FUNGAL CHITOSAN PRODUCTION USING AGRICULTURAL AND INDUSTRIAL WASTES AND HEAVY METAL REMOVAL USING CHITOSAN DERIVED NANO-BIOSORBENTS

A Thesis

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

DOCTOR OF PHILOSOPHY

by

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श्री कृष्ण अर्पणमस्तु

Dedicated to my parents

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DECLARATION

I, hereby declare that the content embodied in this thesis entitled "**Fungal chitosan production using agricultural and industrial wastes and heavy metal removal using chitosan derived nano-biosorbents**" is the result of investigations carried out by me at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of **Prof. Kannan Pakshirajan.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: **M M Tejas Namboodiri**

Place: IIT Guwahati

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Fungal chitosan production using agricultural and industrial wastes and heavy metal removal using chitosan derived nano-biosorbents"** by **M. M. Tejas Namboodiri** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted either in whole or in part elsewhere for a degree.

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ABSTRACT

Chemical extraction methods are currently used widely to derive chitin and chitosan from marine sources is the widely used and accepted route of their production. However, harsh chemical treatments for demineralization and deproteinization result in hazardous wastes and undesired by-products. Moreover, variation in properties of the derived chitosan is a serious drawback of chemical extraction of chitin. On the other hand, fungi contain both chitin and chitosan within their cell wall, which, therefore, negates the harsh demineralization step required in the case of marine sources. Fungi are also known to utilize industrial and agricultural wastes as substrates for their growth, which help in reducing the costs involved in chitosan production. Moreover, properties of the fungal chitosan are consistent and can be controlled by a proper choice of cultivation conditions.

The present thesis aimed at sustainable fungal chitosan production by utilizing different inexpensive raw materials and wastes as substrates.

Two different fungal strains, *Cunninghamella elegans* and *Penicillium citrinum,* were evaluated for their ability to utilise organics present in different wastewater and produce chitosan. Whereas *C. elegans* has been reported earlier for chitosan production, *P. citrinum* was isolated in the study from bamboo shoot and cultured on potato dextrose agar plates. The fungal isolate was later identified based on the sequence variation present in its 18s rRNA which revealed 99% similarity to the genus *Penicillium citrinum*. Domestic, paper mill, and dairy wastewater, were examined as substrates for chitosan production by *C. elegans* and *P. citrinum* which revealed that paper mill wastewater served as very good substrate for *P. citrinum*, and the values of chitosan production was better compared with domestic or dairy wastewater. Wastewater supplementation

with mineral salt media (MSM) containing ammonium chloride as the N-source enhanced the COD removal from paper mill wastewater and chitosan production by *P. citrinum.*

Acetic acid was added at varying concentrations of 20, 50, 80, 100 and 150 mg/L to paper mill wastewater supplemented with MSM in order to study its effect on chitosan production by *P. citrinum*. Addition of acetic acid at 50 mg/L resulted in a maximum chitosan production and COD removal from the wastewater. A similar trend was observed for phenolics present in the wastewater, and in which case ~70% removal efficiency was achieved due to acetic acid addition.. Among the various kinetic models analysed in the present study, Haldane model was found to accurately fit the data with a very high coefficient of determination value (R^2) greater than 0.9 for chitosan production rate by *P. citrinum.* A maximum chitosan production rate of 2.33 mg/L·h was observed with the addition of 50 mg/L acetic acid. The positive effect due to acetic acid addition on the utilization of xylose present in the paper mill wastewater was further confirmed by analysis of XR and XDH enzyme activities.

Fermentation using a bioreactor operated under controlled conditions of temperature, pH, agitation and aeration rate, enhanced COD removal efficiency upto 75% and maximum chitosan production upto 117 mg/L with *P. citrinum*. Fed-batch experiments with the bioreactor resulted in a COD removal of ~70% during the initial 48 h followed by a drastic reduction in COD during the feeding stage. Chitosan production measured up to 160 mg/L during the fed-batch operation and a maximum COD removal of ~ 90% was achieved at the end of 72 h. Moreover, maximum reduction of 86% of phenolics present in the wastewater was achieved after 72 h of fed-batch mode of fermentation along with about 89% decolourization of the paper mill wastewater was achieved using the *P. citrinum* biomass. Under continuous mode of operation with the bioreactor, maximum

chitosan production of ~170 mg/L was observed. However, the COD removal efficiency (83%) was low when compared with the value obtained under fed-batch mode.

