioreactor (AFB), a similar design to ASFB, but where fluidization is performed through a liquid source (wastewater or effluent stream) has drawn considerable attention. Engineering aspects for AFB bioreactors are discussed for few cases that may be adapted for ASFB and other SSF bioreactors. Finally, mathematical modeling and scale-up criteria developed for ASFB has been revisited. Fig. 2.10 shows a schematic diagram of ASFB.

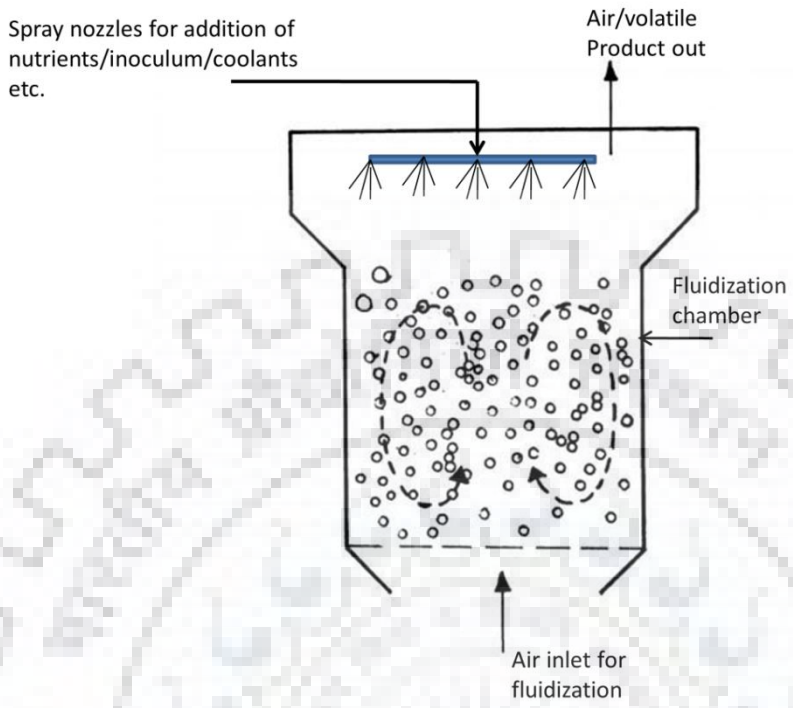

Figure 2.10 Schematic diagram of air-solid fluidized bed bioreactor.

The design consists of a fluidization chamber that has provision for the entry of fluidizing fluids from the bottom, and a nozzle is usually present at the top for spraying the substrate. A base plate may be provided that serves as a support for the particles, and just above it, the stirrer may be present to prevent agglomeration of falling wet particles. Most reports with fluidized bed bioreactors include the production of volatile products such as hydrogen, ethanol, glutathione, and biological treatment of industrial or agriculture waste and cultivation of yeast. Production of volatile products using ASFB is advantageous as they are continuously stripped of the reactor, as a result, there is lower product inhibition and the need for expensive downstream processing is also avoided. Furthermore, high cell densities may be achieved by using solid particles having a high surface area such as granulated activated carbon. The ASFB bioreactor can be operated as fed-batch or continuous, as the substrate can be added through nozzles during the fermentation.

Tanaka et al. [122] used ASFB for cultivating *Saccharomyces cerevisiae* and enzyme production by *Eupenicillium javanicum* using wheat bran as substrate. Excessive supply of O_2, due to fluidization, resulted in a change in yeast physiology (smaller cell size) which led to low glucose consumption as compared to static SSF. With *E javanicum*, amylase and protease productivity was twice than static SSF. However, very high AFR (220 L min^{-1}) were employed to fluidize 150 g of wheat bran in both the cases. Matsuno et al. [123] studied enzyme production in a bench (16 L) and pilot scale (8000 L) ASFB using *Aspergillus sojae*. High cell biomass and enzyme activities were observed than static and SMF operations. Alkaline protease and peptidase activities were 5-10 times higher than static and SMF; however, glutaminase production was fairly poor. Very little performance data was provided, and no information was available for superficial velocities used for fluidization. These workers also developed a vibrating ASFB for the cultivation of *S. cerevisiae*, which was used for reduction of ethyl acetoacetate (EA). The bioreactor was 41.5 cm in height with variable diameter. The lower 11.5 cm length had an internal diameter of 5 cm; this was followed by an expanding diameter region of length 12 cm to finally a length of 18 cm with a constant diameter of 8 cm. Vertical vibrations were induced into the bed through a reciprocating device. A screen was installed at the top to prevent entrainment of substrate particles and yeast. An AFR of 90 L min^{-1} was used for fluidization of 80 g of wheat bran powder with nutrient supplements. As observed with Tanaka et al. [122], AFR was much higher than what is usually used with PBR or RDBs. Furthermore, it was not clear how frequently the bed was fluidized. Yeast cells were harvested from ASFB, static SSF and SMF to compare the reduction rates of EA. Reduction efficiency for cells under the static cultivation was significantly higher than ASFB and SMF. Silva and Yang [124] developed a spouted bed SSF bioreactor for the production of amylases using *Aspergillus oryzae*, where 200 g of brown rice was packed in a plexiglass column with a height and diameter of 73.6 and 7.3 cm, respectively, and a 9 cm working bed height was used. Intermittent spouting at a four-hour interval gave high protein activities that were comparable to PBR and were ten times more than continuous spouting and tray fermentation. Continuous spouting proved deleterious to mycelia, increased the lag time and resulted in a drastic decrease in amylase production. Due to problems encountered in solid handling with PBR, these workers recommended used of spouted bed for large-scale production of amylases. Bed moisture content, spouting velocity and mixing time were observed as critical design parameters. Foong

et al. [125] evaluated the effect of air superficial velocity during fungal fermentation of palm kernel cake in PBR and a lab scale ASFB. In PBR, an AFR of 0.10 m s^{-1} and 0.17 m s^{-1} resulted in early sporulation and low sugar consumption (25-28%) which was attributed to low water activity, air channeling, and O_2 limitation. However, an AFR of 0.60 m s^{-1} doubled cell biomass, maintained working pH and optimum moisture level, and increased sugar utilization (up to 40%). However, for a moderate increase in sugar consumption, 0.60 m s^{-1} AFR appeared to be considerably higher as compared to those usually employed with PBRs.

Process parameters such as the superficial velocity of air, gas retention time, nature of substrate-support, bed porosity are likely to affect the performance of ASFB; however, very little work is available to derive any generalization. AFB, a similar type to ASFB, has attracted many workers for production of gaseous products, mostly for H_2, to be used as fuel [126] [127] [128]. Here, solid particle or carriers which support/immobilize cell biomass remain suspended by drag force of fluidizing liquid. Barros et al. [129] investigated the suitability of polystyrene and expanded clay (EC) as a solid support in a glucose enriched synthetic wastewater. AFB with EC at low hydraulic retention times (HRT) resulted in higher H_2 content and H_2 yield. Marques dos Reis and Silva [121] also used EC in an AFB which was operated continuously (227 d) with synthetic wastewater for H_2 production. It was observed that high superficial velocities of air did not exert any positive effect on H_2 yield and production rate, whereas low HRTs resulted in high H_2 productivity. These workers also emphasized on the estimation of mass transfer parameters for different reactor configuration to derive useful generalization and scale-up guidelines. Alvarado-Lassman et al. [130] used inverse fluidization bed bioreactor for the treatment of brewery waste where the density of support material was less than that of water. Hydrodynamic characterization of two different supports was carried out, and optimum bed expansion as a function of liquid velocity was studied. Liquid velocity and particle size had a significant effect on bed expansion. 25% support volume (of total active bed volume) was determined to be optimum for high organic loading rate and chemical oxygen demand removal. High HRT negatively affected biofilm formation. Similar approaches analyzing the effect on hydraulic parameters on process productivity could also be adapted for the development of ASFB systems.

Röttenbaucher and Bauer [131] used mathematical modeling as a tool to assess the performance of ASFB bioreactor during ethanol production by *S. cerevisiae*. Microbial kinetics, as well as the bioreactor height and diameter, were accounted for in the model. A mass balance on glucose concentration was made to describe its profile inside the cell pellet. Glucose profile was used to balance ethanol, CO_2 and cell mass and corresponding theoretical yield coefficients were also obtained. Experimental validation was done on two laboratory scale bioreactors with diameters of 20 and 55 cm, respectively. Ethanol yield in ASFB was higher than SMF. The model generated yield coefficients correlated well with those obtained experimentally. These workers proposed two parameters for the scale-up of fluidized bed reactors, i.e., circulation time and substrate bed loading rates.

As mentioned above, ASFB has received very little attention and consequently, very few reports are available on it. This is corroborated by its very limited or no reference even in recent reviews on SSF technology [3] [132] [1]. There may be a multitude reasons for less popularity. Firstly, traditional designs are unsuitable for scale-up because of improper mixing and substrate distribution in deep beds. Besides, the procedure for keeping the bed in the fluidized state requires high superficial velocities which mean high operational cost. Moreover, ASFB is suitable for volatile products, and finally the detrimental effect of high shear forces on fungal mycelia and biofilms during reactor operation. Performance comparison of ASFB bioreactor and other SSF bioreactors types is shown in Table 2.3.

2.3 Conclusion

Over the years, SSF bioreactors have been in the process of rapid modifications and advancement so as to maximize the productivity and increase the commercial utilization of SSF processes. It includes the development and exploration of new processes employing different engineering tools to accomplish the desired outputs. With increasing improvement and application of rational methods in engineering, SSF can reach higher levels of industrial standardization. The above-mentioned bioreactors, categorized by their operating mechanisms represent a holistic picture of the existing technology and what can indeed be done to realize its potential at the industrial scale truly. The relevant details clearly suggest that most current available design models neglect many of the important observable facts which are important for

scale-up and to meet safety standards and approval from regulatory authorities. The integration of maximum possible features in a single bioreactor system along with design equations aiding in automation and control of the fermentation parameters, and thereby facilitating its potential to be scaled up to the production level, with complete sterility, is desired to cash in on the immense socio-economic opportunities that SSF technology holds.

Table 2.2 SSF Bioreactors used in various bioprocesses.

SSF Bioreactor types used	Substrate used	Product	Microorganism used	Production level	Reference
Tray bioreactor	Rice bran, wheat bran, soybean meal and wheat flour.	Alkaline protease	*Aspergillus oryzae*	74 U/g ds	[20]
Tray bioreactor	Rice	Conidia	*Beauveria bassiana*	3.92×10^{12} conidia kg^{-1} rice	[25]
Tray bioreactor	Wheat bran	Polygalactouronase	*Aspergillus oryzae*	298 U/g	[133]
Tray bioreactor	Sifted pine sawdust, rice straw and soybean powder.	Manganese peroxidase	*Irpex lacteus*	950 U/L	[134]
Tray bioreactor	Palm kernel cake and Palm pressed fiber	Protease	*Aspergillus oryzae*	319.3 U/g	[135]
Tray bioreactor	Wheat bran, orange peel and lemon peel	Polygalatouronase	*Aspergillus giganteus*	180 U/gds	[136]
Column-tray bioreactor	Lemon peel pomace	Pectinase	*Aspergillus niger*	2181U/L	[21]
Tray bioreactor	Wheat bran and linseed oilcake	Phytase	*Rhizopus oryzae*	148.98U/gds	[26]
Tray bioreactor	Apple pomace	β glucocidase	*Aspergillus niger*	64.18IU/gds	[19]
Tray bioreactor	Tomato pomace	Xylanase	*Aspergillus awamori*	195 IU/gds	[137]
Tray bioreactor	Grape pomace and orange peels	Xylanase CMCase	*Aspergillus awamori*	42.64 IU/gds 2.16 IU/gds	[138]
Static bedbioreactor	Wheat bran	Endoglucanase	*Aspergillus niger*	29.8IU/gds	[139]
Static bed with forced aeration	Wheat bran	Endoglucanase	*Aspergillus niger*	50.2IU/gds	[139]
Packed bed bioreactor	Babassu cake	Exoamylase Endoamylase Protease Xylanases Cellulases	*Aspergillus awamori*	73.4 U/ g 55.7 U/ g 31.8 U/ g 23.8 U/ g 6.2 U/ g	[44]
Packed bed bioreactor	Citrus pulp and sugarcane bagasse	Pectinase	*Aspergillus oryzae*	37 U/ g	[43]
Packed bed bioreactor	Wheat bran and sugarcane bagasse	Pectinase	*Aspergillus niger*	20 U/ g	[42]
Packed bed bioreactor	Pressmud	Inulinase	*Kluyveromyces marxianus*	300.5 U/gds	[140]
Packed bed bioreactor	Copra waste	Inulinase	*Penicillium rugulosum*	239 U/gds	[39]
Packed bed bioreactor	Wheat bran and sugarcane bagasse	Endoglucanase	*Myceliophtora sp I-1D3b*	878 U/gds	[141]
Packed bed bioreactor	Cane molasses and soybean bran	Inulinase	*Kluyveromyces marxianus*	436.7 U/gds	[142]
Packed bed bioreactor	Carob fruits and wheat bran	Ethanol	*Zymomonas mobilis*	60.9 g/kgds	[143]
Packed bed bioreactor	Defatted soybean meal	Iturin A	*Bacillus* iso-1	5.58 g/kgds	[41]
Packed bed bioreactor	Wheat bran and wheat straw	Lovastatin	*Aspergillus terreus*	1.86 mg/gds	[144]

SSF Bioreactor types used	Substrate used	Product	Microorganism used	Production level	Reference
Packed bed bioreactor	Waste bread	Glucoamylase Protease	*Aspergillus awamori*	130.8U/g 80.3 U/g	[37]
Packed bed bioreactor	Cane bagasse	Citric acid	*Aspergillus niger*	55.90g/100 gds	[145]
Packed bed bioreactor	Wheat bran	α-amylase	*Bacillus sp. KR-8104*	473.8U/gds	[146]
Packed bed bioreactor	Wheat bran	Phytase	*Aspergillus ficuum*	87.75 U/gds	[147]
Packed bed bioreactor	Strain immobilised in orange peels	Endo- and exo-Polygalacturonase	*Aspergillus nigerURM 5162*	1.18 U/ml 4.11 U/ml	[148]
Packed bed bioreactor	Polyurethane foam	Tannase	*Aspergillus niger*	7,955 U/l	[149]
Packed bed bioreactor	Sugarcane bagasse and sunflower seed meal	Biodiesel	*Burkholderia cepacia*	95% conversion after 46h	[150]
Glass columns with forced aeration	Citric pulp, sugarcane molasses and methanol	Citric acid	*Aspergillus niger (mutants)*	278.4, 294.9 and 261.1 g CA/kg	[151]
Rotatory drum bioreactor	Soybean meal	Amylase and Protease	*Aspergillus oryzae*	85,000-110,000U/gds, 7800-9000U/gds	[152]
Rotatory drum bioreactor	Defatted rice bran	Amyloglucosidase Exo-PG	*Aspergillus niger*	886.2 U/g-dm 84 U/g-dm	[153]
Rotatory drum bioreactor	Apple pomace	Citric acid	*Aspergillus niger*	220.6 g/kg-ds	[77]
Rotatory drum bioreactor	Sugarcane bagasse	Ethanol	*Kluveromyces marxianus*	24 g/L	[75]
Rotary drum bioreactor	Rice by-product, whey and sugarcane bagasse	Ethanol	*Aspergillus niger*	11.7 g/L	[154]
Rotary drum bioreactor	Empty palm fruit bunch fiber	CMCase Avicelase Xylanase	*Penicillium verruculosum*	6.5 6.8 8.8	[155]
Rotary drum bioreactor	Empty palm fruit bunch fiber	Endoglucanase Exoglucanase β-glucosidase	*Aspergillus niger*	135 U/L 52U/L 161U/L	[156]
Rotatory drum bioreactor	Palm oil lignocellulosic biomass	Cellulase	*Trichoderma harzanium*	8.2 FPA/g-ds	[74]
Rotary drum bioreactor	Grape pomace and orange peels	Xylanase, exopolygalacturonase CMCase	*Aspergillus awamori*	54.4 IU/gds, 8.77 IU/gds, 3.69 IU/gds	[76]
Rotatory drum bioreactor	Coffee husk	Ethyl acetate	*Ceratocystis fimbriata*	28.55µmol/L/gds	[157]
Rotatory drum bioreactor	Grape pomace	Pectinases, xylanases and cellulases	*Aspergillus awamori*	8.77IU/gds 54.42IU/gds 3.69 IU/gds	[76]
Rotatory drum bioreactor	Autohydrolyzed algae	Fucoidanase	*Mucor sp. 3P*	9.62 U/L	[158]
Gas double-dynamic solid-state fermentation bioreactor	-	Cellulase	*Penicillium decumbens JUA10*	18 IU/gds	[67]
Gas double-dynamic solid-state fermentation bioreactor	-	Cellulase	*Trichoderma viride* L3	-	[65]
Solid-state bioreactor with honeycomb loading device (HLD)	Wheat bran/rice bran/Soybean cake powder	Spore	Bacillus cereus DM423	$(1.50 \pm 0.07) \times 10^{11}$ cfu/gds at 40 h	[55]

SSF Bioreactor types used	Substrate used	Product	Microorganism used	Production level	Reference
Air pressure pulsation bioreactor	Rice straw and wheat bran	Feruloyl esterase	*Aspergillus niger*	881mU/g	[60]
Air pressure pulsation bioreactor	Polyurethane foam	Xanthan	*Xanthomonas campestris*	42.62 g/L	[64]
Air Pressure pulsation Bioreactor	Steam exploded wheat straw and wheat bran	Filter paper enzyme activity	*Penicillium decumbens JUA 10*	20.4 IU/g	[58]
Air pressure pulsation bioreactor	Corn cob Wheat bran	Xylanase	*Thermomyces lanuginosus*	8237 IU/g moldy bran	[62]
Intermittently mixed bioreactor	Wheat bran and maize meal	Spores	*Clonostachys rosea*		[159]
Counter-current SSF bioreactor	Sugarcane bagasse	Glucoamylase	*Aspergillus niger*		[160]
Plafractor	Wheat bran	Lovastatin	*Aspergillus flavipes*	-	[103]
Plafractor	Wheat bran	Protease	*Rihzhomucor pusillus*	-	[103]
Plafractor	Wheat bran	Cyclosporin	*Fusarium solanii*	-	[103]
Plafractor	Wheat bran, malto-dextrin	Proteases, betaglucanases, amylases	*Bacillus subtilis*	-	[103]
Plafractor	Soybean	Flavoring agent	*Aspergillus oryzae*	-	[103]
Intermittently mixed modular bioreactor	Pine wood chips and orange peel	Laccases	*Trametes hirsute*	-	[102]

Table 2.3 Performance comparison of SSF Bioreactors.

Performance parameter	SSF Bioreactors Types					
	Tray Bioreactors (TB)	Packed Bed Bioreactors (PBR)	Bioreactors with Air Pressure Pulsation & Forced Air Circulation	Rotating Drum Bioreactors (RDB)	Intermittently Mixed Forcefully Aerated Bioreactors	Air-Solid Fluidized Bed Bioreactors
Heat transfer capability	Primarily through conduction, low thermal conductivity of substrate often leads to inefficient heat dissipation.	Conductive, convective and evaporative heat transfer at play, but at large scale the process is often confronted with bed compaction & air channeling. As a result, maintenance of optimum bed temperature and moisture becomes challenging.	Forced air circulation and periodic pulsation of air pressure employed to achieve high degree of convection. Limited reports are available on heat transfer at industrial scale.	Heat transfer is achieved through conduction, convection and evaporative cooling. At high substrate bed loading temperature control is off limits.	Mode of heat transfer includes conduction, convection and evaporative heat loss. Mixing action increases the surface area of substrate exposed to incoming moist air. This expedites heat transfer.	Efficient heat transfer achieved through forced convection as the bed is kept in a fluidized state by the action of upward flow of fluid.
Scalability	Mostly done by increasing the surface area and the number of trays	Due to issues of bed compaction, air channeling, heterogeneity, traditional PBB are not suited for scale-up.	Scalability is generally obtained by increasing the number and surface area of trays since increasing air pressure beyond a certain limit is detrimental.	The working volume is usually 30% of the reactor volume. RDBs are generally not suited for scale-up.	Potentially scalable if the design and operational strategies are guided by design equations.	The present designs are not amenable to scale-up because of high shear stress and very high energy requirements.
Sterilization/ Containment issues	Not suited for sterile applications and the process is not contained.	In-situ sterilization may be achieved by injecting high pressure steam, however this may not be feasible with high bed loading.	Since most fermentation operations are carried outside the cultivation chamber, current APP-SSF reactors do not provide contained environment.	In situ sterilization may be achieved by injecting high pressure steam. The process can be operated in a contained environment.	In-situ sterilization has been reported with modular bioreactor designs. However in most of the designs, inverse relation between scalability and sterility is observed.	In situ sterilization possible by sending in high pressure steam.
Loading Capacity	Low substrate bed loading due to limitations in operational bed heights.	High loading coefficients are generally not possible due to problems of bed compaction and air channeling.	Impressive loading coefficient of 66.87% has been reported for Honeycomb loading device (HLD).	Low substrate bed loading. Usually useful space for fermentation comprises only around 30% of total volume.	Substrate bed loading generally higher than TB, PBR and RDBs.	Low bed loading, since large reactor volume is needed for fluidization of substrate.
Modularity	A single tray my serve as a module so the system can be modular in nature.	Zymotis among PBR is partly modular in nature.	A single tray my serve as a module so the system can be modular in nature.	RDBs are not modular in nature.	Except PLAFRACTOR most other designs are not modular.	The existing bioreactors under this category are not modular in nature.
In-situ product recovery	May not be feasible	May not be feasible.	May not be feasible	Has been possible in some reactor types.	Has been reported in some reactor types (e.g., PLAFRACTOR).	Possible in case of volatile products.
In-situ residue treatment	May not be feasible.	May not be feasible.	May not be feasible	Has been possible in some reactor types.	Has been possible in some types. (e.g., PLAFRACTOR).	May be possible.
Maintenance requirements	Generally maintenance is not intensive.	Due to its simple design, maintenance is generally not intensive.	Regular check up and maintenance may be necessary as the system involves the use of high air pressure.	Generally not intensive, but may be required in large scale RDB with mixers.	For bioreactors with large ancillary equipments, extensive maintenance may be required by skilled workers.	Continuous check up and maintenance may be required by skilled workers.
Damage to fungal mycelia	No damage to the fungal mycelia due to shear.	No damage to the fungal mycelia due to shear.	Fungal mycelia could be disrupted owing to high air pressure pulsation and circulation velocity and time.	Shear forces come into play. Trade off between the rotational speed and fermentation.	The mycelia may be subjected to disruption during mixing. Optimization of mixing events becomes a critical design parameter.	Intensive shear forces into play, as a result bioreactor is not suited for operation with aseptate fungi.

3 Chapter 3

Design and Mode of Operation of a Novel Solid-State Fermentation Bioreactor

Summary

A novel bioreactor system for solid-state fermentation and a process of operation thereof is proposed. The bioreactor system comprises a plurality of vertically stacked cylindrical modules around a central pipe. The central pipe facilitates the supply of conditioned air to the modules, and also acts as a central shaft for mixing purpose, whereupon, a variable speed motor is fitted. Each module further comprises a lower chamber facilitating the entry of steam or moistened air, a perforated disc between upper and lower chamber(s), substrate bed in upper chamber, one rotating plate at the top of upper chamber fitted with mixing blades, a plurality of spray nozzles for intermittent addition of fluids and a variable diameter exhaust vent. Bioreactor also comprises of an exhaust processing system which facilitates on-line monitoring of process parameters. The bioreactor claims to operate at high substrate bed loading (59.2%, v/v) and performs all the fermentation operations within the module in a highly aseptic fashion.

3.1 Introduction

Despite the resurgence of interest in SSF, a majority of research reports cite the use of flasks, columns, and trays for process development. One possible reason for this may be the dearth of standard SSF bioreactor systems, both at laboratory and pilot scale, which can operate at high substrate bed loading and provide strict aseptic conditions [1]. Moreover, quantitative characterization of heat and mass transfer phenomenon in most SSF process is neglected, or is restricted by the bioreactor design. Inability to do so is most likely to complicate scale-up. For instance, large-scale production in trays is usually achieved by increasing the surface area of the tray and increasing the number of trays [2], rather than the increase in bed height. Even with standard bioreactor designs, such as packed bed and rotating drum bioreactor, scale-up is impeded mainly due to intense heat buildup and heterogeneity across the bed [3]. Moreover, most of the existing bioreactors designs do not operate with complete sterility.

In this chapter, the design of a novel solid-state fermentation bioreactor is proposed which is modular in nature and claims to operate at high substrate bed loading. The bioreactor also claims to perform all the fermentation operations within the module in a highly contained fashion. The bioreactor design is described in detail which is followed by a discussion on the mode of operation, and analysis of substrate bed loading for a single bioreactor module. For the ease of understanding, bioreactor design components are numbered against their description both in the main text and the figures of this chapter. A patent search report on the proposed SSF bioreactor system is shown in Appendix 1.

3.2 Description of bioreactor design

The Bioreactor design (Fig. 3.1) comprising of several cylindrical modules (1) stacked one above the other around a central pipe (2), a mixing assembly along with other embodiments is described in detail.

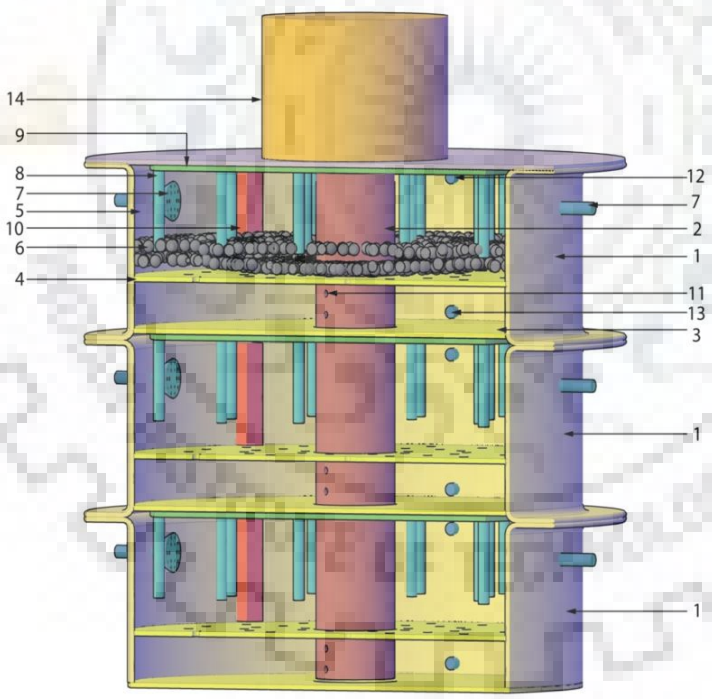

Figure 3.1 Cross section view of the proposed SSF bioreactor.

Each module houses the substrate bed, which is the site of fermentation and is stacked in such a manner that during the entire operation the substrate bed does not come into contact with the

external environment. In this way, the system is modular, i.e., it performs all the fermentation operations of sterilization, substrate pre-treatment, inoculation, cultivation, product removal and post-extraction residue treatment within the same module.

3.2.1 Bioreactor Module

Each bioreactor module (Fig. 3.2) consists of two chambers, the upper and the lower chamber. Lower chamber (3) is essentially closed having two openings. One opening (11) is onto the central pipe through which it receives conditioned air in the radial direction during fermentation, and steam during sterilization. The other opening is onto the chamber wall, i.e., the exit drain vent (13). The top of this chamber is a perforated disk (4) which is the bottom of the upper chamber (5) and holds the substrate bed (6). The upper chamber (5) is the site of fermentation. The moistened air (16) from the lower chamber traverses through these perforations (holes) to the upper chamber vertically. The microbial action occurs in the upper chamber only, where solid substrate bed lies on the perforated plate. The air passes through the substrate bed delivering its content (mainly moisture and oxygen) to the reaction mixture, and the exhaust air comes out through a variable diameter exhaust (12) vent on the upper chamber. Each module also houses revolving plate (9), three or more spray nozzles (7) and mixing blades (8). Baffles (10) may be attached onto the wall of the upper chamber to facilitate mixing, prevent bulk movement and air channeling.

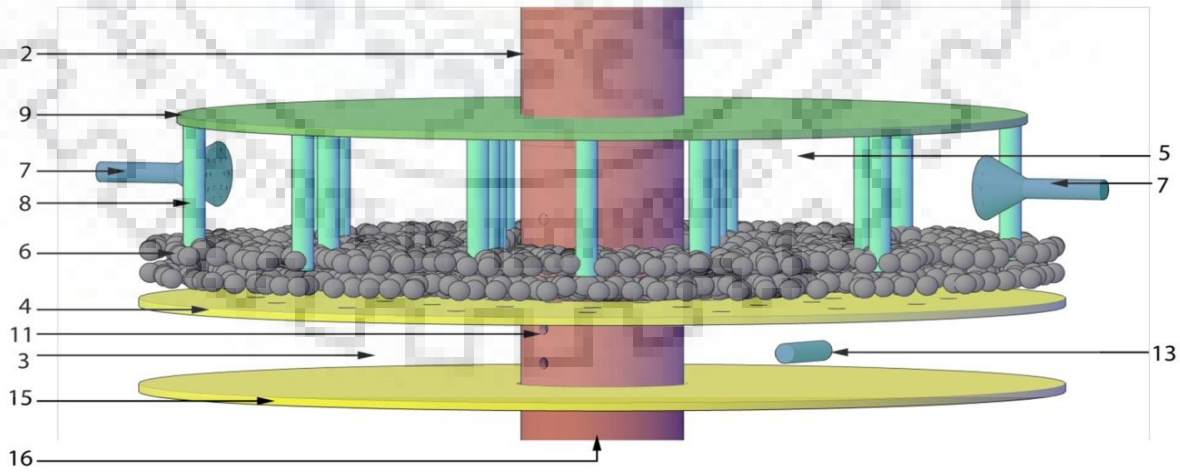

Figure 3.2 SSF Bioreactor module.

3.2.2 Central pipe

Central pipe (2) traverses along the length of the bioreactor and acts as a shaft for the mixing purpose, and also facilitates the supply of conditioned air into the lower chamber of the module. Revolving plate (9), which is present at the top of the upper chamber (5) is connected to the central shaft/ central pipe through a gear system. The top end of this central pipe is connected to a variable speed motor (14). The central pipe itself is modular, i.e., rather than being one long cylindrical pipe, multiple pipe fragments are joined by threading provided at the upper and lower end of the individual pipe fragment. This is essential for the ease of assembling, dissembling, storage, transportation and also for the fact that with the course of time if central pipe develops a snag, the whole system shall not sit idle for its maintenance.

3.2.3 Mixing apparatus

Another aspect of the bioreactor is the mixing apparatus (Fig. 3.3). SSF processes are usually discouraging owing to intense heat buildup and heterogeneity [3, 4] concerning bed temperature and moisture, aeration, cell biomass, and the product. The purpose of mixing is to facilitate the maintenance of optimum bed temperature and ensure homogeneity of heat and mass across the substrate bed. Mixing is accomplished with the help of mixing blades (8) that protrude into the fermentation bed from a rotating plate (9) (Fig. 3.4) situated at the top of the upper chamber (5). This rotating plate is connected to the central pipe (2) by a gear system. The speed of the rotating plate can be varied in the module through a gear system having different gear train or gear ratio. The gear train is the ratio of the angular velocity of the input gear to the angular velocity of the output gear. This ratio can be calculated directly from the number of teeth on the gear. Gear train may be varied according to the desired speed required for a rotating plate, which in turn shall be decided by the degree of shear tolerance of the microorganism. The mixing shaft is connected to a variable speed motor (14) which is mounted on the top of the reactor. The position and shape of the mixing blade can be changed or adjusted. The arrangement of mixing blades is such that forces acting in the bioreactor bed do not allow substrate agglomeration within the bed nor on the top, i.e., between the blades and the revolving plate. The shape and arrangement of mixing blades also restricts the movement of the reactor content in a bulk fashion.

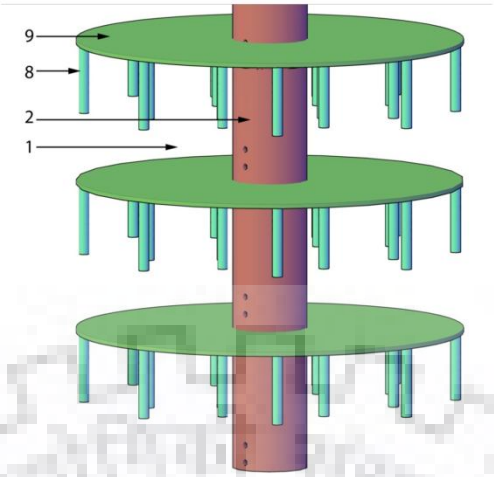

Figure 3.3 Mixing apparatus.

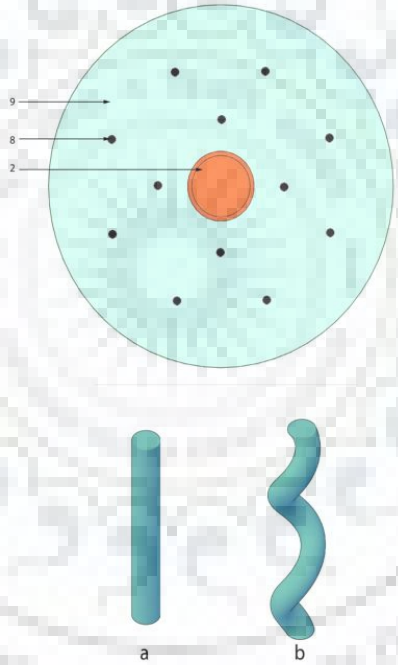

Figure 3.4 Revolving plate and mixing blades (a. Linear blade, b. Helical blade).

3.2.4 Nozzle

Another aspect of the bioreactor design is the spray nozzle (7). Orifice shaped nozzles are present at the top ends of the upper chamber (5). Their main purpose is to increase the fluid surface area by atomization, i.e., breaking the kinetic energy of the liquid to make them into fine droplets and distribute the fluid into the reactor bed. This atomization results in greater

liquid-gas mass transfer area than what is usually observed with unidirectional flow of fluids. Different types of fluid could be sent in through these nozzles, the flow of which shall be controlled by variable speed pumps. For example, inoculation of microorganism on the substrate media can be facilitated through the nozzle. Similarly, during cultivation, nutrients, buffers, and water could be sent it. Nozzles are also used during product extraction and post-extraction treatment of the fermentation bed, wherein, organic solvents or extracting fluids and steam can be sent in, respectively.

3.2.5 Sensors/Probes/Actuator/Control system(s)

The bioreactor also has the facility of sensors for temperature, humidity, pressure and exit gas (CO_2 and O_2) measurement. This is accomplished by installing thermocouples, hygrometer, pressure gauge and exit gas analyzer at the exit stream of the bioreactor system. Thermocouples may also be inserted, aseptically, at different heights on the reactor bed to obtain bed temperature profile. Gauge pressure valve, hygrometer, and exit gas analyzers are present at the exhaust valve. Provision is provided for aseptic sampling of the fermented substrate to facilitate the estimation of microbial biomass, water activity, residual substrate and product concentration through sampling ports provided at different heights along the wall of the bioreactor. This is essential for studying the microbial kinetics for process development at the laboratory scale.

3.2.6 Exhaust Valve

Another feature of this bioreactor system is the exhaust valve (12) which is present at the top end of the upper chamber. Here, the diameter of the vent could be varied by installing a steel disc with an inner aperture. The purpose of this embodiment is to accelerate convection through generation of periods of air pressure pulsations in the reactor bed. Since convection is the major contributor to heat transfer in SSF bioreactors [5], this feature will facilitate the maintenance of optimum and homogenous temperature profiles along the bed. The enhancement of convective heat transfer through air pressure pulsation may potentially increase the critical bed height and diameter of the module, thereby, further enhancing the substrate bed loading.

3.2.7 Exhaust processing system

Another unique design feature is the exhaust processing system (Fig. 3.5) that facilitates trouble free on-line monitoring of respiratory activity in the bioreactor system. Reliable estimates of O_2 and CO_2 concentration in exhaust stream could provide on-line insights into the biomass concentration, metabolic production water, substrate consumption, product formation, etc., which are otherwise very tedious to determine in an SSF process. Spent gases through exhaust pipes (17), from each module, meet into four cylindrical tubes (18) placed around the stacked modules. The exhaust gases then ultimately traverse into a ring-shaped plate (19) situated at the top of the stacked assembly. Then via a single outlet the gases pass through an air filter (20) and are then subjected to temperature, humidity, pressure, O_2 and CO_2 measurement respectively, (21) and the fermentation data is then logged on to a computer (22). The advantage of such a system is that there is no back mixing of exhaust gases, less hardware to sterilize, the system occupies lesser footprint and facilitates on-line monitoring and control of process parameters.

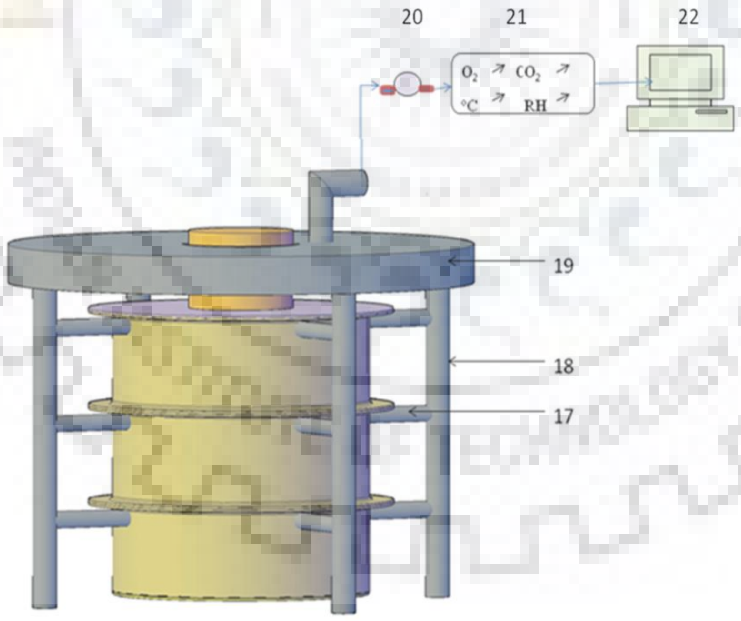

Figure 3.5 Exhaust processing system.

Another important feature of the bioreactor is the vertical arrangement of modules. The vertical arrangement of modules/ tray/ tubes in SSF offers an advantage that they possess more surface

area to volume ratio as compared to horizontal layouts. As a result, higher substrate bed loading, and ultimately higher productivities are possible. However, control of bed temperature along the axial direction is far more challenging than in horizontal configuration. The proposed bioreactor has been designed and experimentally validated (Chapter 4) considering this aspect. After an exhaustive literature survey, it shall be safe to claim that none of the existing SSF bioreactor design offers temperature control by simultaneous synergic effects of: a) inlet of conditioned air through lower chamber (3), b) addition of coolant through spray nozzles (7), c) the mixing apparatus and, d) air pressure pulsation; by varying the diameter of exhaust valve (12).

3.3 Operation of the SSF Bioreactor

First, the moistened substrate is loaded onto a clean module (1) which are then stacked one above the other around the central pipe (2). A variety of substrate such as wheat bran, linseed oil cake, soya beans, apple pomace, orange peel, etc., resulting in high product yield and possessing suitable transport properties may be used. The bed height and the operating variables such as the inlet air flow rate, temperature and humidity shall be optimized using design equations. Sterilization or substrate pre-treatment can be achieved in-situ by sending in high-pressure steam for the desired amount of time. For cooling purposes and to bring the temperature of reactor bed to optimum for fermentation, conditioned air may be passed to the modules through inlet vent (11).

When desired temperature and moisture is reached within the module, the substrate is inoculated in-situ by sending spore suspension through nozzles (7). These nozzles spray the inoculum onto the reactor bed which is then mixed to evenly distribute the spores/inoculum. Different microorganisms, i.e., fungi, yeast, and bacteria giving high product yield and possessing suitable kinetic characteristics may be used. Once the cultivation starts, process parameters such as temperature, inlet air flow rate, inlet air humidity and water activity within the bed can be precisely measured collectively through sensors, actuators and exit gas analyzers. This bioreactor design offers a multitude of control options for different process parameters. For example, heat transfer in the bioreactor is possible through multiple modes; conduction (through the bioreactor wall), forced convection (moist inlet air, air pressure pulsation, and mixing) and evaporative cooling (metabolic heat generated by microbial activity). Another important operation is the intermittent addition of nutrient, buffers, water,

etc., via spray nozzles. Intermittent water addition may be necessary in case the bed is dehydrated by uptake of water by microorganism and metabolic heat generated due to microbial activity, and as a result, water has to be added through nozzles, and also since the water carrying capacity of inlet air is reduced at low temperature.

For product extraction, extracting fluids such as organic solvents may be sent it. These fluids may be allowed to remain in contact with the fermented solids for an optimized time. Once the extract is collected, any trapped fluid may be recovered by applying vacuum at the exit channel (13) on the lower chamber (3) of the module (1). The post-extraction treatment of any residual material may also be achieved in-situ by sending heating or cooling fluids. More often, organic solvents used for the product extraction neutralize the microorganism inside the module; however, to meet stringent sterility requirements, the reactor contents may be sterilized again by sending high-pressure steam. Upon completion of the process, the stack is opened, the residue is disposed from individual modules, and the bioreactor is cleaned and put to use again.

For research and development of a new bioprocess, the bioreactor facilitates the quantitative characterization of heat and mass transfer phenomenon. For this purpose, fermented solid samples may be collected from different bed heights aseptically, which will provide the measure of substrate utilization rate, biomass formation rate, product formation rate and bed water activity. The temperature profile is obtained with the use of thermocouples at different bed heights.

3.4 Substrate bed loading analysis

A major limitation in the majority of the existing SSF bioreactors is that they operate at low substrate bed loading (SBL). SBL or substrate bed loading coefficient is defined as the ratio of the volume of substrate bed to the volume of the bioreactor/ module. High bed loading usually becomes detrimental for fungal growth, especially in trays, packed bed and rotating drum bioreactors due to heterogeneity and development of zones of high temperature. This problem is compounded in the vertical arrangement of bioreactor/ module. Hongzhang et al. [6] reported that temperature gradient increased 5.2 times in vertical arrangement (4.2ºC cm^{-1}) of trays than horizontal configuration (0.8ºC cm^{-1}). However, the vertical arrangement has the advantage that it requires lesser footprint and therefore augurs higher productivity. An analysis of substrate

bed loading for a single bioreactor module is made below during the fermentation of moistened wheat bran and linseed oil cake (1:1) by *Rhizopus oryzae*, for phytase production.

Mass of moistened and autoclaved substrate bed in single module = 0.36 kg.

Density of substrate bed = 516.36 kg m^{-3} [4]

Volume of substrate bed = 6.9718×10^{-4} m^3.

Volume of the cylindrical module = $\pi r^2 h$

Radius of module (r) = 0.075 m

Height of module (h) = 0.066 m

Volume of single bioreactor module = 1.176×10^{-3} m^3

$$\text{Substrate bed loading coefficient (\%)} = \frac{\text{Volume of substrate bed in module}}{\text{Volume of bioreactor module}} \times 100 = 59.2\%$$

Substrate bed loading in rotating drums, packed bed, and intermittently mixed bioreactors varies between 30-50% [7]. High substrate loading is restricted especially with fungal fermentation due to a dense network of mycelia [3, 7]. The proposed bioreactor operated at a relatively high substrate bed loading of 59.2 % (v/v) which is attributed to its design. Chen and He [7] reported a novel 'Honeycomb loading device' (HLD) for the production of *Bacillus cereus* DM 423 spores, with a higher substrate bed loading of 66.87% (v/v). However, a fast growing fungus with abundant aerial hyphae formation is expected to offer greater heat and mass transfer resistance than bacteria. Moreover, the reactor in the present study performed all the fermentation operations in a highly contained fashion which was absent in tray and possibly in HLD too.

3.5 Conclusions

A novel SSF bioreactor design is proposed wherein multiple modules may be stacked around a central pipe in the vertical arrangement. The central pipe not only facilitates the transfer of

conditioned air into the module but also acts as the shaft for mixing apparatus. The exhaust processing system facilitates trouble free on-line estimation of process parameters. The bioreactor claims to operate at high substrate bed loading rate (59.2 %; v/v) and provides strict aseptic environment; where all the fermentation operations can be performed within the module in a highly contained fashion. The proposed bioreactor promises to be an ideal system for development of SSF bioprocess.

Representation of numbers for Figure 3.1 through 3.5

(1) Circular disc(s) or module(s),

(2) Central pipe,

(3) Lower Chamber,

(4) Perforated Disc,

(5) Upper Chamber,

(6) Substrate Bed,

(7) Spray Nozzles,

(8) Mixing Blades,

(9) Revolving Plate,

(10) Inoculation port,

(11) Opening for steam/ moistened air,

(12) Variable diameter exhaust valve/ vent,

(13) Exit channel at lower chamber,

(14) Variable speed motor,

(15) Base of lower chamber,

(16) Moistened air,

(17) Exhaust pipes,

(18) Four cylindrical tubes,

(19) Common ring-shaped plate,

(20) Air filter,

(21) Exit gas analyzer,

(22) Data logger.

4 Chapter 4

Heat Transfer Validation and Optimization of Bed Water Activity in a Novel Solid-State Fermentation Bioreactor

Summary

Heat accumulation and heterogeneity of heat and mass, across the substrate bed, constitutes a major challenge for scale-up of solid-state fermentation (SSF) process. In this regard, a novel modular SSF bioreactor system operating at high substrate bed loading was proposed (Chapter 3) and its heat transfer capability was tested in the present study. A design equation describing the heat transfer phenomenon in the SSF system was experimentally validated at different conditions of inlet air flow rate and relative humidity (RH) during the production of a thermo-tolerant and acid stable phytase, by *Rhizopus oryzae*, using optimized growth media containing wheat bran and linseed oil cake (1:1) as the main substrate. Sensitivity analysis showed that the system's transport and kinetic parameters had a significant effect on critical bed height ($H_{critcal}$). Among the parameters, *R. oryzae* specific growth rate constant had a pronounced effect on $H_{critcal}$. Phytase production was examined in a 0.5 L mini-bioreactor system using synthetic media, in submerged fermentation (SMF) conditions. Phytase yield in SMF was 15.9 times less than SSF. Water activity in the substrate bed was optimized by varying RH of the inlet air stream. Desorption Isotherm for the substrate was used to correlate bed moisture content and water activity. A bed moisture content of 0.55 mL g-dry-solid^{-1} corresponding to bed water activity of ≥0.95 was optimum for phytase production. Effect of bed moisture content on bed temperature, biomass and phytase production was also analyzed.

4.1 Introduction

Unlike the SMF, where the transfer of O_2 to the microorganism is the major factor pertaining to scale-up, in SSF, the maintenance of optimum bed temperature, water activity and to lesser extent oxygen concentration, have proved to be difficult. Among these issues, maintenance of optimum bed temperature has been the most challenging [1]. Due to the low thermal conductivity of the solid substrate, bed temperature has been reported to exceed 20ºC from optimum [2], even for bed heights of 30 cm. This could potentially render 20-30% of biomass

inactive, particularly in the upper regions of the bed [3]. Increasing the inlet air flow rate is one option to avoid development of zones with steep temperature and gas gradients [2, 4]. However, operation with high superficial air velocities often results in bed compaction and air channeling [5, 6], thereby, adversely affecting the productivity of the desired metabolite. Another option is to cool the inlet air, coupled with the intermittent addition of water [4]. Although this would increase the cost of operation, cooling the inlet air may well be unavoidable especially during peak heat generation. In this regard, process design equations have proved to be an indispensable tool which help design operational strategies and economize the process. For instance, information of system's transport and kinetic parameters, and operating variables can be used in a heat transfer design equation to optimize bed heights and decide on the cooling requirements.

SSF involves the growth of microorganisms on a solid substrate in absence or near absence of free water. However, maintenance of optimum water activity (a_w) in fermentation bed is a key parameter and depends upon the nature of the substrate, microbial growth kinetics, addition of water, salts, sugars, alcohols, etc. [7]. The level of a_w in the substrate bed may affect microbial growth and enzyme production in a both positive and negative way. In some cases, low water activity has been reported favorable for enzyme stability [8], where, conformational modification prevented proteolytic degradation in the low aqueous environment. On the contrary, numerous reports are available where low a_w has been proved to be detrimental for phytase [9] [10] [11] and other enzyme production [12]. One major reason for poor a_w in substrate bed is the water loss due to evaporative cooling. To address this issue, the inlet air is usually humidified, or/and the process is operated with intermittent addition of water coupled with mixing. Moreover, evaporative cooling is not the only phenomenon responsible for water loss in the bed. Water uptake by growing cells may constitute a significant proportion of water loss from the bed. Nagel et al. [13] in their modeling studies for on-line control of moisture content in SSF bed showed that water incorporation into new microbial cells would approximately be 45% of water lost to evaporative cooling. Therefore, in addition to address detrimental temperature rise in the substrate bed, maintenance of optimum water activity is also critical for the success of an SSF process.

In the present study, heat transfer validation was performed in an intermittently mixed novel SSF bioreactor, during the production of a thermo-tolerant and acid stable phytase [14], by *Rhizopus oryzae*, utilizing wheat bran and linseed oil cake as the sole carbon source. Effect of

system's transport and kinetic parameters was studied on critical bed height ($H_{critcal}$). Phytase yield under SSF conditions was compared with than obtained with SMF using optimized growth media. Finally, bed water activity was optimized, and the effect of bed moisture content on bed temperature, biomass and phytase production was analyzed.

4.2 Materials and Methods

4.2.1 Microorganism and inoculum preparation

R. oryzae procured from Microbial Type Collection Centre (MTCC), Chandigarh, India, was routinely grown on Potato Dextrose Agar (PDA) for six days at 30ºC. Viable spore suspension (~ 1×10^6 CFU mL^{-1}) was harvested from PDA slants using 0.1% (v/v) Tween 80 solution, to be used as inoculum in subsequent fermentations.

4.2.2 Substrate and media

Wheat bran and linseed oil cake (~ 1mm size) were used as solid substrate and were procured from local retail feedstuff outlet from Roorkee (Uttarakhand, India). The media composition was optimized in a previous work [15] and its composition is as follows: wheat bran and linseed oil cake (1:1) supplemented with 2.05% (w/w) mannitol, 2.84% (w/w) ammonium sulphate, 0.38% (w/w) di-potassium hydrogen phosphate/di-sodium hydrogen phosphate and 20% (v/w) mineral salt solution ((w/w); 0.3% NaCl; 0.3% $MgSO_4.7H_2O$; pH 5.6).

4.2.3 SSF Bioreactor

SSF was carried out in a single module of the bioreactor system. As shown in Fig. 4.1 B, each module consisted of two chambers; separated by a perforated plate. The central pipe facilitated moist air supply and also functioned as a shaft of the mixing apparatus (Fig. 4.1 A and 4.1 B). Moist air was received in the lower chamber, through an opening in the central pipe, and then traversed through the perforated plate into the upper chamber. The upper chamber housed the substrate and was the site of fermentation. The upper chamber also harbored mixing assembly and consisted of a revolving plate to which mixing blades were fixed, to mix the substrate bed. The revolving plate was connected to the central pipe through a gear system. In addition to a variable diameter exhaust vent, three spray nozzles were provided on the wall of the upper chamber for the addition of inoculum, water, extracting fluids, etc. Multiple such modules could be stacked vertically. The proposed bioreactor operated as a packed bed bioreactor (PBR)

when no mixing events were initiated, and tray bioreactor (TB) when both mixing and forced aeration were absent.

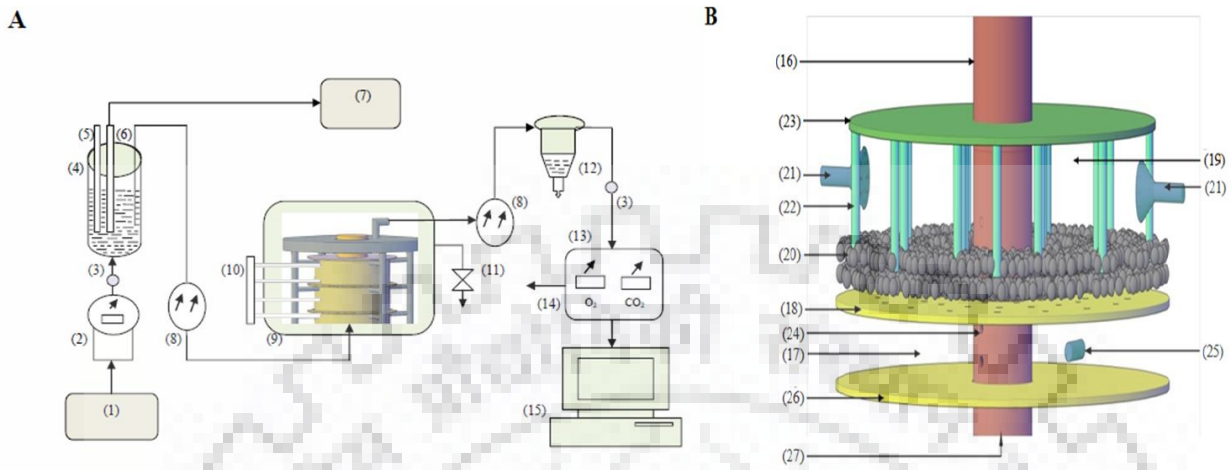

Figure 4.1 Schematic representation of experimental setup and bioreactor module. A. Schematic representation of SSF experimental setup. (1) air compressor (2) air flowmeter (3) air filter (4) humidifier column (5) heating rod (6) cooling coil (7) temperature controller (8) thermo-hygrometer (9) SSF bioreactor(10) thermocouples (11) sampling port (12) moisture remover (13) exit gas analyzer (14) exhaust (15) PC (data logger). **B.** Schematic representation of a single module of SSF bioreactor. (16) central pipe (17) lower chamber (18) perforated disc (19) upper chamber (20) substrate bed (21) spray nozzles (22) mixing blades (23) revolving plates (24) opening for steam/moistened air (25) exit channel at lower chamber (26) bottom plate of module (27) air inlet to central pipe.

4.2.4 Solid-state fermentation: setup and operation

Inlet air and ambient room temperature were maintained at 30ºC. Inlet air at a particular relative humidity (RH) was supplied to substrate bed through the central pipe. Required RH was maintained by passing air through a temperature controlled water column and monitored through a thermo-hygrometer (ZEAL, United Kingdom). Required air flow rate was maintained with a flowmeter (KDH, England). Growth media were sterilized in situ at 121ºC for 30 min. After cooling the reactor contents to room temperature, the media was inoculated aseptically with a spore suspension (~2×10^5 CFU g-dry solid^{-1}) through spray nozzles in the upper chamber via a peristaltic pump (Applikon, Nederlands) and the fermentation was carried out for 72 h. For mixing experiments, bed was continuously mixed for 10 min immediately after inoculation, and thereafter mixing was initiated at 20 rpm, for 1 min, after every one hour. On

the contrary, no mixing events were initiated when the bioreactor was operated as PBR and TB. A working bed height of 3.5 cm was used in module and temperature was recorded axially across the bioreactor at every six hours using thermocouples, with Pt 100 sensors (Nutronics, India), through a sterile opening on the reactor wall. Sketch of the experimental setup is depicted in Fig. 4.1A.

4.2.5 Analyses

4.2.5.1 Biomass estimation

Fungal biomass was estimated by determining *N*-acetyl glucosamine released by acid hydrolysis of chitin, a component specific for fungal cell wall [16]. Here, 0.5 g of dried fermented solid was mixed with 2 mL of concentrated sulphuric acid and kept at 30ºC for 24 h. The concentration of mixture was adjusted to 1N, autoclaved for one hour, neutralized with 1 N NaOH and then made up to 100 mL with sterile water. 1 mL of this mixture was incubated with 1 mL of acetyl acetone reagent at 100ºC for 20 min. After cooling, 6 mL of ethanol was added followed by the addition of 1 mL of Ehrlich reagent and incubated at 65ºC for 10 min. The glucosamine content was quantified by absorbance measurement in a spectrophotometer (Agilent Technologies, USA) at 530 nm. Biomass was expressed as gram of glucosamine per kilogram-dry-solid (g kg-ds^{-1}).