Agro-industry wastes, viz. rice straw (RS), lime peel and paper mill waste sludge (PMWS) were evaluated as solid substrates for chitosan production by *P. citrinum*. Microwave alkaline pre-treatment further enhanced utilization of RS by the fungi. Chitosan production by the fungal isolate on rice straw was investigated by varying the moisture content (50-80%) and particle size. An increase in the moisture content enhanced the fungal growth and chitosan production. Optimum conditions of these parameters were found to be 70% and 4-5 mm, respectively. A lab scale tray fermenter with three trays (12cm×6.5cm) was later employed to carry out the solid state fermentation under controlled condition of relative humidity (65%), which yielded a maximum chitosan production of 9 g/kg of rice straw. The results were similar with paper mill sludge as the substrate, and a maximum chitosan yield of 8.5 g/kg paper mill sludge was achieved at a relative humidity of 60%. MSM containing paper mill wastewater in different concentrations (5%, 10%, 15%, 20% and 25%) were added as a supplement to paper mill sludge further enhanced the chitosan yield up to 10.6 g/kg substrate.

Application of biosorbent based on the fungal chitosan produced in the study was evaluated for heavy metal removal from aqueous solution. Magnetic iron (Fe_3O_4) nanoparticles were first prepared by the co-precipitation method, and later these nanoparticles were coated with chitosan and carboxymethyl chitosan (CMC) derived from the fungus. CMC was prepared by carboxymethylation of chitosan to form N-substituted CMC and tested for removing Cr(VI) and Pb(II) from aqueous solutions. Coefficient of determination (R^2) values for the pseudo-second order kinetics are comparatively high (R^2>0.99) for both the metals and with the two biosorbents.

These results confirm that the rate limiting step for bio-sorption of Cr(VI) and Pb(II) species by CMC nanoparticles is governed by the pseudo second order model which is based on chemisorption. The Langmuir isotherm model gave the best fit for Cr(VI) and Pb(II) biosorption onto CNP at 303 K temperature with theoretical maximum sorption capacity (Q_{LM}) of 30 mg/g and 100 mg/g, for Cr(VI) and Pb(II), respectively. In the case of CMCNP as the biosorbent, Freundlich model gave the best fit with R^2 values of 0.991 and 0.998 for for Cr(VI) and Pb(II). Based on the sorption-desorption results, it could be said that the nano biosorbents could be safely reused for up to 3-4 times without significant loss in metal removal efficiency. This study established an excellent potential of the cheaply produced biosorbent for heavy metal removal from wastewaters.