4.2.5.2 Phytase activity estimation

Phytase activity was determined by the method of Engelen et al. [17]. The reaction mixture consisted of 1 mL of enzyme sample and 2 mL of 5 mM sodium phytate (0.1 M citrate buffer, pH 5.5). The reaction mixture was incubated at 37ºC for 30 min and thereafter stopped by adding 2 mL of freshly prepared stopping reagent. The stopping reagent was prepared by mixing 25 mL of ammonium molybdate solution (10 g ammonium molybdate tetra hydrate, 90 mL distilled water, 1 mL ammonia solution (25%, v/v) and adjusted to 100 mL) with 25 mL ammonium vanadate solution (0.235 g ammonium vanadate, 40 mL distilled water at 60ºC and 2 mL of 65% (v/v) nitric acid before adjusting to 100 mL) and 16.5 mL of nitric acid (65%). The mixture was then cooled to room temperature and the volume adjusted to 100 mL. The color developed as a result of phytase activity was determined by absorbance measurement in a spectrophotometer at 415 nm. In blank, stopping reagent was added prior to the enzyme

sample. A unit of phytase activity was defined as the amount of enzyme required to release one nmol of inorganic phosphate per second under the standard assay condition.

4.2.5.3 Bed moisture estimation

The moisture content of fermented solids was determined by heating them in a pre-dried and weighed pan, which was kept in a hot air oven at 80ºC for 24 h. The weight loss was measured after the sample cooled down to room temperature in a desiccator. Bed moisture was expressed in mL per gram-dry-solid (mL g-ds^{-1}).

4.2.5.4 Respiratory measurements

The exhaust gas from bioreactor was analyzed for O_2 and CO_2 concentration using gas control and analysis system GAS 150 (PSI, Czech Republic). Before analysis in the gas analyzer, moisture was removed from the exhaust stream by passing it through moisture absorber, which condensed the exhaust water from gaseous to the liquid state.

4.2.6 Heat transfer design equation and validation

Heat transfer in the axial and radial direction was included in the design equation which was aimed at predicting temperatures within the bed. It was assumed that the bed and headspace gases were at thermal and moisture equilibrium with each other and bed properties such as thermal conductivity, density, and specific heat, and void fraction did not vary significantly during fermentation. It was also assumed that the microorganism grew uniformly across the bed. Energy transfer in the bioreactor system was attributed to: a) storage of energy due to temperature increase (energy accumulation) b) axial conduction c) radial conduction d) axial convection e) metabolic heat generation.

Considering the above assumptions, the following equations were worked out for a reactor bed of height 'H.'

Energy transfer in the system was attributed due to the following processes:

A. Energy Accumulation (ΔE):

$$\Delta E = M_b.C_{pb}.(\Delta T) = \{\rho_b A(\Delta z)(\Delta r)\}C_{pb}(T_{t+\Delta t}-T_t)$$

B. Axial conduction ($Q_{a,cond}$) :

$Q_{a,cond}=k_b.A.(\Delta(dT/dz)).(\Delta t)\ (\Delta r) = \{k_bA((dT/dz)|_{z+\Delta z}- (dT/dz)|_z)(\Delta t)(\Delta r)\}$

C. Radial conduction ($Q_{r,cond}$):

$Q_{r,cond}=k_b.A.(\Delta(dT/dr)).(\Delta t)(\Delta z)=k_bA(\Delta z)(\Delta t)(\Delta r)\{(1/r)(\partial T/\partial r)\}+k_bA(\Delta z)(\Delta t)\{\Delta(dT/dr)\}$

D. Axial Convection ($Q_{a,conv}$):

$Q_{a,conv} = M_a.C.(\Delta T)= \rho_aV_zA(\Delta t)(\Delta r)C_{pa}(T_{z+\Delta z}-T_z) + \rho_aV_zA(\Delta t)(\Delta r)\ (dH_{sat}/dT)\lambda(T_{z+\Delta z}-T_z)$

E. Metabolic heat generation (Q_x) :

$Q_x= M_x.Y_{q/x}.(\Delta X)= \rho_s(1-\epsilon)A(\Delta z)(\Delta r)Y_{q/x}(X_{t+\Delta t}-X_t)$

Energy Balance:

Energy Accumulation = Energy transfer due to axial and radial conduction – Energy transfer due to convection + Heat generation from growth

$\Delta E= Q_{a,cond} + Q_{r,cond} - Q_{a,conv}+ Q_x$

$[\rho_bA(\Delta z)(\Delta r)C_{pb}(T_{t+\Delta t}-T_t)] = [\{k_bA((dT/dz)|_{z+\Delta z}-(dT/dz)|_z)(\Delta t)(\Delta r)\} + k_bA(\Delta z)(\Delta t)(\Delta r)\{(1/r)(\partial T/\partial r)\}+ k_bA(\Delta z)(\Delta t)\{\Delta(dT/dr)\}] – [\{\rho_aV_zA(\Delta t)(\Delta r)C_{pa}(T_{z+\Delta z}-T_z) + \rho_aV_zA(\Delta t)(\Delta r)\ (dH_{sat}/dT)\lambda(T_{z+\Delta z}-T_z)\}]+[\rho_s(1-\epsilon)A(\Delta z)(\Delta r)Y_{q/x}(X_{t+\Delta t}Xt)]$

On dividing by $A(\Delta z)(\Delta t)(\Delta r)$:

$\rho_bC_{pb}(\partial T/\partial t) = k_b.(\partial^2T/\partial z^2)+K_b.\{(1/r)(\partial T/\partial r)+(\partial^2T/\partial r^2)\} –\rho_a\{C_{pa}+ (dH_{sat}/dT).\lambda\} V_z(\partial T/\partial z) +\rho_s(1-\epsilon)Y_{q/x}(dX/dt)$

Rearrangement of the above equation gives Eq. (4.1), the initial and boundary conditions for which are as follows:

Initial Conditions

i. $T=T_0$ at t=0 for all r and z
ii. $X=X_0$ at t=0 for all r and z

Boundary Conditions

i. $T=T_0$ at z=0 for all r and t

ii. $(\partial T/\partial z)=0$ at z=H for all r and t

iii. $(\partial T/\partial r)=0$ at r=0 for all z and t

iv. $(\partial T/\partial r)= (Bi/R)\ (T_S-T)$ at r=R for all z and t

Where Bi=Dimensionless biot number

$$\frac{\partial T}{\partial t} = \left(\frac{k_b}{\rho_b . cp_b}\right)\left[\left(\frac{\partial^2 T}{\partial z^2}\right) + \left\{\frac{1}{r}\left(\frac{\partial T}{\partial r}\right) + \left(\frac{\partial^2 T}{\partial r^2}\right)\right\}\right] - \left(\frac{\rho_a}{\rho_b . cp_b}\right)\left\{cp_a + f.\lambda\right\} V_Z \left(\frac{\partial T}{\partial z}\right) + \left(\frac{\rho_s}{\rho_b . cp_b}\right).\left((1-\epsilon) Y_{Q/X}.\left(\frac{dX}{dt}\right)\right) \quad (4.1)$$

Numerical solution for Eq. (4.1) was obtained with MATLAB 7.10.0 (R2010a), using implicit method. Substrate density (ρ_s), bed packing density (ρ_b), void fraction (ε) and bed specific heat (c_{pb}) were estimated as described by Mitchell et al. [18]. Substrate thermal conductivity (k_s) was measured using KS-1 probe of thermal property analyzer (KD2 Pro, USA). Values of density of air, specific heat of air and latent heat of vaporization of water were obtained from standard literature [19, 20]. $Y_{Q/X}$ was determined by correlating metabolic heat generation rate (a function of OUR) and biomass concentration of *R. oryzae*. $Y_{Q/X}$ estimation has been discussed in more detail in Chapter 6.

For validation of Eq. (4.1), five experiments were performed in the proposed bioreactor system, where, inlet air flow rate and relative humidity was varied, and the experimentally obtained temperature profiles were compared with the predicted response of Eq. (4.1). Percentage error analysis (% error $= \left|\frac{T_{experiment} - T_{predicted}}{T_{predicted}}\right| . 100$) was used to assess the goodness of fits. Heat transfer validation was shown for 3 cm bed height since in a bottom-aerated bed the upper regions of the bed will reach higher temperature and microbial biomass at that level is more sensitive to inactivation. Five different experimental conditions used were; (A) 80% RH and 4 L min^{-1}, (B) 80% RH and 5 L min^{-1}, (C) 80% RH and 3 L min^{-1}, (D) 70% RH and 4 L min^{-1} and (E) 60% RH and 4 L min^{-1}, respectively. Effect on inlet air flow rate on maximum bed temperature, biomass concentration, and phytase yield was also studied.

4.2.7 Sensitivity analyses

To determine the effect of design equation's transport and kinetic parameters on bed height and diameter in the bioreactor, sensitivity analysis was performed. Working range of the parameters

was selected based on their values reported in SSF literature. Effect of substrate thermal conductivity (0.05-0.20 W m^{-1} K^{-1}), substrate density (500 - 1100 kg m^{-3}), void fraction (0.10 – 0.60), specific growth rate constant (0.06 – 0.20 h^{-1}), maximum biomass concentration (0.05 – 0.13 kg-biomass kg-dry-$solid^{-1}$), and yield of metabolic heat generation per kilogram biomass (0.5 – 3×10^7 J $kg\text{-}biomass^{-1}$), on bed temperature was evaluated one at a time. Eq. (4.1) was used to study the effect of system parameters on maximum possible bed height and to determine the $H_{critcal}$ in the bioreactor. For determination of the $H_{critcal}$, a working range of temperature was defined, where, it was decided that an inlet air temperature below 20°C shall not be suitable for the bioprocess to be economical, and a bed temperature beyond 40°C will be detrimental to microbial growth and the production of the desired metabolite. Effect of system parameters on bed height was evaluated at four different temperatures (30º, 27ºC, 24ºC, and 20ºC) of the inlet and surrounding air.

4.2.8 Submerged fermentation studies for phytase production

Phytase production was tested under submerged fermentation conditions in a mini-bioreactor system, of 0.5 L capacity (Applikon, Nederlands), using synthetic media. The media composition was optimized in a previous work [15]. The basal medium contained (% w/v): Glucose, 1.0; Peptone, 0.5; micronutrient solution (NaCl, 0.15; $MgSO_4.7H_2O$, 0.15; $MnSO_4.H_2O$, 0.005; $FeSO_4.7H_2O$, 0.005 and $CaCl_2.2H_2O$, 0.15) with initial pH adjusted to 5.6. Bioreactor along with media contents except for glucose and micronutrient solution were sterilized in an autoclave for 20 min at 121ºC. Glucose and micronutrient solution were filter sterilized separately using 0.22 µm pore size Nylon-66 membrane (Hi-media, India) in a laminar air flow chamber (Rescholar, India) and were subsequently added to the reactor contents aseptically. Inlet air flow rate, medium pH and temperature were maintained at 0.8 L min^{-1}, 5.5 and 35ºC, respectively. Fermentation was carried out for seven days, and the impeller speed was maintained at 250 rpm. The crude extract was obtained after centrifugation at 10000×*g* for 15 min in a refrigerated centrifuge. The cell-free supernatant was subjected to phytase activity assay. Phytase activity was defined in U mL^{-1}. A unit of phytase activity was defined as the amount of enzyme required to release one nmol of inorganic phosphate per second under the standard assay condition.

4.2.9 Desorption isotherm of substrate

To obtain the relationship between water activity (a_w) and moisture content of the substrate, moisture desorption isotherm was developed at 30ºC. Moistened and autoclaved wheat bran and linssed oil cake (1:1) were transferred onto pre-dried and pre-weighed dishes which were then kept in an air tight chamber containing NaCl solution at different a_w. After few hours, substrate was removed and analyzed for moisture content at the corresponding NaCl solution a_w.

4.2.10 Optimization of bed water activity in substrate bed

To optimize water activity in the fermentation bed, four experiments were conducted in which the relative humidity of inlet air was varied from 50% to 80%. The inlet air flow rate was kept constant at 4 L min^{-1} in all the experiments. The temperature of the inlet and surrounding air was maintained at 30ºC, and the fermentation was carried out for 72 h. The bioreactor was intermittently mixed at 20 rpm, for 1 min, at every one hour. Fermented samples were collected aseptically from the reactor bed, at three different heights (1, 2 and 3 cm), and were analyzed for cell biomass concentration, moisture content and phytase activity after every 12 h, as described in Section 4.2.5. The bed temperature was measured axially at four different bed heights (1, 2, 3 and 4 cm) after every six hours. Effect of moisture content on bed temperature, cell biomass and phytase production was also analyzed.

4.3 Results and Discussion

4.3.1 Heat transfer validation

Experimentally obtained and literature reported values for transport and kinetic parameters used in simulation of Eq. (4.1) are given in Table 4.1. Fig. 4.2 (A-E) shows the validation results, from where it was evident that experimentally obtained bed temperature profiles were well predicted by Eq. (4.1) for all the operating conditions. The percentage error was less than 5% between experimental and the predicted temperature response (Table 4.2). However, experimentally obtained bed temperature values at 24 and 36 h slightly exceeded the predicted response for some of the operating conditions (Fig. 4.2 (A-E)). This behavior was probably due to rapid development of aerial hyphae, early during the exponential phase, which may have moderately changed the bed transport properties. Extensive development of mycelium network

and consumption of substrate resulted in bed compaction that most likely caused an increase in bed density, and decrease in void fraction and bed thermal conductivity, all of which, may have been responsible for slight deviation from the predicted temperature response at 24 and 36 h. Since Eq. (4.1) contains spatial variables, it can be used to predict temperature profile at different positions in the bed. However, here, validation results have been shown for 3 cm bed height. This is because in a bottom aerated SSF bioreactor, upper regions of the bed are more sensitive and prone to high temperature than lower regions exposed to conditioned air. As the moist inlet air traverses axially, it gets warmer and loses its ability to cool the bed.

Table 4.1 Parameter used in the simulation of Eq. (4.1).

Parameters	**Value**	**References**
Temperature in (T_{in})	30°C	Experimentally obtained
Temperature ambient (T_s)	30°C	Experimentally obtained
Air superficial velocity (V_z)	0.35 m s^{-1}	Experimentally obtained
Void fraction (ϵ)	0.19	Experimentally obtained
Density of air $(\rho)_a$	1.1714 kg m^{-3}	19
Density of substrate (ρ_s)	638.29 kg m^{-3}	Experimentally obtained
Thermal conductivity substrate (k_s)	0.074 W m^{-1} K^{-1}	Experimentally obtained
Thermal conductivity air (k_a)	0.0206 W m^{-1} K^{-1}	20
Latent heat of vaporization (Λ)	2.4143E(06) J kg-$water^{-1}$	20
Change in water carrying capacity with temperature (f)	0.00246 kg-water kg-$air^{-1}K^{-1}$	20
Metabolic heat yield ($Y_{Q/X}$)	2.98±0.25(07) J kg-$biomass^{-1}$	Experimentally obtained
Heat capacity of air (C_{pa})	1180 J kg^{-1} K^{-1}	19
Heat capacity of substrate (C_{ps})	4349.8 J kg^{-1} K^{-1}	18

Ghildyal et al. [2] observed that bed temperature exceeded to more than 20ºC than optimum, in a 0.3 m bed height PBR, which could potentially render 20-30% biomass inactive particularly in the upper regions of the bed [3]. Fig. 4.3 shows bed temperature at different heights in the intermittently mixed SSF bioreactor and PBR. It was evident that temperature was relatively homogenous across the bed in mixed SSF, whereas in PBR, the temperature profile was heterogeneous with temperature at 3 bed height being significantly higher than at 1 and 2 cm

during peak heat generation. This observation confirmed the sensitivity of upper regions of the bed to high temperatures and also underscored the importance of mixing events in an SSF bioreactor bed. Mixing events should be optimized to ensure efficient heat transfer and homogeneity across bed, with minimal shear stress on the microbe. Table 4.3 shows the effect of inlet air flow rate on maximum bed temperature, and maximum cell biomass and phytase production. Operation with an air flow rate of 4 L min^{-1} was optimum as further increase in air flow rate did not result in significant change in biomass and phytase production. However, maximum bed temperature was brought down by almost 3°C with 5 L min^{-1} air flow rate.

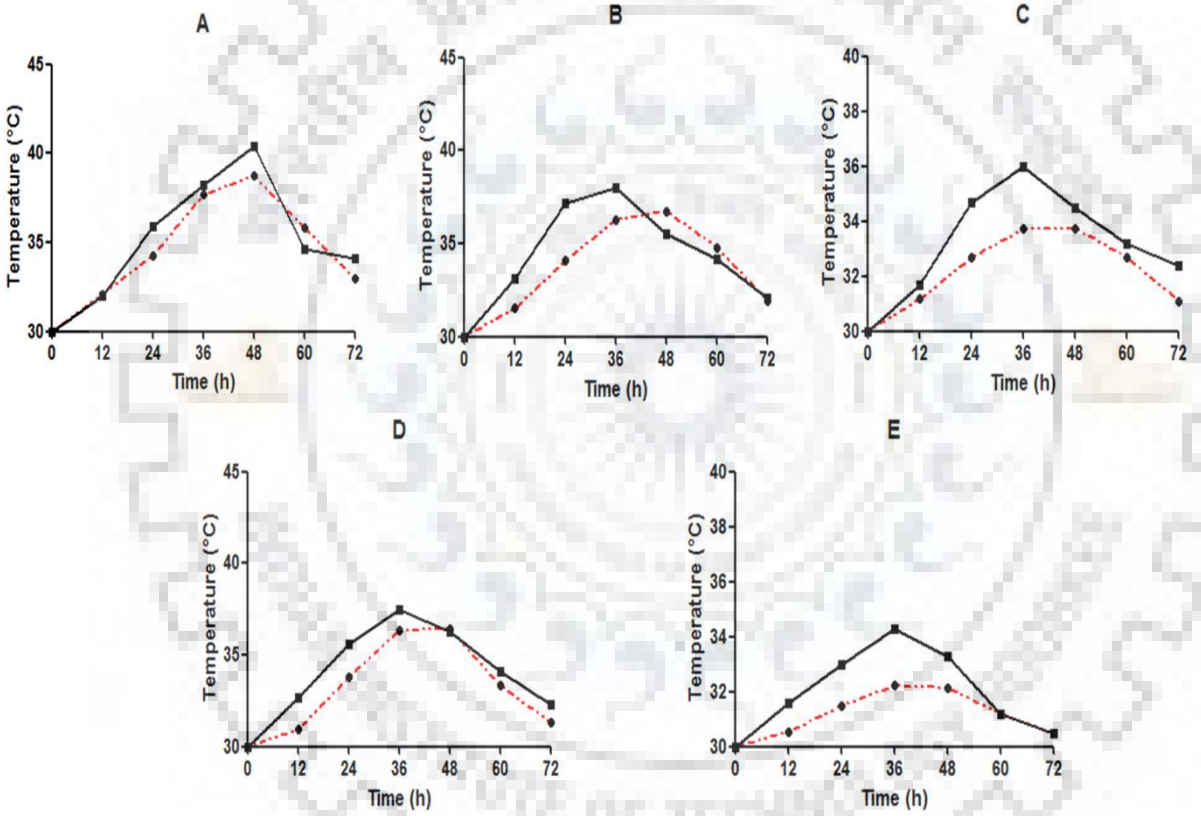

Figure 4.2 Experimental (closed square) and predicted (closed circle) temperature profiles at different operating conditions. **A.** 80% RH and 4 L min^{-1}. **B.** 80% RH and 5 L min^{-1}. **C.** 80% RH and 3 L min^{-1}. **D.** 70% RH and 4 L min^{-1}. **E.** 60% RH and 4 L min^{-1}.

Table 4.2 Percentage error analysis

Operating condition	Percentage error (%)
80% RH and 4 L min^{-1}	2.6
80% RH and 5 L min^{-1}	2.2
80% RH and 3 L min^{-1}	3.8
70% RH and 4 L min^{-1}	3.3
60% RH and 4 L min^{-1}	3.1

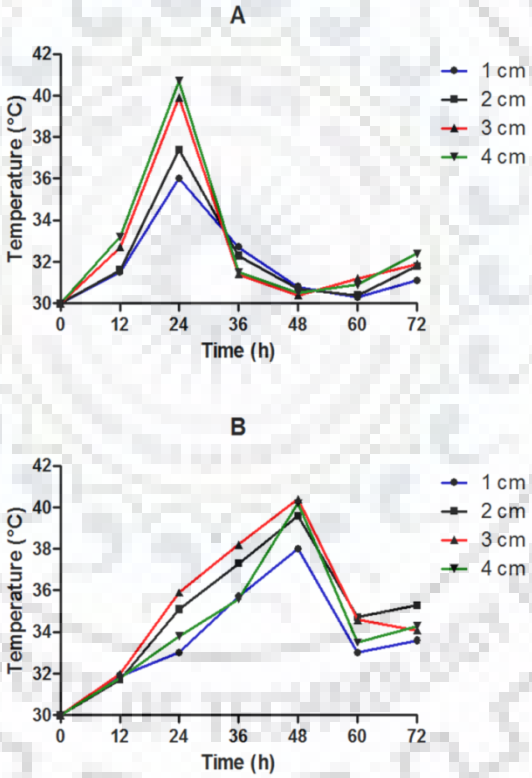

Figure 4.3 Temperature profile in the substrate bed at four different bed heights (1, 2, 3 and 4 cm, respectively) for two bioreactor operations **A.** Packed bed operation (PBR). **B.** Intermittently mixed SSF bioreactor.

Table 4.3 Effect of inlet air flow rate on maximum bed temperature, biomass concentration and phytase yield.

Operating Condition	Maximum bed temperature (°C)	Maximum biomass concentration (g kg-ds^{-1})	Maximum phytase yield (U g-ds^{-1})
80% RH & 3 L min^{-1}	36	43.2	136.5
80% RH & 4 L min^{-1}	40.4	69.84	190.16
80% RH & 5 L min^{-1}	37.5	69.07	188.1

4.3.2 Sensitivity analysis

Tables 4.4 and Table 4.5 show the effect of increase in bed height and diameter on bed temperature respectively, at the inlet and surrounding temperature of 30°C. While increase in bed height had a significant effect on bed temperatures, change in bed diameter did not result in any notable variation in bed temperature. Therefore, increasing the bed height proved critical and sensitivity analysis of system's parameters was studied on bed height in the bioreactor. Fig. 4.4 (A-F) shows the effect of system transport and kinetic parameters on $H_{critcal}$, at four different temperatures (30°, 27°C, 24°C, and 20°C) of the inlet and surrounding air. Among the transport parameters, substrate thermal conductivity (k_s) and substrate density (ρ_s) exerted a significant effect on bed height. With increase in the value of k_s from 0.05 W m^{-1} K^{-1} to 0.10 W m^{-1} K^{-1}, a 45% increase in bed height was predicted when the inlet and surrounding temperature were maintained at 24°C. Under the same operating conditions, a similar decrease in magnitude of substrate density, as with k_s, resulted in 50% increase in bed height. Simulation results identified substrate thermal conductivity and substrate density as important parameters, which could potentially be varied to increase substrate bed loading. Since the maintenance of optimum bed temperature is difficult in SSF, the addition of inert supports to enhance substrate bed transport properties constitutes an interesting strategy. Indeed, addition of inert support such as sugarcane bagasse [6], polyurethane [21], perlite, vermiculite, and glass fiber [7] to the substrate bed have shown to enhance enzyme production in SSF, possibly by facilitating the heat transfer.

Table 4.4 Effect of increase in bed height on maximum bed temperature at inlet and surrounding temperature of 30ºC.

Inlet air temperature (°C)	Bed height (cm)	Maximum Bed temperature (°C)
30	2.0	32.86
30	2.5	34.36
30	3.0	36.12
30	3.5	38.15
30	4.0	40.36
30	4.5	42.72
30	5.0	45.19

Table 4.5 Effect of increase in bed diameter on maximum bed temperature at inlet and surrounding temperature of 30ºC.

Inlet air temperature (°C)	Bed diameter (cm)	Maximum Bed temperature (°C)
30	5	35.53
30	10	37.57
30	22.5	38.41
30	50	38.59
30	100	38.61
30	200	38.62
30	500	38.62

The three kinetic parameters used in the simulation study were found to exert profound effect on bed height. Low specific growth rates constant (< 0.11 h^{-1}), in particular, tremendously influenced substrate bed loading (Fig. 4.4 D). Comparatively, maximum biomass concentration (X_m) and yield of metabolic heat from biomass ($Y_{Q/X}$) had lesser impact. A 2.4 fold increase in bed height was predicted when the specific growth rate constant was decreased from 0.10 h^{-1} to

0.06 h^{-1}, at the inlet and surrounding air temperature of 27ºC. Whereas, at similar operating conditions, doubling X_m and $Y_{Q/X}$ in the operating range, a decrease of 51 % and 44% in bed height was predicted, respectively. Effect of X_m and $Y_{Q/X}$, though significantly less than 'μ', was greater to that of k_s and ρ_s, and therefore, kinetic parameters had a pronounced effect on $H_{critcal}$. A slow growing fungus, but with high phytase yield, utilizing a substrate with high thermal conductivity is likely to be a good prospect. Simulation results showed that when values of all other parameters were kept same but for μ (0.08 h^{-1}) and k_s (0.10 W m^{-1} K^{-1}), and the inlet and surrounding air temperature of 27ºC was maintained throughout, a bed height of 7.2 cm was predicted which is 2.05 times more than the current used bed height.

The importance of selection of appropriate substrate and kinetic parameters for an SSF process is further exemplified in a recent study by Pitol et al. [5] and Biz et al. [6] for pectinase production. Pitol et al. used a 200 L PBR during fermentation of 20 kg wheat bran (WB) by *Aspergillus niger*. However, this led to detrimental temperature rise, air channeling, and heterogeneity, and as a result, 10% of WB was replaced with sugarcane bagasse. The problem of air channeling, heat accumulation, and heterogeneity persisted when substrate loading was increased to 30 kg, which suggested that under the given operating conditions, the bioprocess was not suitable for high substrate bed loading. Biz et al. however successfully countered this problem by using *Aspergillus oryzae* and by enhancing the mechanical properties of substrate support. Use of citrus pulp (51.6%, w/w) and SB (48.4%, w/w) as the substrate, in the same bioreactor system, resulted in a pectinase yield of 37 U g^{-1}, which was higher than that reported by Pitol et al. (20 U g^{-1}). This strategy also circumvented issues of air channeling and detrimental temperature rise and is a classic example which underscores the importance of suitable substrate and kinetic parameters that may potentially affect metabolite yield and productivity.

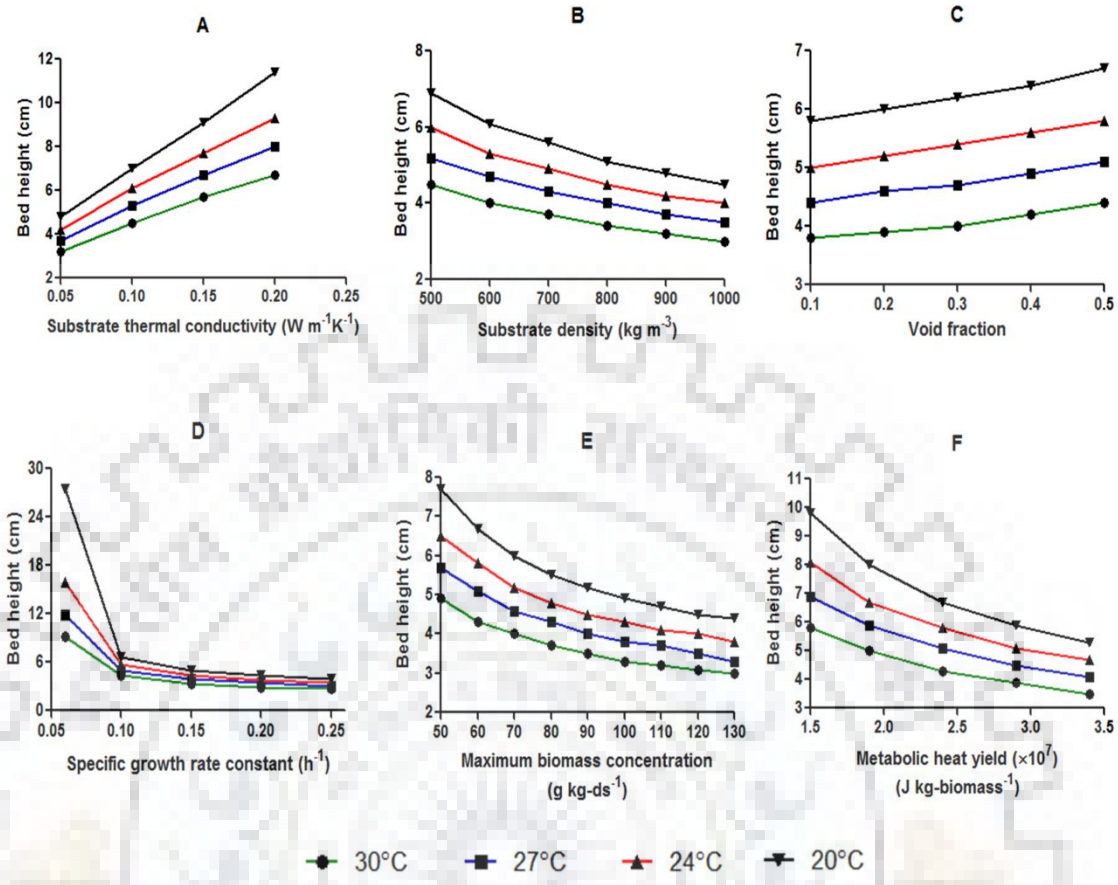

Figure 4.4 Sensitivity analysis of transport and kinetic parameters at different temperatures of inlet and surrounding air (20ºC, 24ºC, 27ºC and 30ºC, respectively) **A.** Substrate thermal conductivity. **B.** Substrate density. **C.** Void fraction. **D.** Specific growth rate constant. **E.** Maximum biomass concentration. **F.** Metabolic heat yield.

With the use of thermophilic microorganisms, the $H_{critcal}$ could further be increased as it shall not be necessary to cool down the inlet air to the level required for mesophilic organisms. Eq. (4.1) was used to optimize working bed height for two reported SSF bioprocess involving thermophilic organisms; *Aspergillus fumigates* producing xylanase from WB and SB at the optimum temperature (T_{opt}) of 45°C [22] and *Bacillus cogular* for α-amylase production utilizing wheat bran at T_{opt} of 50°C [23]. Values of the substrate and kinetic parameters were used from the present study. Simulation results showed that $H_{critcal}$ were enhanced to 5.5 cm for *Aspergillus fumigates* and 6.7 cm for *Bacillus cogular*, respectively. This observation suggested

that thermophilic microorganisms may be a good prospect for enhancing substrate bed loading in the bioreactor module.

4.3.3 Submerged fermentation studies

Phytase production by *R. oryzae* was investigated in submerged fermentation condition using optimized synthetic media in a 0.5 L bioreactor system. Maximum phytase activity was observed on the 7th day of fermentation (Fig. 4.5), and was 15.9 times less than that obtained in the SSF bioreactor. This was expected since solid nature of the substrate simulated the natural environment of the fungus, and most likely, this resulted in higher volumetric productivities of phytase than SMF. A 9.3 fold increase in phytase production, than SMF, was reported by Sapna and Singh [10] when wheat bran was fermented by *Aspergillus oryzae*.

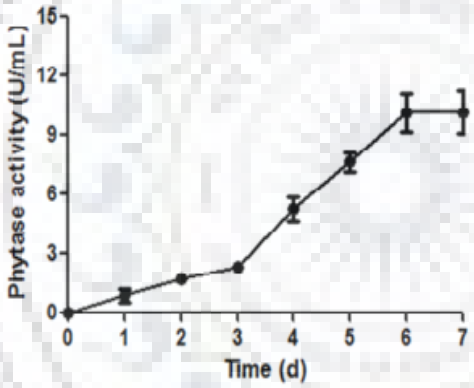

Figure 4.5 Phytase activity profile in submerged fermentation condition using synthetic media.

The tendency of *R. oryzae* to propagate efficiently on a solid support was also evident in the submerged fermentation bioreactor. Fig. 4.6 clearly shows *R. oryzae* biomass (black mass in Fig. 4.6) tightly bound to impeller and shaft assembly. The formation of fungal biomass was also observed on the pH and DO probe. This resulted in faulty DO reading, and as a result, fungal biomass had to be carefully removed from the DO probe after every few hours. The above results showed that SMF resulted in very low yield and productivity of phytase. Comparatively, SSF was much more efficient and was selected as the mode of production.

Figure 4.6 Pictorial representation showing *R. oryzae* biomass (black color mass) tightly bound to bioreactor shaft, impeller and probes.

4.3.4 Adsorption isotherm for substrate

Fig. 4.7 shows the desorption isotherm of moistened and autoclaved substrate at 30ºC. The water activity remained ≥ 0.95 at the bed moisture content of 0.55 mL g-ds^{-1}. This was similar to the findings of Nagel et al. [24], where, a_w in the wheat (substrate) remained very close to 1, as long as the water content of wheat matrix was > 0.5 kg per kilogram dry matter.

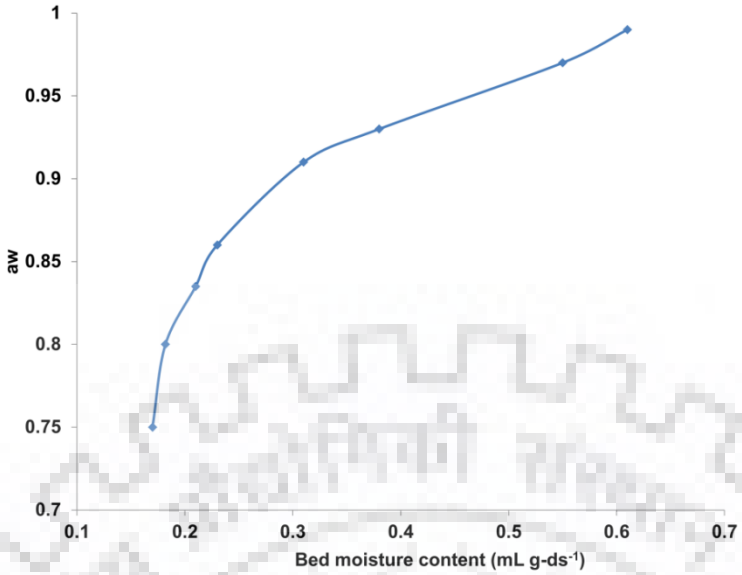

Figure 4.7 Desorption isotherm of moistened and autoclave substrate at 30ºC.

4.3.5 Effect of bed water activity on bioreactor performance

4.3.5.1 Bed Moisture profiles

With bioreactor operation at 50% and 60% RH, bed moisture decreased drastically (Fig. 4.8) which proved detrimental for biomass and phytase production. Under these conditions, the water supplied by the moist inlet air was unable to make up for the loss of water due to evaporation and uptake by the microorganism. *R. oryzae* growth was significantly affected as the bed water activity decreased below 0.90 at 24 h for both 50% and 60% RH, whereas, a_w remained above 0.95 during the whole fermentation for operation with 70% and 80% RH. This resulted in an almost threefold increase in biomass production than with 50% RH and underscored the importance of maintenance of optimum a_w in SSF bed. Numerous reports are available where moderate changes in bed moisture content significantly affected enzyme production in SSF bioreactors [25, 26]. Reactor performance with 80% RH was slightly better than 70% RH since homogenous moisture profiles were obtained and the biomass and phytase production were also higher. From Fig. 4.8 it was evident that operation with inlet air at 80% RH and 4 L min^{-1} was optimum for the process, where $a_w \geq 0.95$ was maintained throughout the process. The bed moisture content of 0.55 mL g-ds^{-1} (Fig. 4.7) corresponded to the water activity of ≥0.95, and therefore, was considered optimum for phytase production.

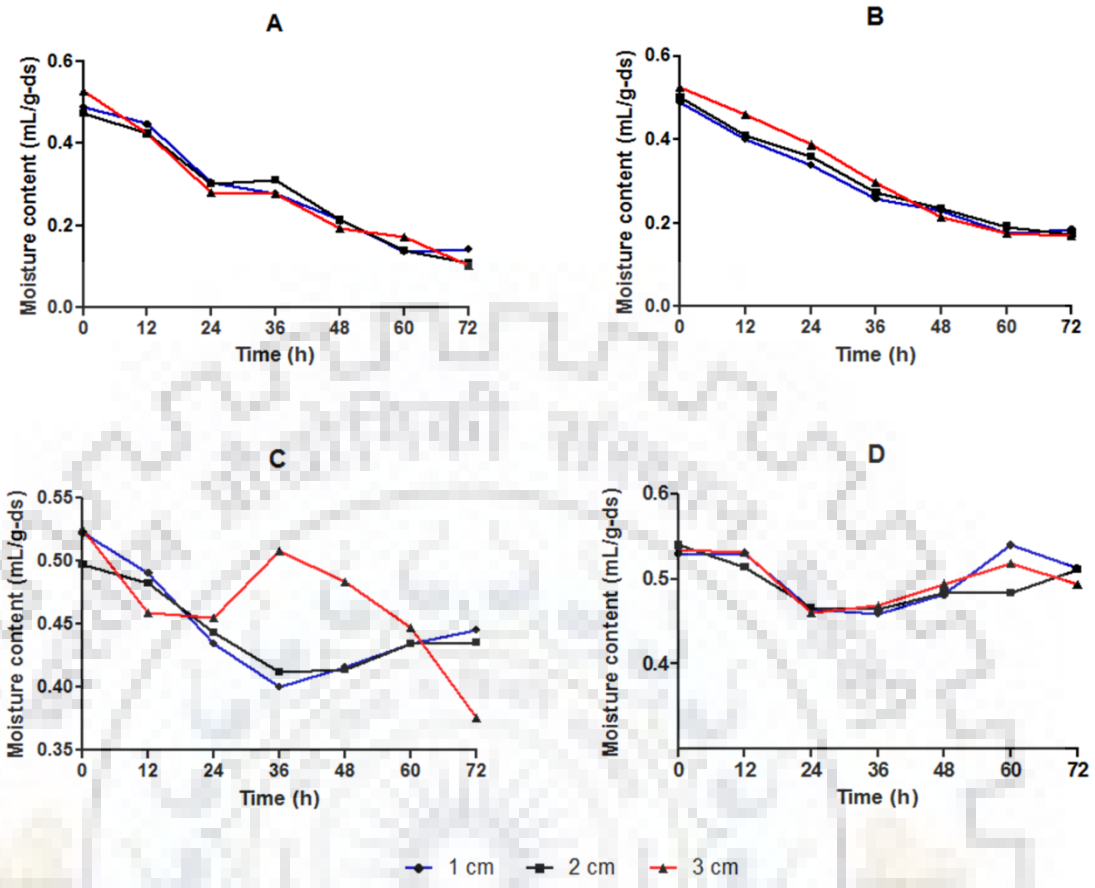

Figure 4.8 Bed moisture profiles at different bed heights (1, 2 and 3 cm, respectively) during the growth of *R. oryzae* under four different experimental conditions. **A.** 50% RH and 4 L min^{-1}. **B.** 60% RH and 4 L min^{-1}. **C.** 70% RH and 4 L min^{-1}. **D.** 80% RH and 4 L min^{-1}.

4.3.5.2 Bed temperature profiles

As a result of low metabolic activity due to poor moisture content, bed temperature did not exceed beyond 34ºC and 37ºC for reactor operation with 50% and 60% RH, respectively (Fig. 4.9). Temperature profiles were however homogenous owing to mixing action. For operation with inlet air RH of 70% and 80%, the bed temperature reached 40ºC at 3 cm bed height. Though temperature profiles were largely homogenous, heterogeneity was observed between 1 and 3 cm bed height during peak heat generation. The axial temperature gradient with 80% RH operation was 2.5ºC, with 3 cm bed height recording the higher temperature. This underscored the importance of optimization of mixing events and use of design equation to decide on the cooling requirements to control the bed temperature.

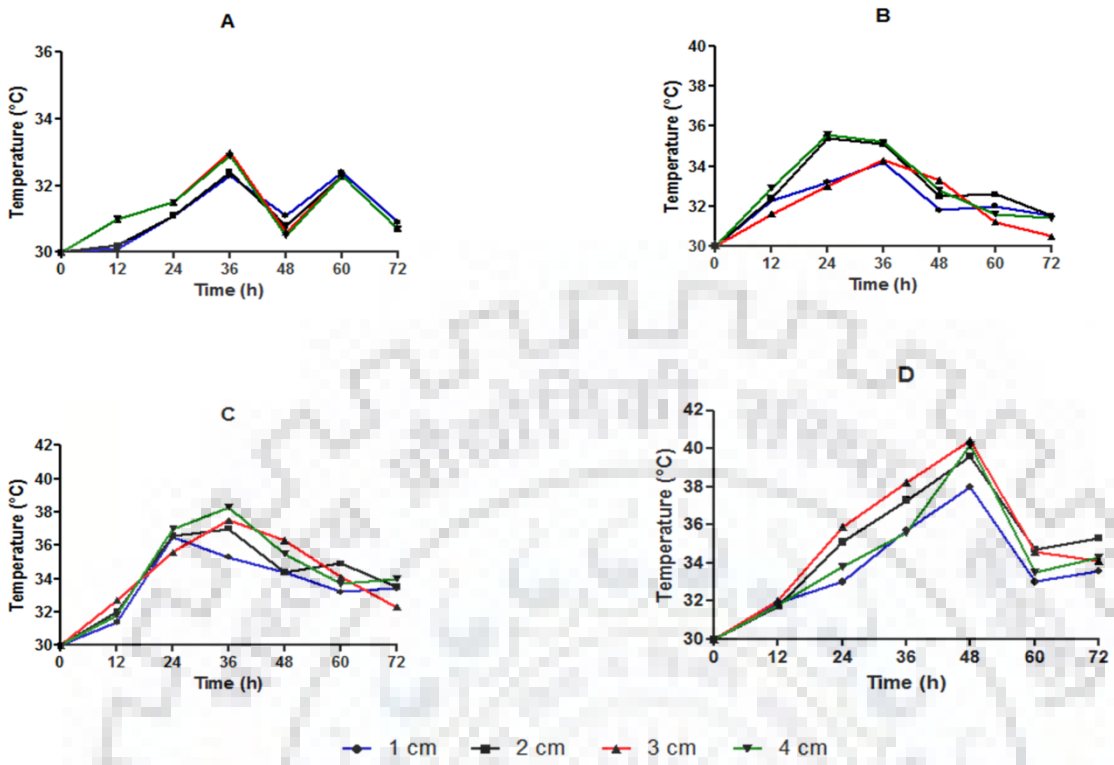

Figure 4.9 Bed temperature profiles at different bed heights (1, 2, 3 and 4 cm, respectively) during the growth of R. oryzae under four different experimental conditions. **A.** 50% RH and 4 L min^{-1}. **B.** 60 % RH and 4 L min^{-1}. **C.** 70% RH and 4 L min^{-1}. **D.** 80% RH and 4 L min^{-1}.

4.3.5.3 Biomass profiles

Poor bed moisture content prevalent with 50% and 60% RH operation adversely affected *R. oryzae* biomass production (Fig. 4.10). Maximum biomass concentration for 50% RH condition was almost threefold less than that obtained with 80% RH operation. Various reports are available in literature where low a_w proved detrimental for biomass production. Oostra et al. [27] reported that even slight decrease in a_w from optimum value of 0.97 resulted in complete inhibition of spore production by *Coniothyrium minitans*. Villena et al. [28] showed that *Aspergillus niger* biomass declined when the water activity of the growth medium decreased below 0.94. In the present bioprocess, biomass production was severely affected below the water activity of 0.90. However, reactor operation with an inlet air flow rate of 4 L min^{-1} and RH of 80% was able to maintain a_w levels greater than 0.95 for the entire process.

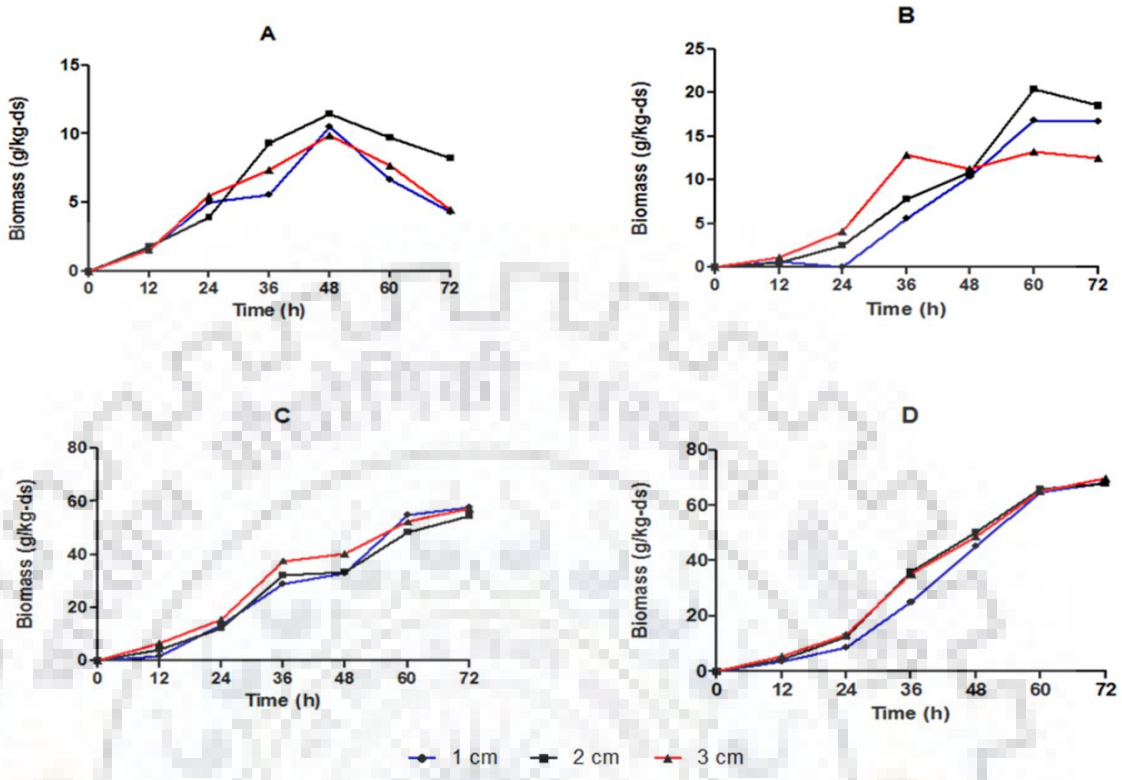

Figure 4.10 Biomass profiles of R. oryzae at different bed heights (1, 2, and 3 cm, respectively) under four different experimental conditions. **A.** 50% RH and 4 L min^{-1}. **B.** 60% RH and 4 L min^{-1}. **C.** 70% RH and 4 L min^{-1}. **D.** 80% RH and 4 L min^{-1}.

4.3.5.4 Phytase profiles

Enzymes are sensitive to changes in water activity and require a hydration layer for their activity [28]. Different studies related to protein stability have highlighted the importance of hydration layer [29, 30]. Various reports are available in literature where low water activity has proved detrimental for the production of phytase [9, 10, 31] and other enzymes [12]. In the present study, phytase production was severely affected at low bed moisture content. Phytase production in bioreactor operation at 50% and 60% RH reduced to almost half than what was observed with 80% RH operation (Fig. 4.11). However, it was not certain if the low a_w alone was responsible for lower phytase production in 50 and 60% RH operation.

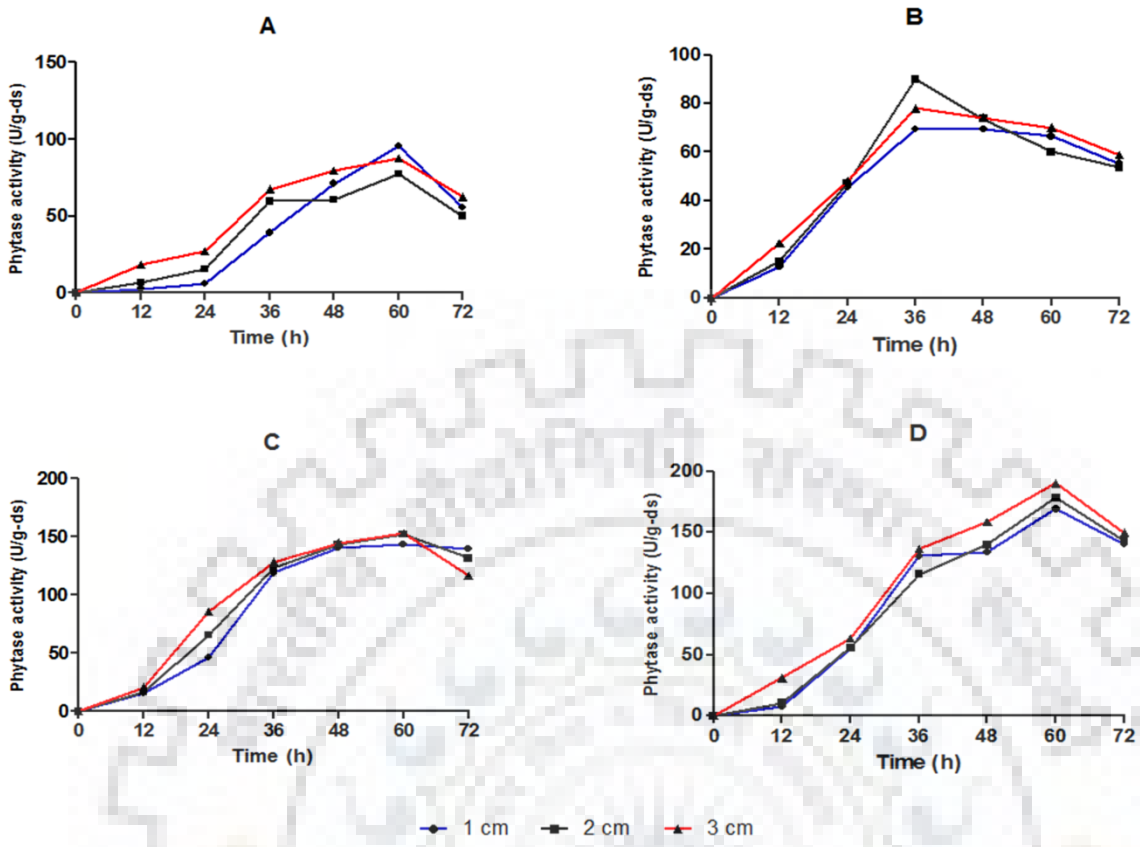

Figure 4.11 Phytase activity profiles of R. oryzae at different bed heights (1, 2 and 3 cm, respectively) under four different experimental conditions. **A.** 50% RH and 4 L min^{-1}. **B.** 60% RH and 4 L min^{-1}. **C.** 70% RH and 4 L min^{-1}. **D.** 80% RH and 4 L min^{-1}.

4.4 Conclusion

A heat transfer design equation was successfully validated for phytase production by *R. oryzae* in a novel modular SSF bioreactor. Simulation results and sensitivity analysis corroborated the utility of process design equations for optimization of substrate bed loading in SSF operation. Inlet air at 4 L min^{-1} with 80% RH gave high biomass and phytase yield and also maintained optimum bed moisture levels. In contrast to SSF, phytase productivity was severely affected under submerged fermentation conditions. Bed water activity of ≥0.95 was favorable for biomass and phytase production. Poor bed moisture content had a detrimental effect on biomass and phytase production.

5 Chapter 5

Effect of Mixing Events on Phytase Production in a Novel Solid-State Fermentation Bioreactor

Summary

Mixing constitutes a critical design parameter in solid-state fermentation (SSF) bioreactor and its effect on heat and mass transport, and microbial growth in substrate bed can significantly influence overall productivity. Effect of mixing events was studied on the production of a thermo-tolerant and acid stable phytase, by *Rhizopus oryzae*, in a novel SSF bioreactor, using optimized growth medium containing wheat bran and linseed oil cake (1:1) as main substrate. A critical mixing phase was identified, in the absence of which fungal growth led to the onset of heat accumulation and subsequent bed drying. The tensile strength of hyphal bonds between two substrate bed particles, at this critical phase, was estimated and related to the mixing intensity in the bioreactor that resulted in an optimum working value of 15 rpm. Effect of mixing time on bioreactor performance was also investigated where a 3 min mixing duration, at every six hours, increased biomass and phytase productivity to 2.2- and 4.5-fold, respectively, in comparison to packed bed bioreactor (PBR) operation. The proposed bioreactor system with intermittent mixing gave superior performance than PBR and tray bioreactor, concerning; maximum bed temperature, axial bed temperature and biomass gradient, average bed moisture content, biomass, and phytase productivity.

5.1 Introduction

In the last two decades, significant effort has been devoted towards heat and mass transfer studies in static SSF processes. Relatively, less information is available regarding the effect of mixing on process productivity, and most of the relevant work has been restricted to a specific reactor type, in particular, the rotating drum bioreactor. Moreover, as the popularity of SSF bioreactor designs tends to shift from conventional tray to modular mixed bioreactor system, the optimization of mixing events, and study of their effect on heat and mass transport holds paramount importance. Static beds are generally preferred for SSF operation, since the microenvironment resembles the natural habitat of mycelial organisms [1], and are often

accredited for high product yield [2]. However, at high substrate bed loading, static beds are prone to intense heat buildup, heterogeneity of heat and mass, bed compaction and shrinkage, high pressure drop across the bed and air channeling [3, 4]. The addition of inert support such as sugarcane bagasse, perlite, vermiculite, polyurethane and glass fiber [5] constitutes an interesting strategy to address these issues, especially if the microorganism is shear sensitive, but this may result in low substrate bed loading. Mixing circumvents these problems by disruption of hyphal bonds and increasing the surface area of fermenting solids exposed to conditioned inlet air [6]. However, aggressive or continuous mixing may be detrimental to microbial growth and result in low productivity [7], substrate agglomeration [8] and energy intensive process. Therefore, at a certain stage during fermentation on a static bed involving a fast growing fungus, a trade-off between the development of mycelial network (kinetic process) and transport processes is highly likely, and as a result, mixing events may have to be initiated and carefully designed to avoid process failure, provided the organism can tolerate mild agitation.

Most reported studies on mixing have been limited to flasks, columns and roller bottles at laboratory scale with few exceptions of rotating drum bioreactors [9-12]. One major reason for this could be the lack of suitable modular mixed SSF bioreactor system at lab and pilot scale. Moreover, conflicting results have often been reported concerning the effect of mixing on enzyme production [13-16]. Profound variations in fungal morphology and nature of substrate make it impossible to select a general mixing strategy for a broad spectrum of bioprocesses. Intermittent mixing, however, is an interesting prospect where optimized gentle agitation may strike a balance between kinetic and transport processes to give high productivity [17-19].

The objective of this work was to evaluate the effect of intermittent mixing on phytase production by *R. oryzae*, in a novel SSF bioreactor. Mixing events were designed based on the tensile strength of *R. oryzae* hyphal bonds, and their effect on bed temperature and moisture, and respiratory profiles were analyzed. The performance of the proposed SSF bioreactor system under optimized mixing regime was compared with tray bioreactor (TB) and PBR operating at same substrate bed loading.

5.2 Materials and methods

Note: Microorganism and inoculum preparation (Section 4.2.1), substrate and medium (Section 4.2.2), SSF bioreactor design (Section 4.2.3), materials and methods for analysis of cell biomass, phytase, bed moisture content and respiratory activity (Section 4.2.5) used in this chapter is the same as described in the respective Section of Chapter 4.