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Abbreviations

AIM: alkali insoluble material

CMC: carboxymethyl chitosan

CMCNP: carboxymethyl chitosan nanoparticle

CNP: chitosan nanoparticle

CO: carbon monoxide

COD: chemical oxygen demand

CTAB: cetyl trimethylammonium bromide

DD: degree of deacetylation

DNA: deoxyribonucleic acid

D-R Dubnin–Radushkevich

EDTA: Ethylenediaminetetraacetic acid

FESEM: field emission scanning electron microscope

FETEM: field emission transmission electron microscope

GlcNAc: N-acetyl glucosamine

ITS: internal transcribed spacer

LPCB: lactophenol cotton blue

MOs: microorganisms

MSM: mineral salt media

PCR: polymerase chain reaction

PDA: potato dextrose agar

PMS: paper mill sludge

PS: particle size

RSH: rice straw hydrolysate

SmF: submerged fermentation

SSF: solid-state fermentation

STR: stirred tank reator

rRNA: ribosomal ribonucleic acid

R-P Redlich-Peterson

RS: rice straw

TSS: total suspended solids

UDP-GlcNAc: Uridine-diphospho-N-acetyl glucosamine

UV-Vis: ultraviolet-visible

XDH: xylose dehydrogenase

XR: xylose reductase

XRD: X-ray diffraction

YPD: yeast peptone dextrose

Notations

d: day	μM: micro mol
°C: degree centigrade	μ: specific growth rate
g: gram	μ_{max}: maximum specific growth rate
g/L: gram per liter	R^2: regression coefficient
h: hour	rpm: rotations per minute
min: minute	s: second
mL/min: milliliter per minute	v/v: volume/volume
mg/L: milligram per liter	w/v: weight/volume
C_0: the initial concentration of adsorbate	k_1: pseudo-first order rate constant
C_t: final concentration of adsorbate	k_2: pseudo-second order rate constant
k_{id}: rate constant for intra-particle diffusion	Q_{LM}: maximum adsorption capacity
L: Langmuir isotherm constant	F: Freundlich constant
Q_m: Dubinin-Radushkevich maximum bio-sorption capacity	K_{DR}: the mean free energy of bio-sorption
ε: Polanyi potential	R: universal gas constant
T: temperature	K_{FK}: Frumkin constant
θ: fractional coverage of sorption sites	α_{FK} = interaction parameter for sorbate-sorbent interaction
Q_{RP}: Redlich-Peterson constant	K_{RP}: Redlich-Peterson constant

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Cellulose and chitin are the most abundant and naturally available biodegradable polymers discovered earlier to cellulose. However, industrial application of chitin and its derivative chitosan could not be realised mainly owing to the economics of its production (Roberts, 2008). Chitin is a polysaccharide made up of β (1,4) linked 2-acetamido-2-deoxy-β-D-glucose, also known as N-acetylglucosamine monomer units. Removal of acetyl groups from chitin leads to the formation of chitosan, which is chemically 2-deoxy-β-D-glucopyranose. Chitosan can be chemically and enzymatically modified due to the presence of free amine group resulting after the deacetylation step. Polycationic nature of the chitosan renders it soluble in acids. These properties of chitosan enable its availability in several forms, such as flakes, beads, powder, membranes, gels, sponges and fibers. It has been used for enzyme immobilization, as a food additive and as an anticholesterolemic, for wound healing and in pharmaceuticals for drug delivery (Amorim et al., 2003; Muzzarelli et al., 1999). Both chitin and chitosan have been used as adsorbents for dye removal from industrial wastewater (Boardman et al., 2017; Mo et al., 2018; Sakkayawong et al., 2005). Owing to its high charge density, reactive amino groups, anti-microbial activity, biocompatibility and biodegradability, chitosan is used in diverse areas such as environmental, food and beverage, pharmaceutical and agricultural sectors (Aranaz et al., 2009; Hadi, 2013; Muzzarelli, 2009; Ngah et al., 2011; Pitakpoolsil and Hunsom, 2014; Rinaudo, 2006).

Till date, the major source of chitosan production is based on chitin obtained from the shell of crustaceans, which is a waste from the seafood industry. Chitin extraction from crustacean wastes involves a combination of concentrated alkali and acid treatments, which, however, results in discharge of highly hazardous wastes. Moreover, seasonal as well as limited availability of the marine source and highly variable property of chitin associated with it are other major drawbacks of the conventional chitin/ chitosan production method. Furthermore, chitosan obtained from marine sources require a series of downstream operation steps to attain the standard commercial quality for its application at an industrial scale. Compared with the marine sources fungi seem to be more attractive to the production of chitosan. Also, inexpensive feedstock for the fungal chitosan extraction may help keep the process economically viable.