5.2.1 Solid-state fermentation: setup and operation

Inlet air and ambient room temperature were maintained at 30ºC. Inlet air with 80% relative humidity (RH) was supplied to substrate bed through the central pipe. Desired RH was maintained by passing air through a temperature controlled water column and monitored through a thermo-hygrometer (ZEAL, United Kingdom). Optimized air flow rate of 4 L min^{-1} was maintained with a flowmeter (KDH, England). Growth media were sterilized in situ at 121ºC for 30 min. After cooling the reactor contents to room temperature, the medium was inoculated aseptically with a spore suspension (~2×10^5 CFU g-dry-$solid^{-1}$) through spray nozzles in the upper chamber using a peristaltic pump (Applikon, Nederlands) and the fermentation was carried out for 72 h. For mixing experiments, bed was continuously mixed for 10 min immediately after inoculation, whereas, no mixing events were initiated when the bioreactor was operated as PBR and TB. A working bed height of 3.5 cm was used in the module and temperature was recorded axially across the bioreactor at every six hours using thermocouples (Pt 100 sensors (Nutronics, India)), through a sterile opening on the reactor wall. Sketch of the experimental setup is given in Fig. 4.1 A.

5.2.2 Hyphal bonds: Tensile strength measurement and mechanical properties

The tensile strength of *R. oryzae* hyphal bonds was determined with Bose tensile strength tester, using a modified method of Schutyser et al. [9]. Here, 90 g of the moistened and autoclaved substrate bed was inoculated and aseptically transferred in two Petri dishes, in a laminar air flow chamber (Rescholar, India), such that base of both Petri dishes made the top and rear end of the setup (Fig. 5.1). To mimic fungal growth, as in a PBR, the rear end of the lower Petri dish was perforated (1mm diameter) to facilitate the supply of moist air within the substrate bed. An exit vent served as the gas outlet on the upper Petri dish. To facilitate the development of hyphal bonds between the substrate particles, a gap of 1 mm was provided between surfaces of the substrate in the two Petri dishes. This was done by keeping substrate

bed height in lower Petri dish 1 mm shorter than the wall height, whereas, bed height in the upper Petri dish was kept equal to its wall height. This whole setup was kept in a sterile jar at 30°C, 80% RH and an air flow rate of 1 L min^{-1} was maintained. To determine hyphal bond strength at different fermentation times, setup of Petri dishes were subject to tensile strength tester at 14, 16, 18 and 20 h of incubation, respectively. As the fermentation progressed, each setup was carefully transferred onto tensile strength tester where the upper Petri dish was pulled upwards via hook, at a constant velocity of 0.1 mm s^{-1}, while the lower one rested on a base plate (Fig. 5.1). The force applied to detach the two Petri dishes until complete separation was recorded to develop stress versus strain curve. This curve was used to determine mechanical properties of the hyphal bonds, namely; tensile strength, yield strength, modulus of elasticity, ductility, fracture stress and elastic recovery. The tensile strength of hyphal bonds was then translated to the force required to separate two individual substrate bed particles in the bioreactor. This was done by multiplying the area of an individual particle with the tensile strength of hyphal bonds. In static bed fermentations without process control, bed temperatures can exceed significantly from that required for optimum microbial growth, and this may also affect hyphae mechanical properties. To study the effect of temperature on hyphal bond strength, a thermocouple was inserted into a separate set up of Petri dishes containing substrate bed.

Figure 5.1 Pictorial representation of the experimental setup for tensile strength measurement of *R. oryzae* hyphal bonds.

5.2.3 Effect of mixing intensity and mixing time on bioreactor performance

The critical phase for mixing corresponded to the period just before oxygen uptake rate (OUR) ceased to increase, and this was assumed to be the beginning of heat and mass transfer limitation in substrate bed. The tensile strength of the hyphal bonds at critical mixing phase, obtained from Section 5.2.2, was related to operating mixing intensity in the bioreactor with the aim to afflict minimum damage to fungal mycelia and reduce energy requirements. The force (F) obtained from the hyphal bond tensile strength estimation was correlated to the mixing intensity in the bioreactor, by Eq. (5.1):

$$F = n.m.\omega^2.r \tag{5.1}$$

Where, n is the number of blades on the revolving plate (n= 10), m is the mass of one blade (m= 0.005 kg), ω is the angular velocity of plate and r is the mean distance of blades to the centre of the plate (r= 0.0375 m). To study the effect of mixing time on bioreactor performance, four different mixing times of 1, 2, 3 and 5 min were employed, respectively, with all operating at same mixing intensity. Effect of excessive mixing was also studied when the substrate bed was mixed aggressively for 1 min at 20 rpm with a frequency of 10 min. This condition was referred to as ‘un-optimized mixing regime.'

In mixed SSF operations, information on bed particle size distribution during actual fermentation may throw insights into optimum mixing regime. This information may particularly be useful while scaling up mixing operations in different bioreactor configurations using mechanistic models (e.g. discrete particle simulations). To determine optimum bed particle size range in the present study, distribution of particle size obtained after first mixing (at 16 h) for different mixing duration was determined using four sieves of sizes of 0.85, 1.18, 2.36 and 4.72 mm, respectively, in separate experiments. The particle size range with maximum biomass concentration was considered to be optimum for the process.

5.2.4 Performance comparison with Tray and Packed bed bioreactor system

The performance of the proposed bioreactor system under optimum mixing regime was compared with TB and PBR, all operating at same substrate bed loading. Inlet air, with a flow rate of 4 L min^{-1} with RH of 80% was fed to the PBR system, and exit gas was analyzed for CO_2 and O_2 concentration. The bioreactor operated as a TB when it was kept in a sterilized incubator (New Brunswick, USA) with its top plate removed to facilitate the exchange of gases. Ambient temperature for both TB and PBR was maintained at 30ºC, operating bed height was 3.5 cm, and the fermentation was carried out for 72 h. Fermented samples were removed aseptically from different bed heights, i.e., 1, 2 and 3 cm, respectively, to determine biomass, phytase activity, and bed moisture content, as described in Section 4.2.5. Thermocouples were inserted along bioreactor height to obtain the temperature profile. Maximum bed temperature, axial temperature and biomass gradients, maximum biomass concentration, biomass and phytase productivities and bed average moisture content were selected as performance parameters.

5.3 Results and Discussion

5.3.1 Effect of bioreactor operation on *R. oryzae* respiratory activity

In packed bed fermentation, oxygen uptake rate (OUR) increased exponentially after 12 h and recorded a maximum of 0.11 mol h^{-1} at 18 h, after which it remained almost constant up to 36 h (Fig. 5.2). A similar pattern was observed with the carbon dioxide evolution rate (CER) where it increased exponentially after 10 h, peaked around 18 h and then, however, declined steadily (Fig. 5.3). This sudden surge in respiratory activity, early during fermentation, may be attributed to the extensive development of aerial hyphae since the latter has been attributed to

account for the bulk of oxygen uptake [20]. Fig. 5.4 (B) shows that this phase also corresponded to a spike in temperature profile, which was expected since oxygen uptake is proportionally related to metabolic heat generation [21].

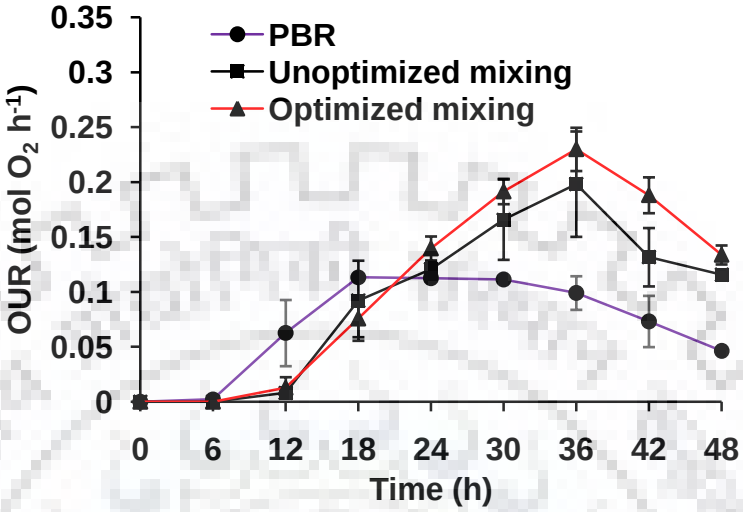

Figure 5.2 Oxygen uptake rate (OUR) profiles of *R. oryzae* in three different bioreactor operations.

Bed compaction and air channeling were prominent after the initial surge in microbial activity and were most likely responsible for poor growth later. This was probably due to the synergistic effect of *R. oryzae* growth, substrate consumption and water loss from the bed which caused the bed to shrink and moist inlet air to escape along the walls of the reactor. Consequently, this led to drop in respiratory activity. Mixing circumvented the problem of bed compaction and air channeling presumably by disruption of hyphal bonds and increasing the surface area of the substrate exposed to incoming conditioned air. With mixing at 20 rpm for 1 min at a frequency of 10 min (un-optimized mixing regime), a progressive increase in OUR was observed from 12 to 36 h (Fig. 5.2). However, moderate increase in metabolic activity was observed when the bed was mixed at a lower intensity of 15 rpm with a mixing time of 3 min at every 6 h (optimized mixing regime). This implied that increasing the mixing intensity did not have a profound effect on organism's metabolic activity. As shown in Fig. 5.3, CER also followed a similar pattern to OUR in the mixed system. Under the optimized mixing conditions, CER increased to reach a maximum of 0.086 mol h^{-1} at 34 h and thereafter declined slightly to remain constant around 0.05 mol h^{-1}. Moderate decrease in respiratory activity after

36 h was maybe because mycelia were pressed onto the substrate particles, possibly due to mixing, since temperature and water activity continued to remain favorable in mixed SSF.

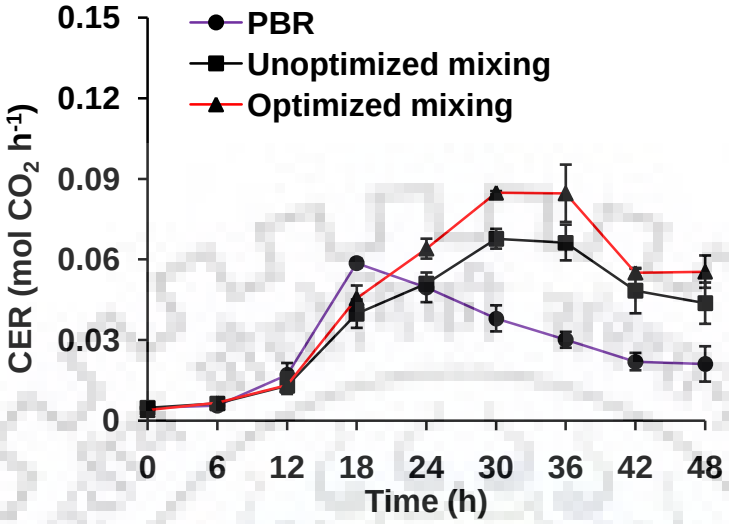

Figure 5.3 Carbon dioxide evolution rate (CER) profiles of *R. oryzae* in three different bioreactor operations.

Respiratory studies implied that mixing had an overall positive effect on metabolic activity, which was attributed to efficient heat transfer. Fig. 5.4 (B-D) shows that at 18 h, bed temperature remained below 36°C in mixed SSF, which unlike in PBR exceeded 40°C. Moreover, moisture content in both PBR and mixed SSF did not fall considerably below the optimum level of 0.55 mL g-ds^{-1} till 18 h (Fig. 5.5), which implied that the failure to mitigate intense heat buildup was primarily responsible for low metabolic activity in PBR.

5.3.2 Effect of bioreactor operation on bed temperature profiles

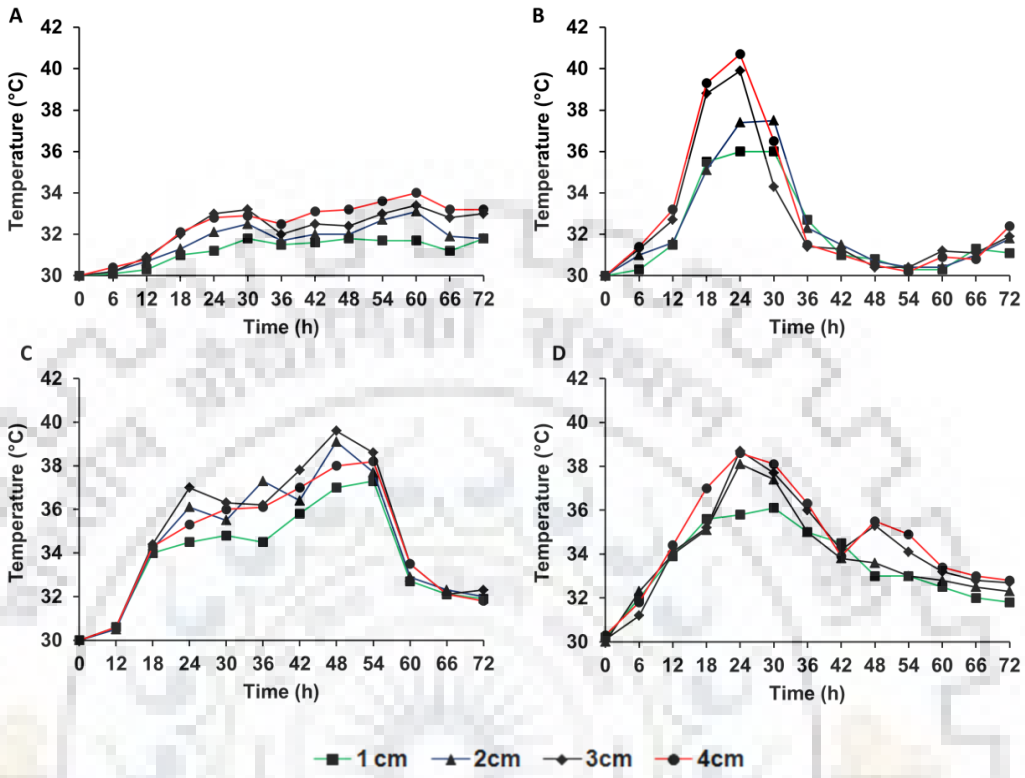

Figure 5.4 Temperature profiles at different bed heights (1, 2, 3 and 4 cm, respectively) during the growth of *R. oryzae* under in different bioreactor operations. **A.** Tray bioreactor. **B.** Packed bed bioreactor (PBR). **C.** Bioreactor operation with unoptimized mixing. **D.** Bioreactor operation with optimized mixing.

The initial surge in metabolic activity in PBR led to a steep increase in bed temperature up to detrimental levels (Fig. 5.4 (B)) which affected fungal growth and phytase productivity (Table 5.1). Moreover, an axial temperature gradient of 4.7°C was observed, which is significant in a small bed height of 3.5 cm. In the absence of mixing, this heterogeneity was probably due to unequal distribution of inoculum, which was confirmed by a heterogeneous biomass profile (Fig. 5.6 (B)). This substantiated the importance of the first mixing event for uniform distribution of mass, even for static bed operations. As illustrated in Figs. 5.4 (C) and 5.4 (D), mixing circumvented temperature rise to reach detrimental levels and also maintained a high degree of homogeneity across the bed. Due to low metabolic activity in TB, the average bed

temperature did not exceed 32ºC which implied that substrate bed height of 3.5 cm was highly unproductive. A working bed height of 1.5-2 cm is usually employed in trays [22-24], although this is certainly not a thumb rule and bed height may vary depending upon the system transport and kinetic parameters, bioreactor configuration, and operating conditions.

5.3.3 Effect of bioreactor operation on bed moisture profiles

Following the steep rise in bed temperature, bed moisture content in PBR started to decline rapidly after 18 h (Fig. 5.5 (B)). Initially, heat accumulation marked by subsequent rise in bed temperature (Fig. 5.4 (B)) accentuated drying of the substrate with moist inlet air unable to prevent it. This problem was compounded by bed compaction and air channeling due to which surface area of substrate exposed to moist air reduced considerably. Pitol et al. [3] observed with PBR that as the fermentation progressed, substrate consumption and microbial growth resulted in shrinkage of substrate bed along the wall of the bioreactor, which resulted in air channeling. Heterogeneity in bed moisture, in fixed bed, has also been reported by other workers [4, 25]. A similar phenomenon was observed in the present study where rapid microbial growth (as evident from respiratory profile) and rapid consumption of substrate made the bed highly compact that most likely offered resistance for moist air to traverse between the bed particles. The decrease in bed moisture content eventually led to low metabolic activity which was corroborated by a sharp decline in bed temperature after 24 h and cessation in the increase of OUR (Fig. 5.2). However, mixing facilitated the maintenance of optimum moisture level by increasing the surface area of substrate exposed to moist air, thus circumventing bed drying. As inferred from Table 5.1, the average bed moisture content in un-optimized and optimized mixing regime was 0.58 and 0.59 mL g-ds^{-1}, respectively, which was favorable for biomass and phytase production, whereas, in PBR, it dropped to 0.28 mL g-ds^{-1} which adversely affected fungal growth. Few workers have argued that intermittent water addition to fermentation media through nozzles or sparger may be useful in replenishing moisture content in a static bed [26, 27], however, to ensure its homogeneous distribution in an SSF bed, mixing is most likely to be essential [28].

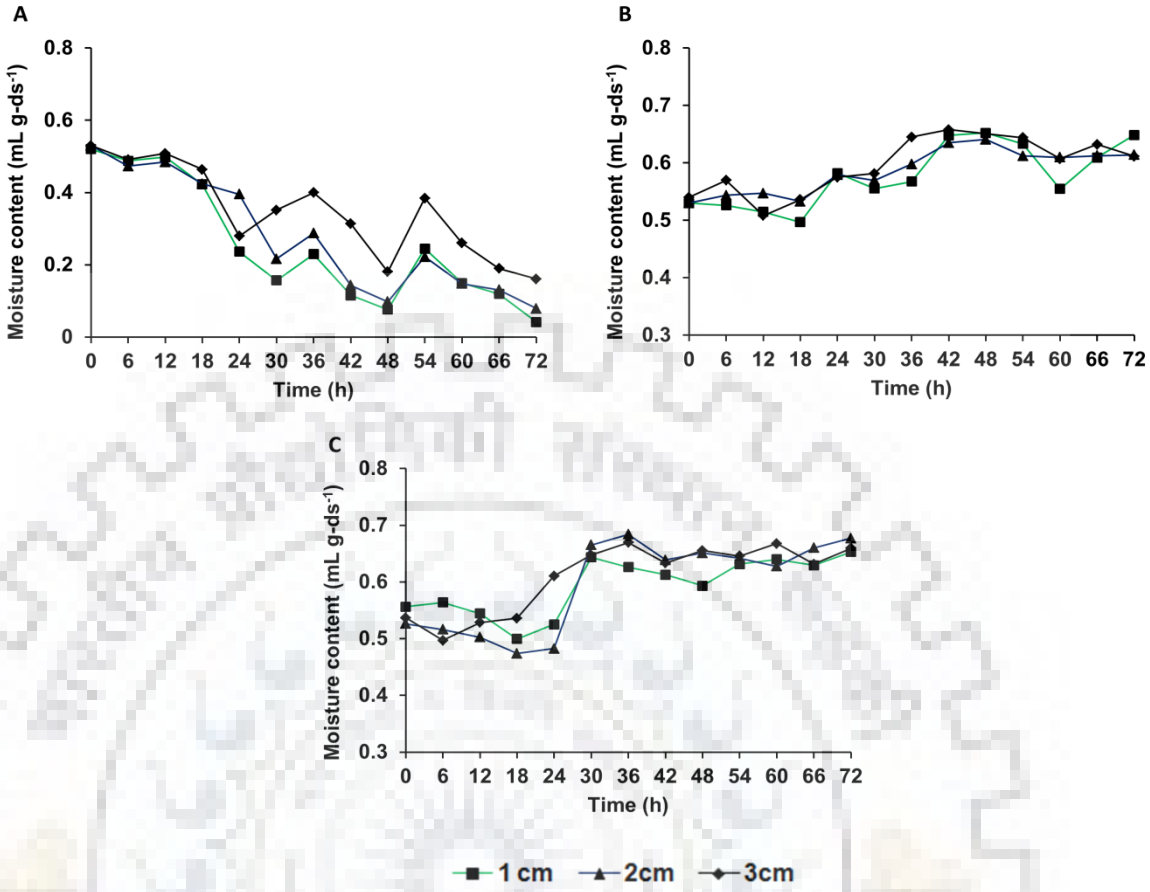

Figure 5.5 Bed moisture profiles at different bed heights (1, 2 and 3 cm, respectively) during the growth of *R. oryzae* in three different bioreactor operations. **A.** Packed bed bioreactor (PBR). **B.** Bioreactor operation with unoptimized mixing. **C.** Bioreactor operation with optimized mixing.

5.3.4 Effect of bioreactor operation on R. oryzae biomass

An investigation into early fermentation period (0-24 h) of PBR and aggressively mixed SSF (unoptimized mixing) revealed significant differences in specific growth rate constant (μ). Using logistic growth model, μ for PBR and un-optimized mixing regime were found to be 1.10 and 0.57 h^{-1}, respectively. This implied that the initial static period was highly favorable for biomass production until intense heat buildup followed by low bed moisture content slowed down fungal growth after 18 h. Lower μ with unoptimized mixing was most likely due to the damage inflicted onto the under-developed mycelial network, early during fermentation. This coincided with the observation of Fung and Mitchell [29] that mixing may be damaging to hyphae, especially during the initial germination period. As a result of the above observation,

the bioreactor was initially (up to 16 h) operated as a static bed to allow development of *R. oryzae* hyphal bonds, which was followed by intermittent mixing to avoid heat accumulation and facilitate the maintenance of optimum water content in the substrate bed. As shown in Fig. 5.6 (B), lower biomass production was observed in PBR, which was expected since unfavorable conditions of bed temperature (Fig. 5.4 (B)) and moisture (Fig. 5.5 (B)) were prevalent, along with high degree of heterogeneity across bed height (Table 5.1).

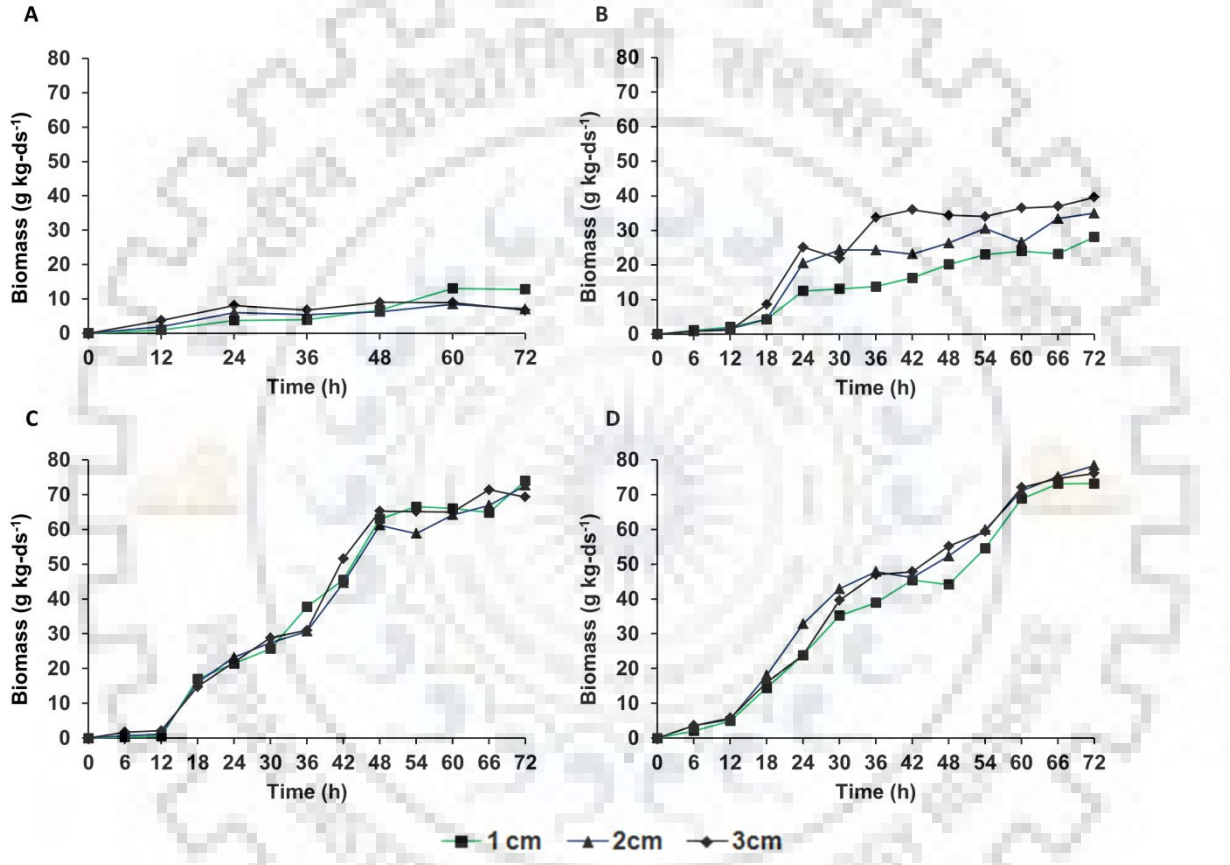

Figure 5.6 Biomass profiles of *R. oryzae* at different bed heights (1, 2 and 3 cm, respectively) in four different bioreactor operations. **A.** Tray bioreactor. **B.** Packed bed bioreactor (PBR). **C.** Bioreactor operation with unoptimized mixing. **D.** Bioreactor operation with optimized mixing.

On the contrary, biomass distribution in mixed SSF regimes was relatively uniform with axial biomass gradient reduced to 3.09 g kg-ds^{-1} with optimized mixing conditions as compared to 9.08 g kg-ds^{-1} in PBR. This gradient was further reduced to 1.69 g kg-ds^{-1} with unoptimized mixing regime; however, maximum biomass concentration also reduced slightly to 73.98 g kg-ds^{-1}. Increasing the mixing intensity beyond optimum mixing regime did not prove detrimental,

but such a strategy would be highly energy intensive, and therefore, undesirable. Fig. 5.6 (A) shows that biomass activity in TB was largely subdued possibly due to O_2 limitation and CO_2 accumulation. As a result, a maximum biomass concentration of only 13.04 g kg-ds^{-1} was obtained at 3 cm bed height which was six times less than what was obtained with optimized mixing regime.

Table 5.1 Performance comparison of intermittently mixed SSF bioreactor (mixed for 1, 2, 3 and 5 min, respectively) with Tray bioreactor and Packed bed bioreactor (PBR).

Performance parameter	Tray bioreactor	PBR	Mixed SSF			
			1 min	2 min	3 min	5 min
Maximum bed temperature (ºC)	34	40.7	41	40.1	38.7	38.9
Axial temperature gradient (ºC)	2.3	4.7	6.3	3.8	2.9	1.9
Maximum biomass conc. (g kg-dry solid^{-1})	13.04	39.60	58.34	69.34	78.38	75.30
Axial biomass gradient (g kg-dry solid^{-1})	1.36	9.08	4.92	7.20	3.09	3.68
Biomass productivity (g kg-dry solid^{-1} d^{-1})	2.95	11.40	18.08	20.64	25.29	24.56
Phytase productivity (IU kg-dry solid^{-1} d^{-1})	396.64	794.4	2652	3062.4	3621.3	3354.4
Average bed moisture (mL g-dry solid^{-1})	-	0.28	0.38	0.56	0.59	0.52

5.3.5 Tensile strength and mechanical properties of hyphal bonds

Fig. 5.7 shows stress vs. strain profile of hyphal bonds at different fermentation times of 14, 16, 18 and 20 h, respectively. Maximum tensile strength of 405.61 N m^{-2} and 555.64 N m^{-2} were obtained at 16 and 18 h, respectively (Table 5.2), which however drastically reduced to 287.90 N m^{-2} at 20 h. This was most likely due to weakening of hyphal bonds due to overheating and subsequent bed drying. As a consequence of overheating during static bed operation (PBR), the biomass concentration after 20 h remained almost stagnant for the next 16 h, and bed moisture content also dropped significantly (Fig. 5.4 (B) and 5.5 (B)). At 16 and 18 h, fermentation bed exhibited elastic characteristics suggesting strong interparticle mycelial bonds, whereas at 14 h, the bed appeared brittle in nature possibly due to scant fungal growth, as corroborated by low biomass concentration (Fig. 5.6 (B)) and respiratory activity (Fig. 5.2 and 5.3). Moistened wheat bran and linseed oil cake possessed sticky nature that resulted in substrate

agglomeration, and therefore, to determine particle diameter, it was necessary to study the effect of substrate agglomeration on particle size. This was done by passing the autoclaved substrate bed through sieves of different sizes. The diameter of 82% of the substrate bed particles was found to vary between 2.36 and 2.96 mm, respectively. Average of the two values (i.e., 2.66 mm) was assumed as the effective diameter of bed particle (D_e). Using D_e of 2.66 $\times 10^{-3}$ m, the area (π r^2) of single bed particle was calculated to be 5.55×10^{-6} m^2. To ensure satisfactory dissociation of hyphal bonds, tensile strength of 600 N m^{-2} multiplied with the area of individual bed particle gave the force (3.33 mN) required to disengage hyphal bonds between two particles.

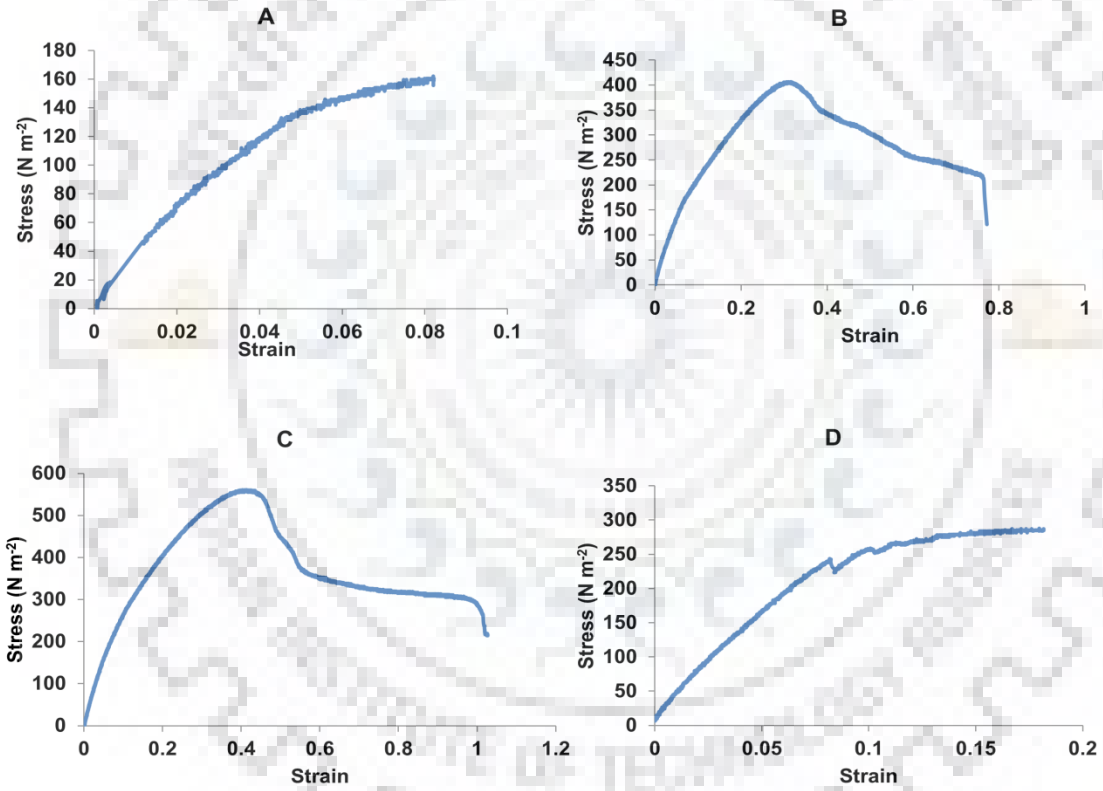

Figure 5.7 Stress vs. Strain profile for *R. oryzae* hyphal bonds at different fermentation times. **A.** 14 h. **B.** 16 h. **C.** 18 h. **D.** 20 h.

A tensile strength of 600 N m^{-2} was more than the maximum tensile strength (555.6 N m^{-2}) at 18 h but was employed to ascertain complete disengagement and facilitate proper heat and water transfer. As evident from biomass, respiratory and temperature profiles of PBR, the system experienced rapid and extensive growth between 14 to 18 h, and it was during this

period when fungal growth became limiting to heat and water transport that subsequently resulted in heat accumulation and low water activity. This implied that it is important to identify a 'critical mixing phase' to avoid process failure and ensure efficient heat and mass transfer. This observation was consistent with the findings of Schutyser et al. [9] where tensile strength of aerial hyphae of *Aspergillus oryzae* increased rapidly from around 250 N m^{-2} to over 850 N m^{-2}, in a short span of 2 h (23.50-25.30 h), in a rotating drum bioreactor. After 25 h, growth was affected and OUR remained relatively constant for the next 10 h, which substantiated the importance of mixing time and intensity in SSF operations. Similar approaches, as the one developed in the present study, may be adapted for other SSF bioreactor processes, where determination of tensile strength of hyphal bonds and other mechanical properties could guide the design of mixing regime. With the use of mechanistic modeling tools such as the discrete particle simulation, this information may be useful in scale-up operations as well [30]. Table 5.2 shows the mechanical properties of hyphal bonds at 14, 16, 18 and 20 h of fermentation, respectively.

Table 5.2 Mechanical properties of hyphal bonds of *R. oryzae* at different fermentation times of 14, 16, 18 and 20 h, respectively.

Mechanical property	**14 h**	**16 h**	**18 h**	**20 h**
Yield stress (N m^{-2})	46.77	154.83	212.90	243.54
Tensile strength (N m^{-2})	160.48	405.61	555.64	287.90
Modulus of elasticity (kPa)	1.93	1.90	2.87	2.93
Fracture stress (N m^{-2})	159.67	221.7	297.5	287.9
Ductility (%)	4.3	74.5	88.42	10.92
Elastic recovery (Δl/l)	0.03	0.09	0.09	0.06

5.3.6 Optimum mixing regime in SSF bioreactor

From the analysis of respiratory profiles (Fig. 5.2 and 5.3) and tensile strength results (Table 5.2), the fermentation time of 16 h was selected as critical for initiation of mixing events. However, to ensure satisfactory dissociation of hyphal bonds, a tensile strength of 600 N m^{-2} was used to determine operating mixing intensity. Using Eq. (5.1), a mixing intensity of 15 rpm

was obtained which would exert sufficient force (4.59 mN) between two substrate bed particles, to ensure dissociation of hyphal bonds. Agitation at lower intensities led to inefficient mixing which resulted in clumps of substrate bed with 59% and 35% of mass fractions greater than 4.76 mm, at 5 rpm and 10 rpm, respectively. Mixing at 15 rpm for 3 min reduced this mass fraction to 26.2% and increased the 1.18-2.36 mm fraction to 42.2%. As evident from Fig. 5.8, bed particle size range of 1.18-2.36 mm augured high biomass concentration and most likely facilitated efficient heat and water transfer than other mass fractions.

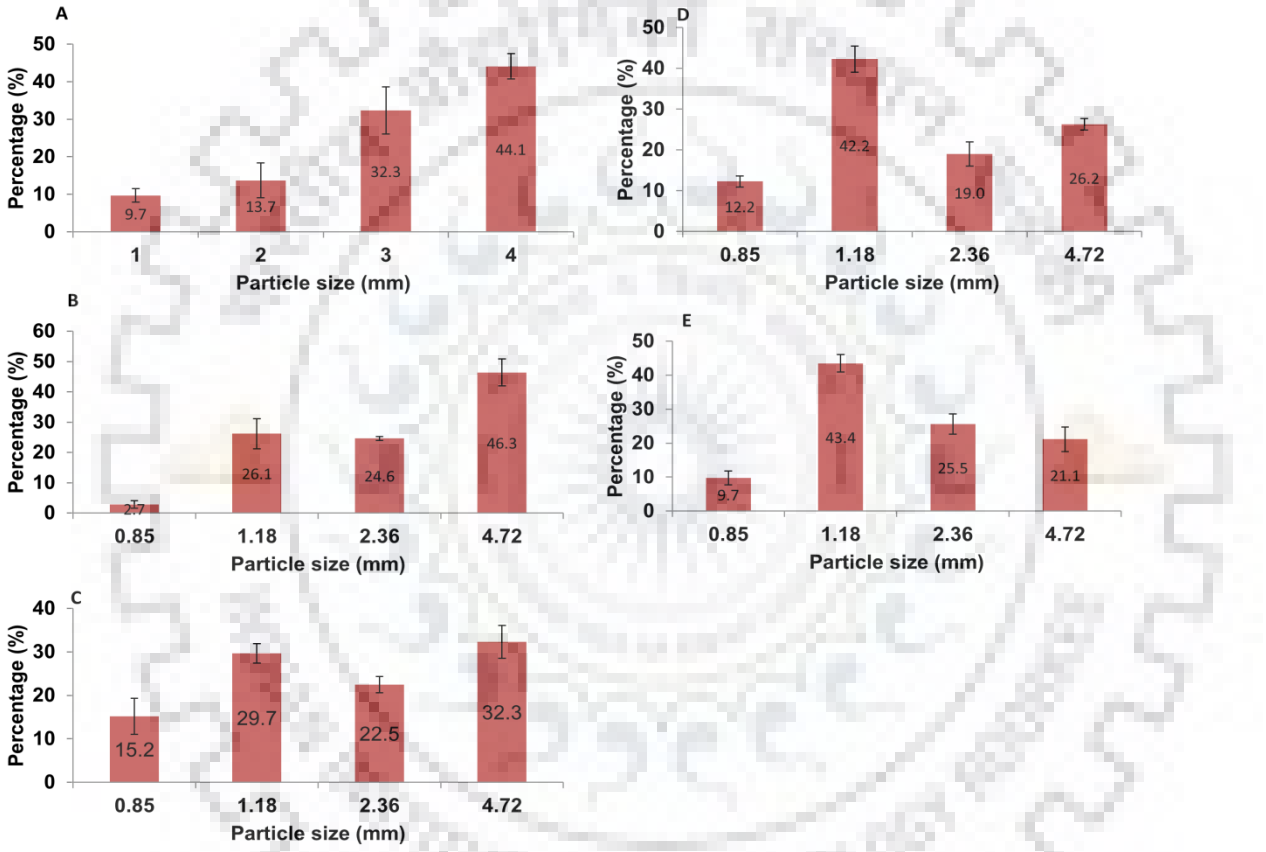

Figure 5.8 Particle size distribution under various mixing regimes in the SSF bioreactor. **A.** PBR (no mixing). **B.** 1 min mixing. **C.** 2 min mixing. **D.** 3 min mixing. **E.** 5 min mixing.

Operation with high mixing intensities, i.e., 20 and 30 rpm did not result in significant change in mass fractions. Effect of mixing time on bioreactor performance was studied while the mixing intensity was fixed at 15 rpm. Bed particle size, in the range of 1.18-2.36 mm, increased in the mass fraction as mixing time increased from 1 to 3 min and remained relatively constant with 5 min rotation (Fig. 5.8). As Table 5.1 shows, axial temperature gradients with 3 and 5

min of agitation were reduced to 2.9ºC and 1.9ºC respectively, from 6.3ºC observed with 1 min rotation. Also, a decrease in maximum bed temperature of 2.3ºC was observed with 3 min mixing as compared to 1 min. Average moisture content in the substrate bed remained relatively constant near the optimal value of 0.55 mL g-ds^{-1} at 2, 3 and 5 min rotation, respectively, but reduced to 0.38 mL g-ds^{-1} with 1 min rotation which was not favorable for microbial growth. No significant difference in bioreactor performance was observed with 3 and 5 min mixing (Table 5.1), and therefore, a 3 min mixing time was optimum for the present operation. Perhaps one of the most significant outcomes in comparison to the unoptimized mixing regime was the considerable reduction in energy requirement, with mixing frequency reduced from 6 min h^{-1} to 0.5 min h^{-1}. Similar approaches with different bioreactor configurations could help economize an SSF operation where hundreds of kilograms of fermenting solids are subject to mixing. It is also pertinent to note that while mixing did not significantly affect microbial growth, static operation (PBR) and low intensity mixing (1 and 2 min rotation) led to inefficient operation which resulted in poor biomass and phytase activity (Table 5.1). Therefore, for an SSF process to run efficiently, it is not only important to identify critical mixing period but also to determine optimum mixing intensity and mixing time.

5.3.7 Performance comparison of novel SSF bioreactor with tray and PBR

Table 5.1 shows the performance comparison of proposed SSF bioreactor system with TB and PBR. For mixed SSF operations, a mixing time of 3 min was optimum and a further increase in mixing time did not significantly enhance the performance. Maximum biomass concentration at optimum mixing increased 6.01 and 1.97 folds as compared to tray and PBR, respectively. Moreover, fermentation was homogenous to a great extent, with axial temperature gradient reduced from 4.7ºC to 2.9ºC, average bed moisture content increased to 0.59 mL g-ds^{-1} from 0.28 mL g-ds^{-1}, and axial biomass gradient reduced to almost three times in comparison to PBR. Maintenance of optimum moisture content in bed was essential for enzyme production, as it can be noted that while biomass productivity increased 2.21 times with mixed operation (Table 5.1), the phytase productivity was severely affected in PBR, possibly due to a combination of high bed temperature (>40ºC), low water activity (<0.90) and poor O_2 level in the substrate bed. Metabolic activity in TB was subdued possibly due to O_2 limitation, CO_2 accumulation, unequal inoculum distribution and therefore lower axial temperature and biomass gradients were insignificant. With optimized mixing, phytase productivity increased 9.12 and 4.55 times than TB and PBR (Fig. 5.9), respectively, which was attributed to the

uniform distribution of moist air and inoculum during mixing thus preventing bed compaction, air channeling and assuring a high degree of homogeneity of process variables.

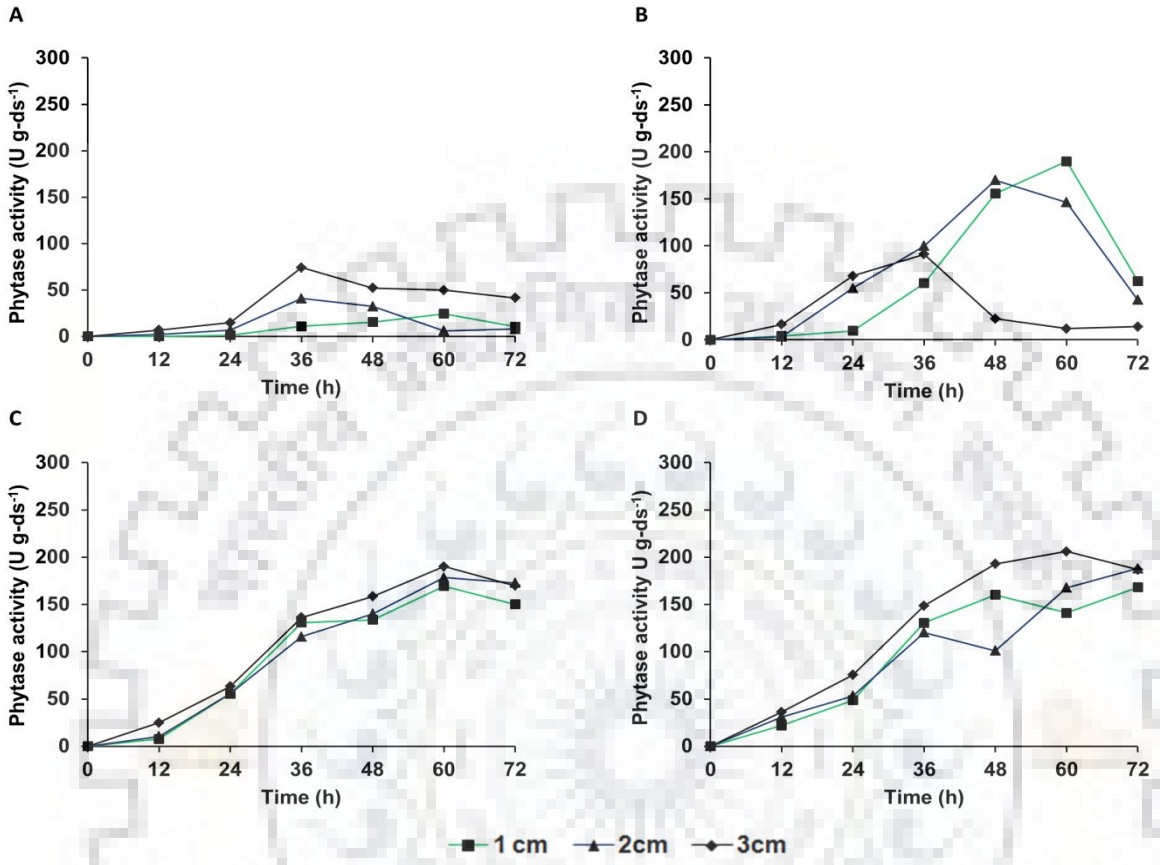

Figure 5.9 Phytase activity profiles of *R. oryzae* at different bed heights (1, 2 and 3 cm, respectively) in four different bioreactor operations. **A.** Tray bioreactor. **B.** Packed bed bioreactor (PBR). **C.** Bioreactor operation with unoptimized mixing. **D.** Bioreactor operation with optimized mixing.

5.4 Conclusion

Mixing events were optimized in a novel SSF bioreactor for the production of phytase by *R. oryzae*. The results emphasized the importance of intermittent mixing to enhance enzyme productivity and reduce the energy input. Using respiratory analysis and hyphal bond strength of *R. oryzae*, the importance of critical mixing phase to facilitate efficient kinetic and transport (heat and water) operations was shown. Information on mechanical properties of hyphal bonds and particle size distribution in an optimally mixed SSF bioreactor may be useful for designing mixing regime in large scale operations. The proposed bioreactor performed better than tray

and packed bed bioreactors, operating at same substrate bed loading. The proposed bioreactor system promises to be an ideal system to study the effect of mixing on process productivity in SSF.

6 Chapter 6

Oxygen Uptake Rate as a Tool for On-line Estimation of Cell Biomass and Bed Temperature in a Novel Solid-State Fermentation Bioreactor

Summary

Direct measurement of cell biomass is difficult in an SSF process involving filamentous fungi since the mycelium, and the solid substrate are often inseparable. However, respiratory data is rich in information for real-time monitoring of microbial biomass production. In this regard, a correlation was obtained between oxygen uptake rate (OUR) and biomass concentration (X) of *Rhizopus oryzae*, during phytase production, in an intermittently mixed novel SSF bioreactor. To obtain the correlation, various models describing sigmoidal growth were tested, namely the logistic, Gompertz, Stannard, and Schnute models. Regression analysis of experimental results, at different operating conditions of inlet air flow rate and relative humidity suggested that OUR and X were correlated well by the logistic model ($R^2 > 0.90$). To corroborate the use of respiratory data for on-line measurement of metabolic activity, OUR was related to metabolic heat generation rate (R_q), and the logistic model was found to satisfactorily correlate R_q and X as well. The model parameter, $Y_{Q/X}$, when substituted into a heat transfer design equation, along with the values of other parameters and operating variables, gave reliable estimates of bed temperature. The correlations developed in the present study, between respiratory activity and biomass concentration may be extended on to other SSF processes for further validation and real-time monitoring of cell biomass and bed temperature.

6.1 Introduction

Biomass is a critical parameter in monitoring and control of a solid-state fermentation (SSF) process but is difficult to estimate on-line. The problem is compounded especially with filamentous fungi, where the hyphae penetrate and bind tightly to the solid substrate. However, numerous indirect methods are available, some of which include estimation of cell-specific components such as ergosterol [1, 2], glucosamine [3], proteins and nucleic acid [4, 5] and spores [6]. Biomass has also been correlated with biological activity, which includes estimating ATP [7, 8], respiratory activity [9, 10], enzyme activity [11], metabolite production and

nutrient consumption [12]. A major disadvantage associated with some of the above-mentioned methods, especially indirect methods, is that they require samples to be withdrawn continuously from the reactor bed which may compromise sterility and also make the analysis time consuming and costly. Moreover, in pilot and industrial scale operation, it is difficult to obtain well mixed or homogenous samples, or otherwise, the sample volume and the number should be increased substantially.

Over the years, various direct and indirect methods for biomass estimation have been developed for submerged fermentation (SMF). Some of these methods have been extended to SSF processes. For example, Fourier transform near-infrared spectroscopy was applied for determining time-related changes during fermentation of wheat straw [13]. Digital image analysis coupled with kinetic models was used to correlate *Penicillium decumbens* biomass grown on a lignocellulosic substrate [14]. However, such methods are more likely to be suitable for static bed operation and specific for a particular microorganism and substrate. For modular mixed bioreactor system, wherein hundreds of modules may be stacked together, measurement and analysis of respiratory activity present a more pragmatic and economic alternative. Exhaust gases are rich in information about organism metabolic activity and in general bioreactor performance. O_2 uptake rate (OUR) and CO_2 evolution rate (CER) are of particular interest, as they provide the most convenient way of on-line monitoring of microbial activity in an SSF bioreactor [15].

In the present study, a correlation has been proposed between OUR and cell biomass concentration for the entire fermentation duration, under optimized growth conditions. For this, the applicability of various models describing sigmoidal growth was investigated. The correlation was tested during the production of a thermo-tolerant and acid-stable phytase, by *Rhizopus oryzae*, in a novel SSF bioreactor system wherein multiple modules may be stacked vertically around a central pipe. As the popularity of SSF bioreactor design shifts from conventional tray to modular mixed systems, wherein multiple modules are stacked horizontally or vertically, on-line estimation of bed temperature can also be useful. Thermocouples may limit mixing, and as a result, it may not be possible to monitor and control the entire fermentation bed effectively. For this purpose, metabolic heat generation rate (a function of OUR) was correlated with biomass concentration. Parameters obtained from the correlation were substituted in a heat transfer design equation to give real-time bed temperature.

6.2 Materials and Methods

Note: Microorganism and inoculum preparation (Section 4.2.1), substrate and medium (Section 4.2.2), SSF bioreactor design (Section 4.2.3), materials and methods for analysis of cell biomass, phytase, bed moisture content and respiratory activity (Section 4.2.5) used in this chapter is the same as described in the respective Section of Chapter 4.

6.2.1 Microbial growth models for biomass estimation

The experimentally obtained biomass concentration of *R. oryzae* at different operating conditions and that of F2 were fitted to the following models predicting sigmoidal growth:

I. Logistic model [15]

$$X = \frac{a}{\left(1-\left(1-\frac{a}{b}\right)exp\,(-c.t)\right)} \tag{6.1}$$

II. Gompertz [22]

$$X = a.exp\left(-exp(b-c.t)\right) \tag{6.2}$$

III. Stannard [22]

$$X = a\left\{1+exp\left[\frac{-(l+k.t)}{p}\right]\right\}^{(-p)} \tag{6.3}$$

IV. Schnute [22]

$$X = \left\{x_1{}^b + \left(x_2{}^b - x_1{}^b\right).\frac{1-exp\left[-a[t-\tau_1]\right]}{1-exp\left[-a[\tau_2-\tau_1]\right]}\right\}^{\left(\frac{1}{b}\right)} \tag{6.4}$$

In the above equations, a, b, c, v, k, *l*, p, $\varkappa_1$, $\varkappa_2$, τ_1, and τ_2 are mathematical parameters in the respective growth models.

6.2.2 Correlation between OUR and cell biomass

Respiratory activity was used for predicting the cell biomass concentration. OUR is associated with growth rate and biomass concentration by Eq. (6.5) [15]:

$$OUR = \frac{1}{Y_{X/O}}\frac{dX}{dt} + m_o X \tag{6.5}$$

In Eq. (6.5), $Y_{X/O}$ is the yield of biomass from O_2 (kg-dry-biomass mol-O_2^{-1}), and m_o is the maintenance coefficient for O_2 (mol-O_2 kg-dry-biomass^{-1} h^{-1}). Differential of the four equations (Eqs. 6.1 to 6.4), representing sigmoidal growth (dX/dt) were substituted into Eq. (6.5), to derive the mathematical relationship between OUR and X.

For example, differential form of the logistic equation (Eq. (6.6)) could be substituted into Eq. (6.5) to derive a quadratic relationship between OUR and X:

$$\frac{dX}{dt} = \mu . X \left(1 - \frac{X}{X_m}\right) \tag{6.6}$$

Eq. (6.7) shows that a quadratic polynomial theoretically related OUR and X:

$$OUR = \frac{-1}{Y_{X/O}} \mu \frac{X^2}{X_m} + \left(\frac{1}{Y_{X/O}} \mu + m_O\right) X \tag{6.7}$$

Using Eq. (6.7), experimentally obtained OUR and biomass concentration data were fitted using the curve fitting tool of MATLAB 7.10.0 (R2010a). Following similar methodology, other correlations corresponding to different growth models were developed and fitted.

6.2.3 Correlation between metabolic heat generation rate and cell biomass

The rate of metabolic heat generation (R_q) is directly proportional to OUR according to Cooney et al. [16]. The metabolic heat generation rate, in turn, is correlated with the biomass concentration and the growth rate according to Eq. (6.8) [15]:

$$R_q = Y_{Q/X} \frac{dX}{dt} + m_Q X \tag{6.8}$$

Following a similar methodology as that described in Section 6.2.2 with OUR, here, the differential forms of the logistic, Gompertz, and Stannard models were substituted into Eq. (6.8), to derive the mathematical relationship between R_q and X. Eq. (6.9) shows that R_q and X were theoretically related by a quadratic polynomial in the case of substitution into logistic growth model:

$$R_q = -Y_{Q/X} . \mu . \frac{X^2}{X_m} + \left(m_Q + Y_{Q/X} . \mu\right) X \tag{6.9}$$

Using Eq. (6.9), R_q (derived through experimentally obtained OUR data) and biomass concentration data were fitted using the curve fitting tool of MATLAB 7.10.0 (R2010a).

Following similar methodology, other correlations corresponding to different growth models were developed and fitted.

6.2.4 Bed temperature estimation

Kinetic parameters obtained from the regression analysis of Eq. (6.9) were substituted into a heat transfer design equation (Eq. (6.10)); an energy balance on the current SSF bioprocess in the bioreactor that included both axial and radial heat transfer [17]. The development of the design equation (Eq. 6.10), assumptions, initial and boundary conditions, the solution technique and the percentage error calculation, is discussed in detail in Section 4.2.6.

$$\frac{\partial T}{\partial t} = \left(\frac{k_b}{\rho_b \,.\, Cp_b}\right)\left[\left(\frac{\partial^2 T}{\partial z^2}\right) + \left\{\frac{1}{r}\left(\frac{\partial T}{\partial r}\right) + \left(\frac{\partial^2 T}{\partial r^2}\right)\right\}\right] - \left(\frac{\rho_a}{\rho_b \,.Cp_a}\right)\left\{c_{p_a} + f.\lambda\right\} V_Z \left(\frac{\partial T}{\partial z}\right) + \left(\frac{\rho_s}{\rho_b \,.Cp_b}\right).\left((1-\epsilon) Y_{\frac{Q}{X}}.\left(\frac{dX}{dt}\right)\right)$$

(6.10)

Substrate density (ρ_s), bed packing density (ρ_b), void fraction (ε) and bed specific heat (c_{pb}) were estimated as described by Mitchell et al. [15]. Substrate thermal conductivity was measured using the KS-1 probe of a thermal property analyzer (KD2 Pro, USA). Values of the density of air, specific heat of air and latent heat of vaporization of water were obtained from standard literature [18, 19]. Heat transfer validation has been shown here for 3 cm bed height since in a bottom-aerated bed the upper regions of the bed will reach a higher temperature, and microbial biomass at that level is more sensitive to inactivation.

6.2.5 Other fungi

To extend the validation of correlation between respiratory activity and biomass concentration, other phytase producing fungi were required. For isolation and screening of phytate hydrolyzing fungi, soil samples (n=15) were collected from Khanjarpur, Roorkee. Serial diluted samples were plated on Phytase Screening Media (PSM) containing, per litre: sodium phytate, 0.4 g; glucose, 1 g; KCl 0.05 g; NH_4NO_3 0.5 g; $CaCl_2.2H_2O$, 0.5 g; $MgSO_4.7H_2O$, 0.05 g; $MnSO_4.H_2O$, 0.01 g; $FeSO_4.7H_2O$, 0.01 g; and agar, 15 g and the medium was adjusted to pH 5.6 [20]. Isolates were selected on the basis of the formation of a clear halo. Further screening was carried out by assessing the extracellular phytate-degrading activity. F2 showed the highest extracellular phytate-degrading activity among all isolates, but more importantly, it showed comparatively significant lower aerial hyphae formation and was subsequently selected

to study the effect of fungal morphology on the relationship between respiratory activity and biomass concentration. The development of correlations between OUR and biomass concentration and R_q and biomass concentration were extended on to a fungal isolate (F2) with distinct form than *R. oryzae*. F2 was grown using the same growth media composition and culture conditions as for *R. oryzae*, and similar analytical methods were followed as described in Section 4.2.5.