Worldwide concerns regarding water resource scarcity have led to the formulation of strict environmental regulations and due to which sustainable utilization and management of water in industries, such as paper mill, is imperative. Approximate water consumption in paper mills is estimated to be 100-250 m^3 per tonne of paper produced along with a huge amount of sludge (Birjandi et al., 2013; Tewari et al., 2009), and about 80-90% of the input water is released into the environment following its treatment, even if the effluent standards are not met in some cases (Hong and Li, 2012; Kamali et al., 2016; Wiegand et al., 2011). Technological advances directed toward sustainable process development have resulted in the reduction of waste generated as well as recovery of resources from waste (Asghar et al., 2008). For example, black liquor is concentrated and burnt to produce a smelt of sodium carbonate and sodium sulphide, which is used for the separation of lignin from cellulose in the industry (Sainlez and Heyen, 2013). The paper mill sludge has been employed for various resource recovery operations such as, vermicomposting (Elvira et al., 1996), biogas and methane production (Kamali et al., 2016; Veluchamy et al., 2018) and energy recovery (Zhang et al., 2010). In general, physico-chemical treatment methods have been applied for the treatment of such industrial effluent prior to its discharge into the environment (Ashrafi et al., 2015). On the contrary, biological treatment involving fungi or bacteria is of great importance in this regard due to its low cost and efficient removal of organics present in such industrial wastewaters. Furthermore, owing to its ability to produce a wide array of extracellular enzymes, fungi seem to be highly suited for biological treatment of effluents. In addition, certain fungi are capable of producing chitin in their biomass, which is of commercial importance as it could be converted to chitosan. An integrated process to simultaneously utilize both paper mill sludge as well as wastewater for chitosan production for achieving sustainable fungal chitosan production would be an attractive area to focus on.

Global production of rice straw is in the range of 800-1000 million metric tons per year, out of which Asia accounts for almost 80% of the total production rate (Satlewal et al., 2018). India is the largest producer of rice in South Asia with an average production of ~153 metric tons per year which results in 195.6 million metric tons per year of rice straw. Only 20% of rice straw generated is utilized for bioethanol production or as animal fodder and almost 80% is burnt in the open or utilized for mulching by farmers for cultivating subsequent crops in the year. In most of the cases, environmental awareness and shortage of labour are the major reasons why the farmers resort to such ineffective methods of straw disposal. Open burning of 1 ton of rice straw results in emission of 34.7 kg of carbon monoxide (CO) along with 3.1 kg NO_X, 0.7 kg of SO_X, and 3.7 kg of PM-10 (Kadam et al., 2000). These emissions exacerbate the already deteriorating air quality across the world resulting in various health risks especially respiratory ailments. Rice straw management is, therefore, a critical area of focus not only for a developing country like India, but also across Asia and other parts of the world (Streit et al., 2009).

On the other hand, fermentation of rice straw is very interesting as it is composed of cellulose (~40%), hemicellulose (~20%) and lignin (~12%) which are bound together by covalent and hydrogen bonds. Another important advantage of using rice straw as a feedstock for fermentation is that the percentage of lignin, which is considerably difficult to degrade by microorganisms, is very low in rice straw among all other lignocellulosic feedstocks. The cellulose and hemicellulose component of rice straw is inaccessible to enzymes produced by the microbes due to the lignin acting as a binding agent among these fibres.

Fungal biomass can be produced by either solid-state fermentation (SSF) or submerged fermentation (SmF). SmF is advantageous as it facilitates easy control of fermentation parameters, such as pH, temperature and nutrient concentration in the fermentation medium (Arcidiacono and Kaplan, 1992). SSF, however, is known to produce larger quantities of biomass than SmF. Recently, studies have been carried out on utilization of inexpensive carbon sources, such as bio-waste for culturing fungi for chitosan production (Berger et al., 2014; Cardoso et al., 2012; Khalaf, 2004; Nwe et al., 2002; Ray and Ghangrekar, 2016; Streit et al., 2009; Tai et al., 2010; Zamani et al., 2007). However, there is still a need to explore more such cheap and novel substrates for fungal chitosan production to keep the production cost low. Fermentative production of chitosan by culturing fungi on inexpensive bio waste means searching for a plentiful and economical source. Considering the significant amounts of fungal-based waste materials accumulated from biotechnological and pharmaceutical industries and the cost involved in managing the wastes, extraction of highly functional value-added products, such as chitosan may provide a lucrative solution to these industries.

Hydroxyl and amino groups on chitosan are involved in binding with various dyes and heavy metal ions from solution, which make it an excellent biosorbent for removing these pollutants from wastewater. Owing to its sensitivity towards pH, several studies have cross-linked chitosan with other compounds such as polyurethane, activated clay, poly vinyl alcohol etc., to improve its sorption capacities (Chang and Juang, 2004; Lee et al., 2009; Zhu et al., 2010). Fungal chitosan based biosorbents for heavy metal removal, therefore, is an attractive option for its application at industrial scale.