6.3 Results and discussion

6.3.1 Cell biomass estimation

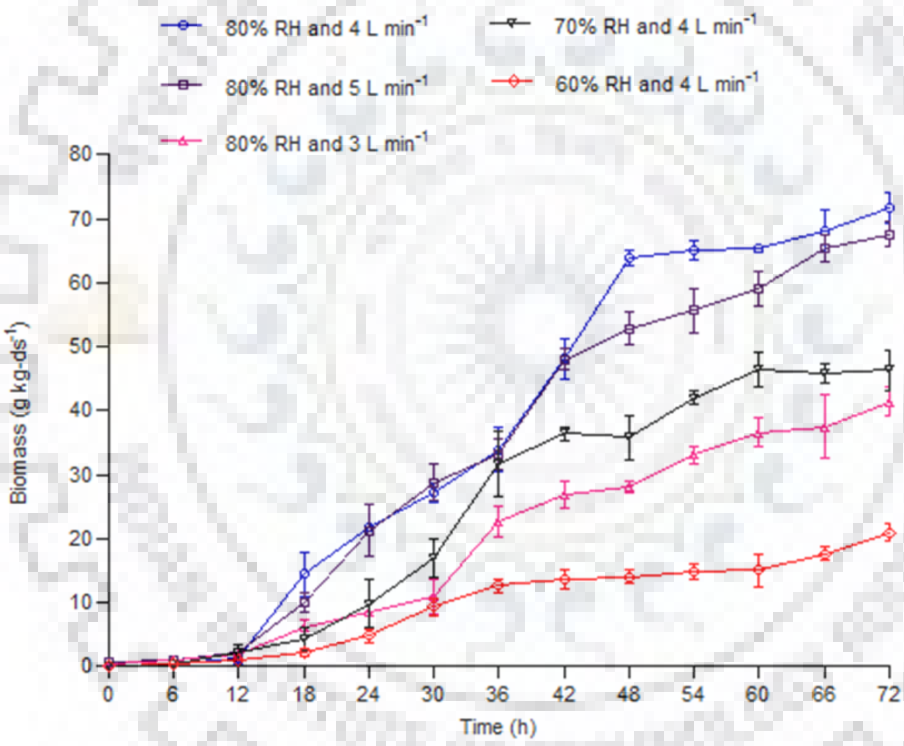

Figure 6.1 Biomass profiles of *R. oryzae* at different conditions of inlet air flow rate and relative humidity in SSF bioreactor.

Fig. 6.1 shows biomass profiles for *R. oryzae* at different operating conditions of inlet air flow rate and relative humidity (RH), obtained through glucosamine method. Maximum biomass concentration of 71±2 g kg-ds^{-1} was obtained for operation with 4 L min^{-1} and 80% RH, whereas biomass production was severely limited when the bioreactor was operated at the same air flow rate but at 60 % RH. The reduction in microbial growth was due to poor moisture

content in the substrate bed. The bed moisture content was near to optimum i.e., 0.55 mL g-ds^{-1} for 4 L min^{-1} and 80% RH operation for the entire fermentation, whereas, this dropped to ~ 0.20 mL g-ds^{-1} (at 48 h) for operation with 4 L min^{-1} and 60% RH. Fig. 6.2 compares biomass and phytase production of *R. oryzae*, which forms abundant aerial hyphae, with isolate F2, which forms sparse aerial hyphae. Extensive formation of aerial hyphae in *R. oryzae* most likely resulted in higher biomass and phytase titre. This was consistent with the findings of Rahardjo et al. [21], where development of aerial hyphae in *Aspergillus oryzae*, early during fermentation, facilitated higher biomass and extracellular enzyme production.

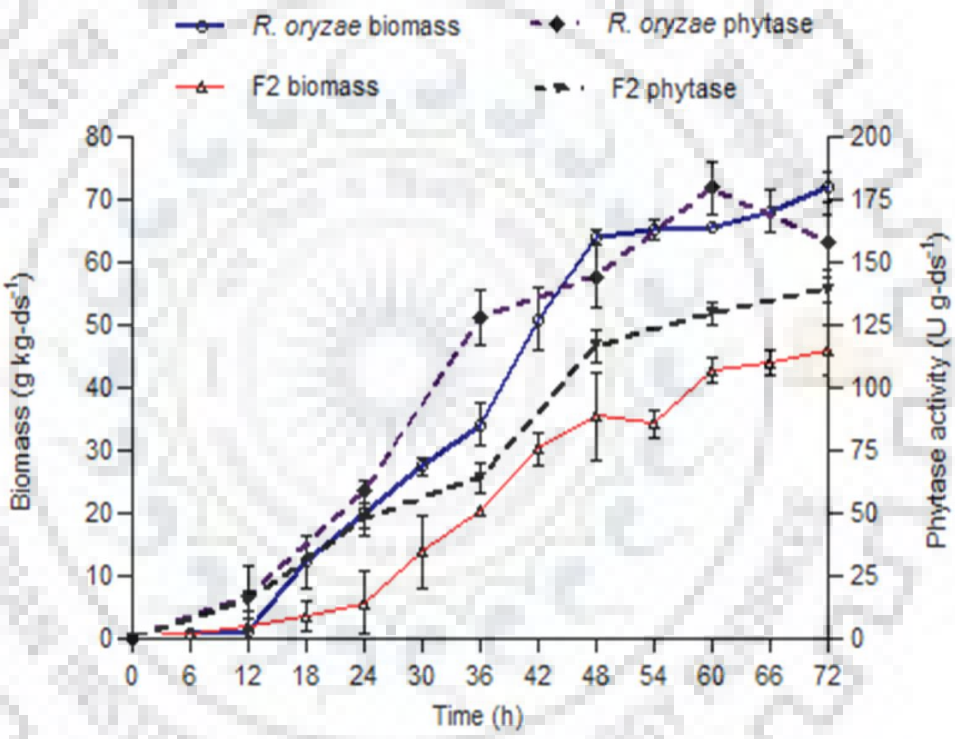

Figure 6.2 Biomass profile of *R. oryzae* and F2 and phytase profile of *R. oryzae* and F2 at 80% RH and 4 L min^{-1} in SSF bioreactor.

6.3.2 Correlation of cell biomass with microbial growth models

Table 6.1 shows the values of coefficients of determination (R^2), for fits between cell biomass profiles and growth models (Eqs. (6.1) to (6.4)) for *R. oryzae* and F2 at different operating conditions of inlet air flow rate and humidity. Logistic, Gompertz and Stannard models fitted to the fungal growth profiles with high coefficients of determination ($R^2 \geq 0.95$). In case of

Schnute, the model yielded a complex value and the fit could not be computed. This was consistent with the observation of Zwietering et al. [22], that it is difficult to calculate 95% confidence intervals for biological parameters if they are not estimated directly in the equation but have to be calculated from mathematical parameters. An investigation with more data points (18), for schnute model, showed a significant correlation (R^2=0.88) with *R. oryzae*, whereas with fewer data points (12), the fit could not be computed.

Table 6.1 Values of coefficient of determination between cell biomass profile, at different experimental conditions with logistic, Gompertz and Stannard model.

Experimental conditions	**Growth model**		
	Logistic	Gompertz	Stannard
R. oryzae (4 L min^{-1} & 80%RH)	0.985	0.985	0.985
R. oryzae (5 L min^{-1} & 80% RH)	0.988	0.993	0.991
R. oryzae (3 L min^{-1} & 80% RH)	0.983	0.989	0.989
R. oryzae (4 L min^{-1} & 70% RH)	0.988	0.99	0.99
R. oryzae (4 L min^{-1} & 60% RH)	0.95	0.972	0.962
F2 (4 L min^{-1} & 80% RH)	0.988	0.99	0.99

6.3.3 Correlation between OUR and cell biomass

Fig. 6.3 shows the OUR profiles of *R. oryzae* under different operating conditions in the SSF bioreactor. To establish a relationship between OUR and X, differential forms of the logistic, Gompertz and Stannard growth models were substituted in Eq. (6.5), individually. Among the three growth models, fitting with logistic model was found to be satisfactory with high coefficient of determination (> 0.90) for all the operating conditions, except for 4 L min^{-1} and 60% RH (R^2=0.88) (Table 6.2). Fig. 6.4 (A-F2) shows the fits of OUR and biomass concentration under different operating conditions in the SSF bioreactor. Moreover, the values for $Y_{X/O}$ and m_o, obtained through logistic fitting, had biochemical significance and were comparable to those reported for fungal microorganisms in other SSF process [15]. The models of Gompertz and Stannard, in contrast to the logistic model, did not satisfactorily correlate

OUR and biomass concentration for all the experimental conditions (Table 6.2). This showed the utility of Eq. (6.7), derived using logistic model, to provide reliable real-time estimates of biomass concentration using OUR data. The correlation obtained (Eq. (6.7)) in this study may be extended and tested on other microorganisms following sigmoidal growth in SSF bioreactor operations for further validation.

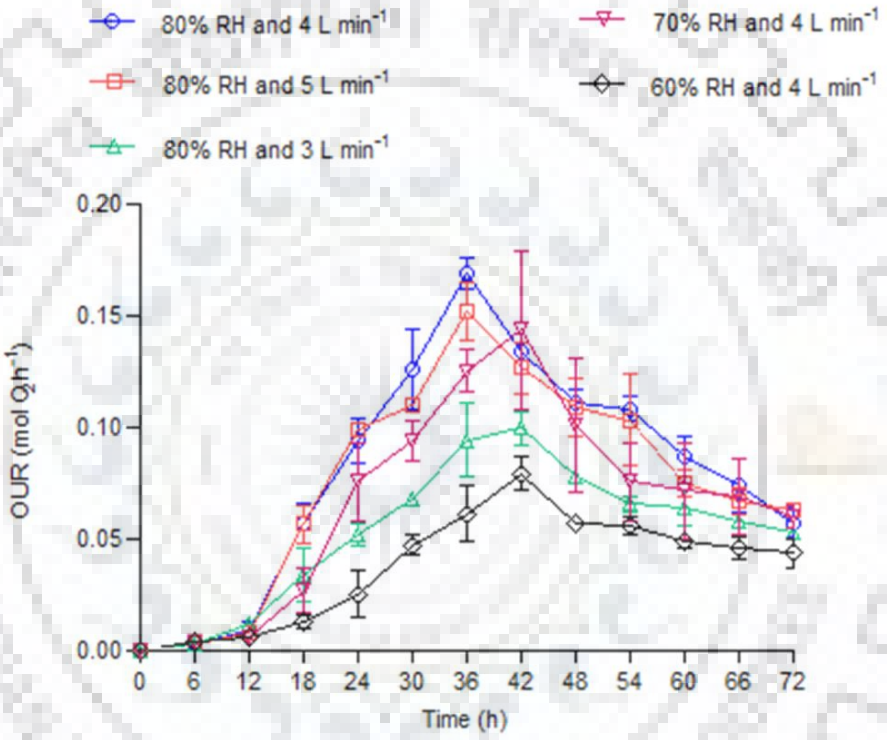

Figure 6.3 Oxygen uptake rate profiles of *R. oryzae*, at different conditions of inlet air flow rate and relative humidity in the SSF bioreactor.

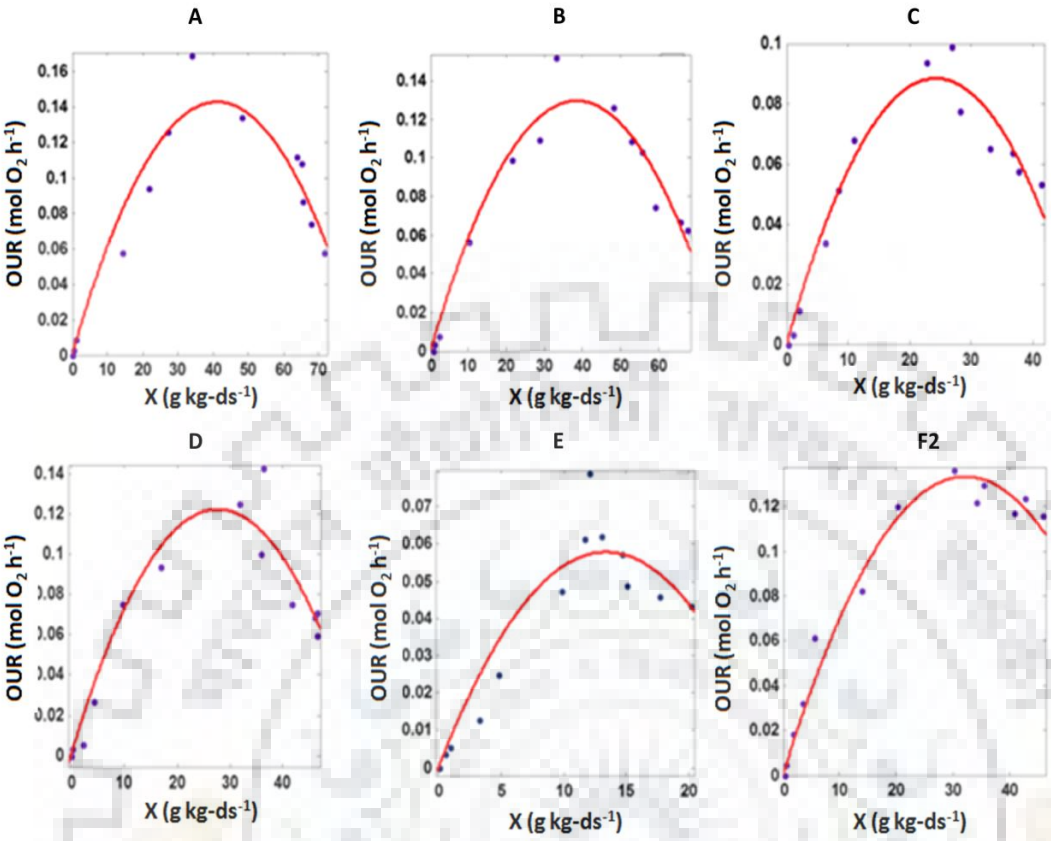

Figure 6.4 Experimental (closed circle) and predicted (solid line) profiles for OUR and biomass production (X) using logistic model approach (Eq. (6.7)). **A.** *R. oryzae* at 80% RH and 4 L min^{-1}. **B.** *R. oryzae* at 80% RH and 5 L min^{-1}. **C.** *R. oryzae* at 80% RH and 3 L min^{-1}. **D.** *R. oryzae* at 70% RH and 4 L min^{-1}. **E.** *R. oryzae* at 60% RH and 4 L min^{-1}. **F2.** Fungal isolate (F2) at 80% RH and 4 L min^{-1}.

Table 6.2 Fitting parameters for Logistic, Gompertz and Stannard model relating OUR and biomass concentration

Experimental conditions	Model parameters								
	Logistic Model			Gompertz Model			Stannard Model		
	$Y_{X/O}$ *	m_{O**}	R^2	$Y_{X/O}$ *	m_{O**}	R^2	$Y_{X/O*}$	m_{O**}	R^2
R. oryzae (4 L min^{-1} & 80% RH)	0.568	2.5×10^{-5}	0.92	0.52	2.8×10^{-5}	0.87	0.50	3.1×10^{-5}	0.92
R. oryzae (5 L min^{-1} & 80% RH)	0.6	2.8×10^{-5}	0.95	0.44	1.8×10^{-5}	0.91	0.50	2.5×10^{-5}	0.89
R. oryzae (3 L min^{-1} & 80% RH)	0.512	3.1×10^{-5}	0.95	0.18	6.2×10^{-5}	0.65	0.46	1.5×10^{-5}	0.79
R. oryzae (4 L min^{-1} & 70% RH)	0.612	3.1×10^{-5}	0.92	0.14	20.6×10^{-5}	0.51	0.35	5.3×10^{-6}	0.73
R. oryzae (4 L min^{-1} & 60% RH)	0.48	6.2×10^{-5}	0.88	0.14	1.8×10^{-5}	0.64	0.09	28×10^{-5}	0.86
F2 (4 L min^{-1} & 80% RH)	0.69	6.2×10^{-5}	0.97	0.60	5.9×10^{-5}	0.73	0.62	3.1×10^{-5}	0.73

* $Y_{Q/X}$ (J g-dry-biomass^{-1})**m_Q (J g-dry-biomass^{-1} h^{-1})

6.3.4 Correlation between metabolic heat generation and cell biomass

To corroborate the use of respiratory data for on-line measurement of metabolic activity, OUR was correlated to metabolic heat generation rate [16], and the latter was then used to estimate biomass concentration and bed temperature. Similar to Section 6.2.2 with OUR, here, differential forms of the logistic, Gompertz and Stannard growth models were substituted in Eq. (6.8), individually. As expected, logistic model was found to satisfactorily correlate metabolic heat generation rate (R_q) and biomass production (X) (Table 6.3) since R_q was directly proportional to OUR (519 kJ/mol-O_2). Gompertz and Stannard models however did not satisfactorily correlate R_q and X for all the experimental conditions.

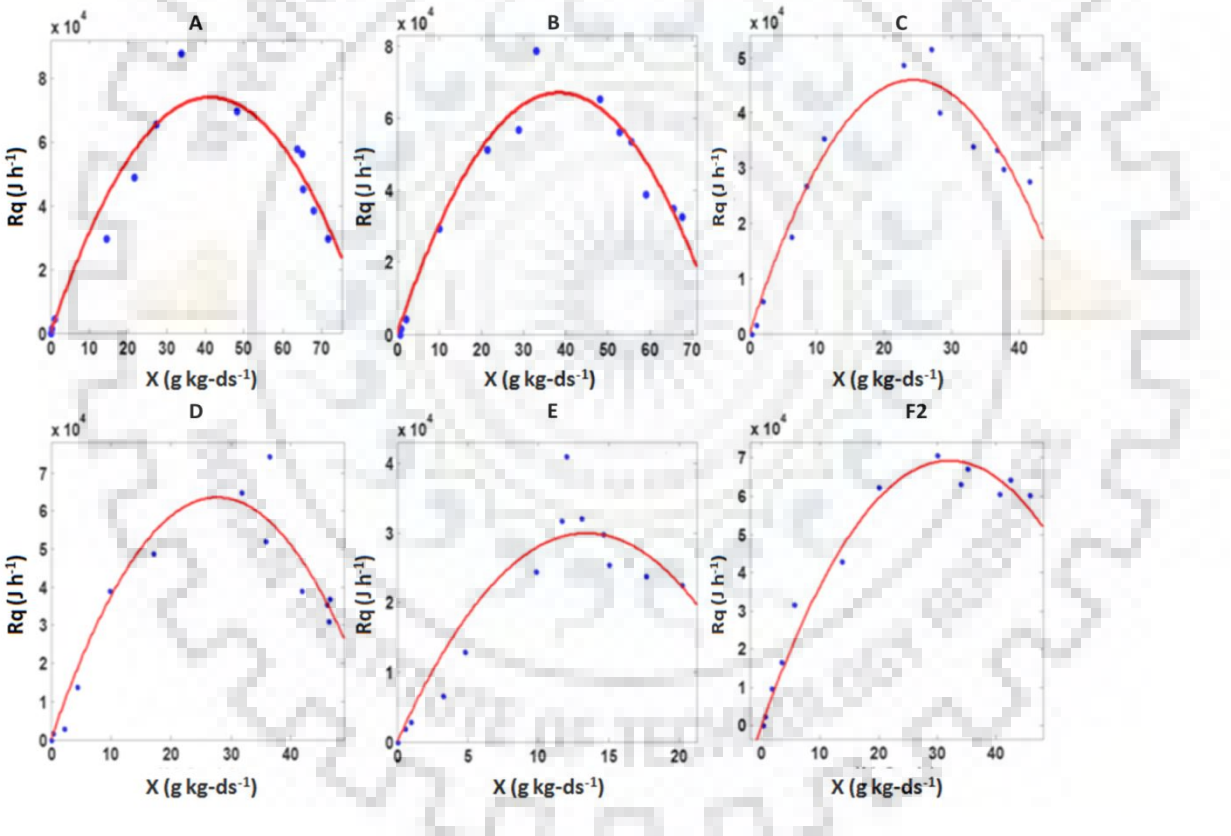

Figure 6.5 Experimental (closed circle) and predicted (solid line) profiles relating metabolic heat generation (R_q) and biomass production (X) using logistic model approach (Eq. (6.9)). **A.** *R. oryzae* at 80% RH and 4 L min^{-1}. **B.** *R. oryzae* at 80% RH and 5 L min^{-1}. **C.** *R. oryzae* at 80% RH and 3 L min^{-1}. **D.** *R. oryzae* at 70% RH and 4 L min^{-1}. **E.** *R. oryzae* at 60% RH and 4 L min^{-1}. **F2.** Fungal isolate (F2) at 80% RH and 4 L min^{-1}.

Fig. 6.5 (A-F2) shows the fitting of R_q and biomass concentration under different operating conditions in the SSF bioreactor. Schiraldi [23] used a similar approach, where, the first derivative of Gompertz model was used to fit the isothermal calorimetric trace obtained from a growing microbial culture, for three organisms; *Saccharomyces cerevisiae*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The fit proved to be statistically relevant for *L. bulgaricus* and *S. thermophilus* and was rather inadequate for the remaining one. The predictive modeling used by Schiraldi was not specifically for solid-state fermentation but for the growth of microbial contaminants in foods, which simulated SSF. This corroborates the fact that predictive modeling tools, such as the one developed in the present study can be used for monitoring metabolic activity.

Table 6.3 Fitting parameters for Logistic, Gompertz, and Stannard relating metabolic heat generation rate and biomass concentration.

Experimental conditions	**Model parameters**								
	Logistic Model			Gompertz Model			Stannard Model		
	$Y_{Q/X}$ *	m_Q **	R^2	$Y_{Q/X}$*	m_Q**	R^2	$Y_{Q/X}$*	m_Q**	R^2
R. oryzae (4 L min^{-1} & 80% RH)	2.89×10^4	0.46×10^3	0.92	2.30×10^4	0.60×10^3	0.80	1.85×10^4	0.70×10^3	0.75
R. oryzae (5 L min^{-1} & 80% RH)	3.02×10^4	0.48×10^3	0.95	2.29×10^4	0.57×10^3	0.79	1.84×10^4	0.66×10^3	0.74
R. oryzae (3 L min^{-1} & 80% RH)	3.17×10^4	0.63×10^3	0.95	2.27×10^4	0.55×10^3	0.40	1.82×10^4	0.70×10^3	0.65
R. oryzae (4 L min^{-1} & 70% RH)	2.67×10^4	0.82×10^3	0.92	2.26×10^4	0.55×10^3	0.36	1.82×10^4	0.73×10^3	0.58
R. oryzae (4 L min^{-1} & 60% RH)	3.45×10^4	1.40×10^3	0.88	2.20×10^4	1.46×10^3	0.60	1.74×10^4	2.20×10^3	0.64
F2 (4 L min^{-1} & 80% RH)	2.38×10^4	1.39×10^3	0.97	2.21×10^4	1.12×10^3	0.72	1.76×10^4	1.24×10^3	0.72

* $Y_{Q/X}$ (J g-dry-biomass^{-1})**m_Q (J g-dry-biomass^{-1} h^{-1})

6.3.5 Bed temperature estimation

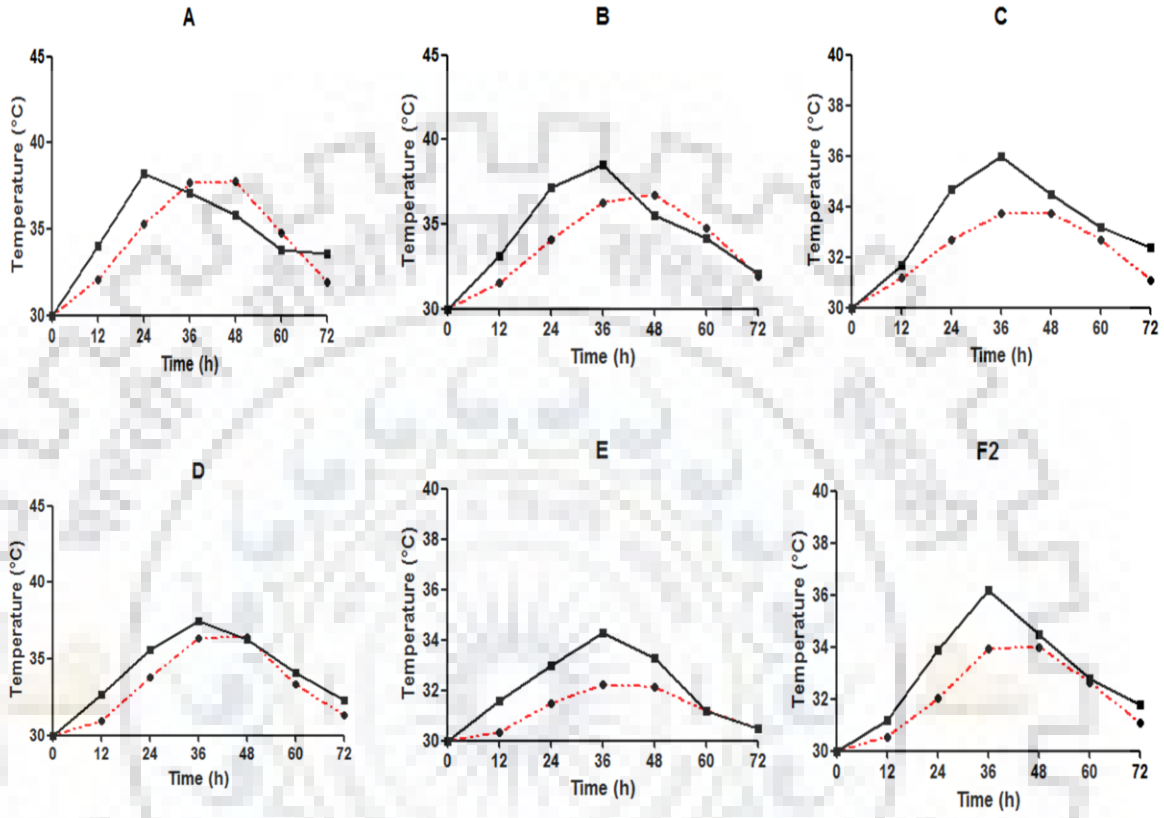

Figure 6.6 Experimental (closed square) and predicted (closed circle) temperature profiles obtained from heat transfer design equation (Eq. (6.10)). **A.** *R. oryzae* at 80% RH and 4 L min^{-1}. **B.** *R. oryzae* at 80% RH and 5 L min^{-1}. **C.** *R. oryzae* at 80% RH and 3 L min^{-1}. **D.** *R. oryzae* at 70% RH and 4 L min^{-1}. **E.** *R. oryzae* at 60% RH and 4 L min^{-1}. **F2.** Fungal isolate (F2) at 80% RH and 4 L min^{-1}.

As evident from Fig. 6.6, experimentally obtained bed temperature profiles correlated well with the predictions of heat transfer design equation (Eq. (6.10)). Percentage error analysis (% error $= \left|\frac{T_{experiment} - T_{predicted}}{T_{predicted}}\right| .100$) showed that the error was less than 10% for all the experimental conditions. The essence of the present work lies in the fact that OUR was related to an equation that gave reliable estimate of biomass concentration in the bioreactor using

predetermined kinetic parameters. The kinetic parameters were, in turn, substituted into a heat transfer design equation which gave a real-time estimation of bed temperature. Here again, experimentally obtained temperature values at 24 and 36 h slightly exceeded the predicted response for most cases (Fig. 6.6 (A-F2)). This behavior was probably due to rapid development of aerial hyphae during the early exponential phase which might have moderately changed the bed transport properties. For instance, an increase in bed density, and decrease in void fraction and bed thermal conductivity, during this period, may have been responsible for deviation from predicted temperature profile. This deviation was however not significant after 36 h, which was most likely due to less abundant aerial hyphae and the mixing action. During the course of fermentation, mixing action may have damaged the mycelia network and also increased the surface area of substrate exposed to moist inlet air, thereby, facilitating temperature control. Experimentally obtained and literature reported values for transport and kinetic parameters used in simulation of Eq. (6.10) are given in Table 4.1

Measurement of bed temperature in most SSF bioreactors involves the use of temperature sensors. However, this may be undesirable in practice for an industrial modular mixed SSF system where hundreds of modules may be stacked since it may not be possible to monitor entire bed due to mixing requirements. Therefore, the present model-based determination of bed temperature may be useful, especially, where it is not possible or undesirable to measure the bed temperature directly.

6.3.6 Applicability of on-line correlations on other fungi

The effect of fungal morphology on the applicability of the correlation between respiratory activity and biomass concentration was studied with a fungal isolate (F2) known to form fewer aerial hyphae than *R. oryzae*. Both Eqs. (6.7) and (6.9), derived from the logistic model, gave high values for the coefficient of determination (R^2), and gave reliable estimates of yield coefficients as shown in Tables 6.2 and 6.3**.** This implied that the proposed correlations might apply to organisms with distinct morphology exhibiting sigmoidal growth; however, extensive testing with experimental data is required before any generalization could be made. Moreover, Eq. (6.9) should also be tested where R_q, the heat production rate, is derived experimentally

through calorimetric studies. Bioreaction calorimetry is a sensitive technique which involves the use of high precision calorimeters that measure thermal power produced as a result of biochemical reactions in biological samples. The technique has been used by few workers [24-26] to correlate biomass activity in fungi. Experimental data derived from such techniques, for different microorganisms, shall be used for further validation of the correlations developed in the present work for future studies. As shown in Fig. 6.7, the oxygen consumption curve for *R. oryzae*, at different operating conditions, peaked during rapid vegetative growth but significantly declined after 36 h. However, with F2, the OUR declined very slightly after peaking at 42 h and remained relatively flat thereafter.

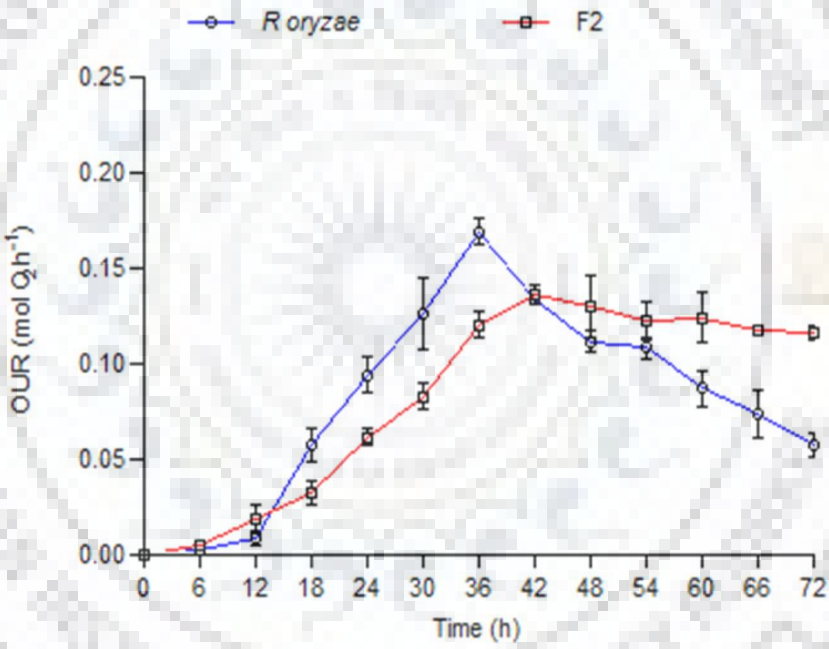

Figure 6.7 Oxygen uptake rate profiles of *R. oryzae* and fungal isolate (F2) at 80% RH and 4 L min^{-1}.

OUR profiles, similar to those obtained for *R. oryzae*, have been reported in previous studies [4, 10, 21, 27] involving *Aspergillus oryzae* and *Rhizopus oligosporus*, which form extensive aerial hyphae. One possible reason for distinct OUR profiles of *R. oryzae* and F2 could be the early appearance of aerial hyphae (12-16 h onwards) in *R. oryzae*. Rahardjo et al. [21]

provided experimental evidence that aerial mycelia account for 75% of oxygen uptake, early during growth, due to rapid diffusion of oxygen in gas-filled spaces between the aerial hyphae and are primarily responsible for biomass and extracellular enzyme production. The extensive network of aerial hyphae in *R. oryzae*, early during fermentation, most likely trapped and condensed the water vapor, in the spaces between aerial hyphae to liquid form which consequently caused a decline in OUR after 36 h. Although there is no visual microscopic evidence of the presence of liquid water between the pores, this may be the likely reason for the decline in OUR.

6.4 Conclusion

A correlation was established between *R. oryzae* OUR and biomass concentration using logistic model. The correlation was tested at different operating conditions of inlet air flow rate and relative humidity in a novel solid-state fermentation bioreactor for phytase production. Use of respiratory data was extended to correlate metabolic heat generation rate and biomass concentration. The correlation derived from the logistic model equation successfully correlated R_q and X. $Y_{Q/X}$, a parameter of the model equation, when substituted in the heat transfer design equation, for the SSF bioprocess, gave reliable estimate of bed temperature. The correlations developed in the present study could be extended on to other SSF bioprocesses for further validation, facilitating in real-time estimation of cell biomass concentration and bed temperature using OUR data.

7 Chapter 7

Concluding Remarks and Future Perspectives

7.1 Concluding remarks

Solid-state fermentation will become of ever increasing importance not only for the production of specialty products [1] but also for solid organic waste treatment [2]. The need to amicably treat the solid waste that we generate will increase as the increasing world population puts pressure on the environmental resources. However, to address both the production of specialty products and treatment of organic waste; we need large scale efficient bioreactors and optimized operating strategies. Chapter 2 discussed in detail on how most current available bioreactor designs neglect many of the relevant observable facts which are important for scale-up and to meet safety and approval from regulatory authorities. There is a dearth of suitable bioreactor designs that can operate both at high substrate bed loading and work under strict aseptic conditions [3, 4]. Moreover, most SSF operations are not backed by design equations which otherwise could guide reactor design and operating strategies. As a result, detrimental bed temperature rise and heterogeneity still impede commercial exploitation of SSF for a broad spectrum of bioprocess applications. The majority of process development still takes place in flasks, columns, and trays with few exceptions of rotating drum bioreactors and other intermittently mixed systems, where mixing events, if any, are most often random and unoptimized. Optimized mixing may potentially enhance the metabolite productivity and reduce the energy input. Moreover, lack of on-line monitoring of microbial activity in SSF systems hinders scale-up and development of suitable control strategies.

In this thesis, a novel solid-state fermentation bioreactor has been proposed which is modular in nature and wherein multiple modules may be stacked around a central pipe. The central pipe not only facilitates the supply of conditioned air into the module but also acts as the shaft

of mixing apparatus. Bioreactor performance was tested for the production of a thermo-tolerant and acid stable phytase by *Rhizopus oryzae* utilizing solid agricultural wastes. Wheat bran and linseed oil cake (1:1) constituted the solid agro-waste and were the sole source of carbon [5]. The bioreactor operated at a high substrate bed loading (59.2%; v/v) and under strict aseptic conditions such that all the fermentation operations could be performed in a single module in a highly contained fashion. In this regard, a heat transfer design equation; incorporating heat transfer due to conduction, convection and evaporative heat loss in axial and radial direction was experimentally validated at different experimental conditions of inlet air flow rate and relative humidity. The equation successfully predicted bed temperature profile and was further subjected to sensitivity analysis. Kinetic parameters and substrate thermal conductivity were found to exert a profound effect on critical bed height in the bioreactor. Phytase production in SSF was compared with that conducted under submerged fermentation (SMF) condition using optimized synthetic growth media in a 0.5 L mini-bioreactor system. Phytase yield in SMF was 15.9 times less than SSF. Bed water activity was optimized ($\geq$0.95) for the phytase production by operating the bioreactor at different inlet air humidity. An inlet air flow rate of 4 L min^{-1} with 80% relative humidity was optimum for the process. Effect of mixing events on bioreactor performance was examined with the objective to facilitate efficient transport and kinetic process. To achieve this, mixing events were designed based on the hyphal bond strength and the respiratory profile of *R. oryzae*. Intermittent mixing at 15 rpm, 3 min at every 6 h was found optimum for the process. With optimized mixing, biomass and phytase productivity increased 2.2- and 4.5-fold, respectively, in comparison to packed bed bioreactor (PBR) operation. Particle size distribution at optimum mixing suggested that bed particle size in the range 1.18-2.36 mm augured maximum biomass concentration. Using mechanistic modeling tools such as the discrete particle simulation [6], this information shall be useful for scale-up of mixing operations. To facilitate on-line monitoring of microbial activity, a mathematical correlation was established between oxygen uptake rate (OUR) and cell biomass concentration. Use of OUR data was extended for determining on-line bed temperature. For this purpose, OUR was related to metabolic heat generation rate and the latter was then correlated with cell biomass. Y_{QX}, a parameter obtained from the correlation between R_q and X, when substituted into the heat transfer design equation

returned reliable estimates of bed temperature. Experimental validation and performance analysis of the proposed bioreactor system strongly encouraged its use for the scale-up of phytase production and also for the development of new SSF processes.

7.2 Future perspective

7.2.1 Scale-up studies

Heat transfer design equation, experimentally validated in Chapter 4, shall be used for scaling up bioreactor operation for phytase production. In this regard, bioreactor configuration, substrate bed loading, and operation strategy shall be governed by the design equation. For mixing events, mechanistic modeling tools such as the soft-sphere discrete particle model, which predicts the forces acting and subsequent motion of individual particle, shall be used to simulate the mixing behavior at large scale. The proposed SSF system backed by appropriate design equations may also be extended for the development of new bioprocess.

7.2.2 Online monitoring of microbial activity

In Chapter 6, correlations were developed between OUR and cell biomass, and metabolic heat generation rate (R_q) and cell biomass, where, R_q was derived from OUR data. The correlations established in Chapter 6 shall be validated with other microorganisms exhibiting sigmoidal growth, and R_q shall be estimated experimentally using a bioreaction calorimeter [7]. Experimental validation of the correlations developed in the present study in various other SSF processes will give more credibility for their use in on-line monitoring and control of SSF bioreactors.

7.2.3 APP coupled with intermittent mixing in proposed SSF Bioreactor

Section 2.2.4 described in detail on the potential of air pressure pulsation and forced air circulation to facilitate efficient transfer of heat and mass within the bed without disruption of mycelia. However, almost all reported studies of APP are limited to fermentation in perforated trays supported in a stainless steel cylinder. This arrangement is similar to tray bioreactor, subject to APP, in a closed environment, which may be undesired owing to low substrate bed

loading and non-sterile conditions. A thought that triggered during the literature review was the prospect of AAP coupled with intermittent mixing at low frequency and intensity in a contained environment. In this regard, provision of a unique design feature was made later in the proposed bioreactor; a variable diameter exhaust vent, with inner aperture, in the upper chamber of the module. The diameter of the exhaust vent could be controlled to generate periods of air pressure pulsation in the substrate bed, while maintaining full sterility. To test the ability of APP to enhance the *R. oryzae* biomass and phytase production, few experiments were conducted in a different setup for SSF. The experimental setup, materials and methodology used, and the results obtained are briefly discussed below.

7.2.3.1 Effect of air-pressure pulsation on phytase production using SSF

To overcome heterogeneity of heat and mass, and enhance the biological activity by outside stimulation; the efficacy of APP was tested for phytase production in a different setup designed for APP-SSF. The fermentation vessel consisted of a plastic jar which had two openings, on opposite sides, to facilitate moist air inlet and exhaust gas outlet. Inside the jar, a perforated Petri plate was placed which contained the fermentation media (Fig. 7.1 A). A bed height of 2 cm was used. A thermocouple was inserted into the substrate bed through the top cap which was tightly sealed with parafilm. Inlet air was passed through a humidifying column and monitored through a hygrometer. The inlet air flow rate, humidity and temperature were maintained at 0.3 L min^{-1}, 80% RH and 30ºC, respectively. The exhaust gases were subjected to gas analyzer after moisture removal. To generate pulsations of air pressure inside the reactor, air mass flow controller of a 1 L mini-bioreactor system (Applikon, Nederlands) was used. In case of no APP, the reactor functioned as a packed bed bioreactor (Fig. 7.1 B). Using SCADA software, a control algorithm (Fig. 7.2 A) was generated which enabled the periodic increase in the inlet air flow rate (into the jar), for 1 min, at set time intervals (Fig. 7.2 B). The exhaust vent was connected to a variable diameter valve and the latter was adjusted so as to generate air pressure pulsation of 0, 10, 25, 40 and 60 mBar respectively, inside the fermentation jar. In this way, five experiments were carried out to examine the reactor performance at five different pulsation pressures (0, 10, 25, 40 and 60 mBar). Pulsation period of 1 min at 20 min interval was initiated during the lag phase (0-12 h)

and then for 12 to 48 h (log phase), a pulsation period of 1 min was activated at every 10 min. Atmospheric pressure was maintained during the non pulsation period (Fig. 7.1 B). A Pressure gauge was installed at the exhaust vent (Fig. 7.1 A). After 48 h, fermented samples were collected from the jar and analyzed for biomass concentration, phytase activity and bed moisture content as described in Section 4.2.5 [8, 9].

Figure 7.1 A. SSF experimental setup for the APP (pulsation event). **B.** SSF experimental setup for APP (non pulsation event).

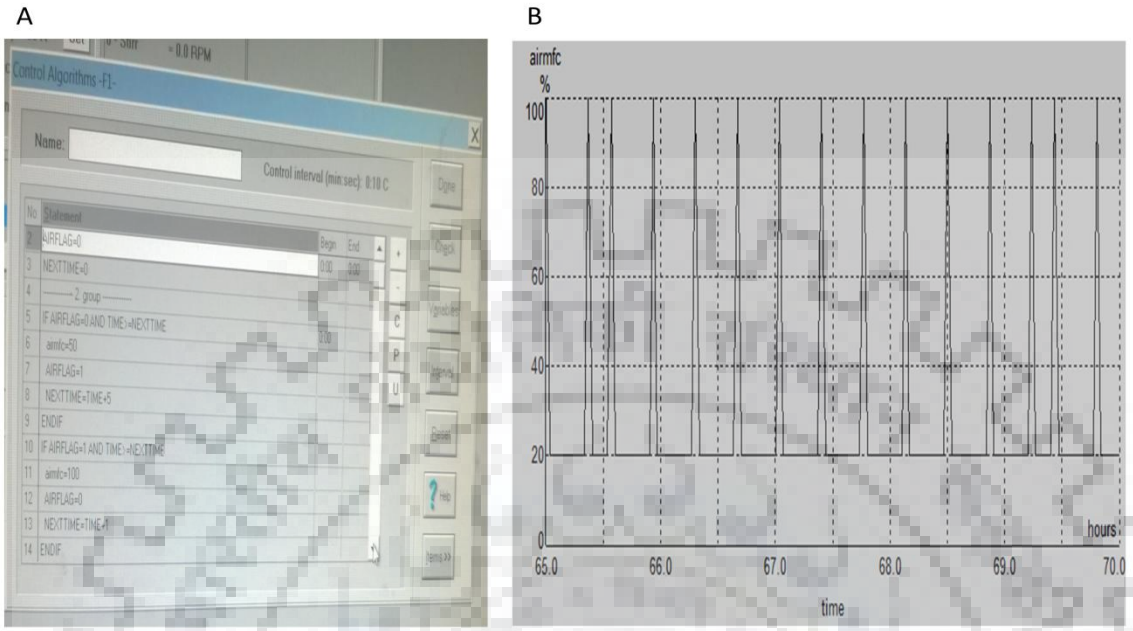

Figure 7.2 A. Control algorithm for APP. **B.** Graphical representation of pressure pulsations at set time interval.

APP significantly enhanced heat and mass transfer and stimulated biological activity. With APP, bed temperature was fairly close to optimum for growth, whereas, the bed temperature reached to almost 40ºC with PBR (Fig. 7.3). For operation with APP, bed moisture also followed a similar suite to bed temperature and remained near the optimum level for the entire fermentation. Pressure pulsation action held the substrate bed loose and therefore simulated mixing action. As a result of better heat and mass transfer with APP, biomass and phytase yield improved (Table 7.1). Biomass and phytase production at air pressure pulsation of 60 mBar increased 1.43 and 1.90 times respectively than PBR. The plastic jar could not sustain further increase in pulsation pressure.

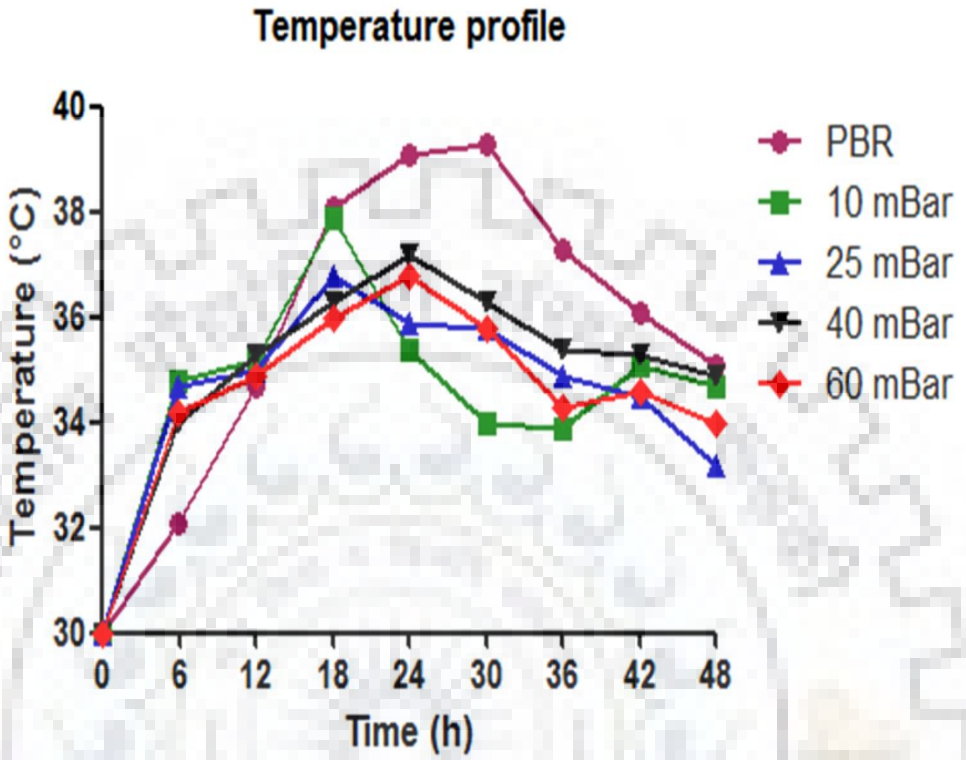

Figure 7.3 Bed temperature profile in PBR and under different operating conditions of APP.

Overall APP had a positive effect on phytase production which also led to few interesting questions. For example, what maximum bed heights could be used with APP? Can intermittent mixing at low frequency and intensity coupled with APP further increase substrate bed loading and reduce the energy requirement. These curiosities shall be answered in the proposed SSF bioreactor in future studies.

Table 7.1 Performance analysis of APP in SSF experimental setup.

Performance parameter	Operating conditions				
	PBR	APP (10 mBar)	APP (25 mBar)	APP (40 mBar)	APP (60 mBar)
Biomass productivity (g kg-ds^{-1} d^{-1})	15.65	18.65	19.38	20.71	22.53
Phytase productivity (IU kg-ds^{-1} d^{-1})	1486.7	2239.44	2360.39	2475.6	2831.8
T_{max} (ºC)	39.1	37.9	36.8	37.2	36.8
Bed moisture content (mL g-ds^{-1})	0.52	0.60	0.60	0.54	0.56

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Appendix

Prior art search report

The novel aspects of the proposed bioreactor design were validated. For this purpose, searches were conducted to include patents and non-patent literature with the global coverage. The search were conducted using various patent databases including Thomson Innovation, USPTO, Espacenet, Google patents, WIPO etc. and non-patent databases like Pubmed, Google scholar, Elsevier and Science direct. The keywords were derived based on the features of present invention. Following are the representative keywords that were used for carrying out the broad search:

Bioreactor, fermenter, SSF, solid state fermentation, stacked modules/disk, modular bioreactor.

Based on the search results, five patents and two non patent literature were obtained which were relevant to the bioreactor design reported in this thesis. The following sections explains in detail how the proposed design differs from each patent and non patent literature (prior art). For detailed information regarding the design and description of the bioreactor in prior art, it is requested to refer to the concerned document, the details of which are mentioned here.

1. **US6197573B1**

Application Number	US 09/193,384
Title	Solid state fermentation
Assignee	Biocon
Inventor	Suryanarayan Shrikumar and Mazumdar Kiran

- In the cited patent (US6197573B1), all modules have been provided with a separate mixing arm connected to two concentric shafts and two independent motors placed outside the modular assembly. **The proposed bioreactor design uses a central pipe**

that not only acts as the shaft of the mixing apparatus, but at the same time also facilitates supply of conditioned air and is a distinguished feature. This also led to substantial increase in substrate loading coefficient (v/v), to 59.2%, in case of phytase production using wheat bran and linseed oil cake as substrates, which is highly unlikely in the cited patent (Chapter 3). Furthermore, the design and mode of operation of a single module in the proposed design is very different from that of the cited patent. The cited patent claims heat transfer through conduction, however, **heat transfer modeling, simulation and validation studies (Eq. 4.1 and Fig. 4.2) in the proposed design provide concrete proof that heat transfer synergistically occurs through conduction, convection and evaporative cooling, which are much efficient than conduction alone.** Furthermore, for the supply of liquids during operation, the proposed design employs spray nozzles which are mounted on the wall of upper chamber in bioreactor modules. The supply of liquids in the cited patent occurs through the communication channels at the bottom of the plate, the same channels that supply conditioned air. When spray nozzles are used for water/moisture supply, the vertical movement of air and horizontal flow of sprayed water result in atomization effect. This atomization results in greater liquid-gas mass transfer area than what is observed in a unidirectional flow of fluids as shall be the case in the cited patent. As a consequence, higher substrate bed loading are possible. This again is a direct manifestation of design the novel SSF bioreactor which is absent in present cited literature.

2. **WO2010032260A1**

Application number	PCT/IN2009/000441
Title	Stacked basket bioreactor for solid state fermentation
Assignee	Kulkarni Sameer Sudhir
Inventor	Kulkarni Sameer Sudhir et. al

The bioreactor design is different in a major way due to presence of a mixing assembly, where, the central pipe acts as shaft for mixing assembly and also facilitates air supply, and is a distinguished feature and is absent in the cited patent (WO2010032260A1). This has direct advantage of increase in substrate bed loading and reduction in heat and mass heterogeneity across bed. The bioreactor in the cited patent resembles more like a packed bed reactor (PBR) which is very different from the proposed design. Packed bed bioreactors, even at low substrate bed loading, are prone to bed compaction and air channeling which negatively affects productivity. Mixing circumvents these problems by increasing the surface area of substrate to conditioned air. Table 4.1 illustrates comparative data, between the proposed design and other designs including PBR and tray bioreactor, in terms of maximum bed temperature (ºC), axial temperature gradient (ºC), maximum biomass concentration (g kg-ds^{-1}), axial biomass gradient (g kg-ds^{-1}), biomass productivity (g kg-ds.d^{-1}), phytase productivity (IU kg-ds.d^{-1}), etc. Optimum mixing (every 6 h for 3 min) was found to reduce temperature and moisture heterogeneity by 60%, increased biomass productivity by a factor of 2.21 and phytase by 4.55, respectively, than PBR.

3. US6620614B1

Title	Compositions comprising Salmonella Porins and uses thereof as adjuvants and vaccines
Assignee	Prophyta Biologischer Lflanzenschutz GMBH
Inventor	Lueth Peter et. al

The design in the cited patent (US6620614B1) is different from the proposed design in the following ways:

Firstly, the bioreactor modules have been stacked around a central pipe which is connected to the upper chamber via revolving plates (attached to mixing blades) and opens up in the lower chamber for supply of conditioned air. The upper chamber also has a provision for supply of fluids through spray nozzles and has a variable size exhaust vent, all of which are absent in the cited patent (US6620614B1).

Secondly, cooling in the cited patent is achieved solely through cooling plates/ coils (i.e., through conduction) below the base which is different from the proposed design and has the following limitations:

a. The mode of heat transfer here is primarily through conduction which is the least contributor to heat transfer.
b. It undermines substrate bed loading of bioreactor.
c. Extra hardware relates to more maintenance, high energy requirement for steam sterilization, hence affects cost effectiveness of the overall process.
d. Differential micro-environment among modules (heterogeneity).

The proposed bioreactor claims to overcome the above limitations, as explained in Section 1 and 2 of this appendix, respectively.

4. WO2013184800A2

Application number	PCT/US2013/044312
Title	Solid State Bioreactor adapted for automation
Assignee	NOVOZYMES BIOAG AS
Inventor	Andersen Claus et al

The proposed design in principle is different from the above cited patent (WO2013184800A2). In the cited patent, it is understood that some (not all) substations (bioreactors) may be arranged in a stacked configuration. **More importantly, it is not modules, but whole bioreactors, that may be stacked. However, in the proposed**

design the central pipe traverses through modules, acting as shaft for mixing operation and also facilitates air supply, and therefore, all modules are in fact stacked and this stacked system comprises the bioreactor. Therefore, the bioreactor proposed in this thesis is truly modular in nature. Furthermore, the water sparger or nozzle appears to be centrally located in the cited patent, whereas, in the proposed design it is located along the walls of the bioreactor. All these features results in higher substrate bed loading rates in proposed design.

In the cited patent (WO2013184800A2), the arrangement of mixing assembly is different from the proposed design in following ways

- First, a revolving plate is connected to the shaft via a gear system, to which mixing blades of different shapes may be attached (Fig. 4.3).
- Second, the position of these blades is different to that in the cited patent (WO2013184800A2), where, the agitator blades appear to cut the substrate across a single plane. However, in the proposed design, the mixing blades cut the substrate perpendicular to it and not across a single plane (Fig. 4.4).

In view of the above clarifications with regard to differences with present cited patent (WO2013184800A2), there are differences in design especially with respect to modularity, arrangement of modules around the central pipe, mixing assembly etc. However enough experimental data is not available for the cited patent to further compare the performance of bioreactors.

5. US8222026B2

Application number	US12/258,180
Title	Stacked array bioreactor for conversion of syngas components to liquids products

Assignee	Tsai Shih-Pering et. al
Inventor	Tsai Shih-Pering et. al

Apart from the axially stacked nature of modules of the bioreactor in the cited patent (US8222026B2), the design and operation of the bioreactor proposed in this thesis is entirely different.

Non-patent literature

1. **Current industrial practice in solid state fermentation for secondary metabolite production: the Biocon India experience**

Authors	Shrikumar Suryanarayan
Source	Biochemical Engineer Journal, Volume 13, Issues 2-3, Pages 189-195
Publication year	March 2003

The differences and advantages of the proposed design have already been mentioned in Section 1 of the patent literature.

2. **Design aspects of bioreactors for SSF: A review**

Authors	H. Kh. Q. Ali and M.M.D. Zulkali
Source	Chem. Biochem. Eng. Q. 25 (2) 255-266
Publication year	2011

The design of bioreactor designs as described in the above paper (Design aspects of bioreactors for SSF: A review) is very different from the proposed design as already explained above. Tray and packed bed bioreactor mostly do not operate aseptically, are prone to heat accumulation and heterogeneity and operate at low substrate bed loading rates. As shown in Table 1, all these drawbacks have been critically analyzed and have been taken care of in the proposed design of SSF bioreactor.

From the above results and clarification, it is evident that bioreactor design proposed in present study is very different from the ones that are reported in literature. The stacked nature of modules around a central pipe, which not only facilitates the supply of conditioned air but also acts as shaft of the mixing apparatus is a distinguished feature and is not present in any of the existing designs. This feature along with the design of individual module significantly increases the substrate bed loading. Furthermore, the design of individual module with its arrangement of mixing apparatus, nozzles, variable diameter exit vent etc. is very different from prior art.