1.2. Aim and objectives

The main aim of this study is fungal chitosan production using cheaply and abundantly available waste substrates.

To accomplish this aim, the following objectives were formulated:

1) Screening of different fungal species for chitosan production
2) Submerged fermentation of wastewater from different sources for fungal chitosan production
3) Solid state fermentation of agro-industrial wastes for chitosan production
4) Application of fungal chitosan and its conjugates as aa biosorbent for heavy metal removal from aqueous solution

1.3. Organization of thesis

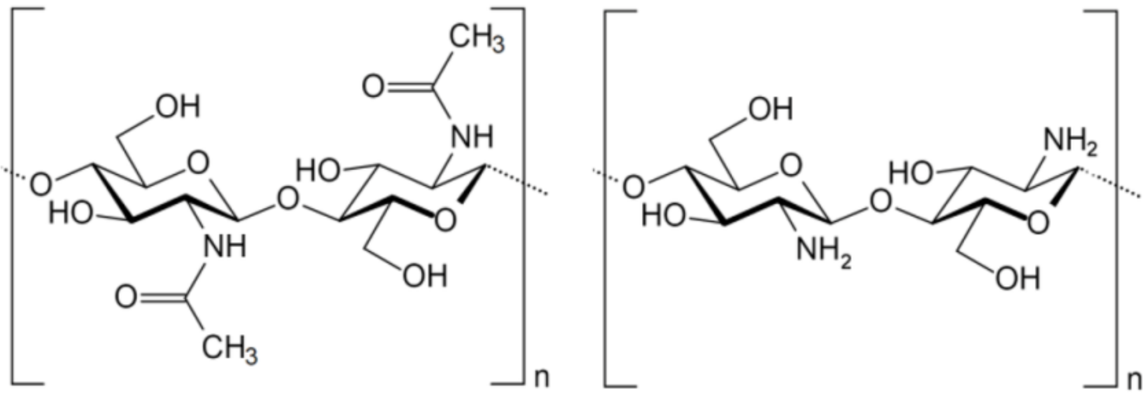
The present work has been divided into seven chapters. The first chapter gives a general introduction, aim and objectives of this work. Chapter 2 presents the introductory literature review of the work carried out, which provides an overview of chitin, chitosan, its properties and applications; metabolic pathway of chitin and chitosan; sources of chitin, viz. crustaceans, insects and fungi; extraction methods and bioreactor configurations for chitosan production. Details of procedure followed for screening and optimization of substrates for chitosan production using different wastewaters and agro-industrial substrates, induction effect of acetic acid along with kinetics and bioreactor studies have been entailed in Chapter 3. It also provides the methodology fungal chitosan based nano-biosorbents and describes heavy metal sorption kinetics and isotherm investigations. Chapter 4 deals with the results of fungal chitosan production from different wastewater and hemicellulose hydrolysates, particularly paper mill wastewater, mechanism of induction by acetic acid with kinetics and bioreactor studies. Results of solid state fermentation of agro-industrial wastes, such as rice straw and paper mill sludge were further discussed in this section along with the application of fungal chitosan based nano-biosorbent for heavy metal removal from aqueous solution. Chapter 5 draws summary and appropriate conclusions based on this work. This chapter also provides some useful recommendations for future research in the relevant area.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Cellulose and chitin are the two most abundant naturally available biodegradable polymers. Despite its earlier discovery than cellulose, industrial application of chitin could not be realised mainly owing to the economics of its production (Roberts, 2008).



Chitin **Chitosan**

Fig. 2.1 Structure of (a) chitin and (b) chitosan

Chitin is found in crustaceans, mollusca, fungi, roundworms and insects, of which the cuticle and external are of major importance. It is a polysaccharide made up of β (1,4) linked 2-acetamido-2-deoxy-β-D-glucose, also known as N-acetylglucosamine monomer units (Fig. 2.1). Chitin occurs in three forms- α, β and γ chitin based on their degree of hydration, unit cell size and chitin chains per cell (Fig.2.2) (Carlström, 1957). Among these forms of chitin, α-chitin is the only extractable and most abundant form, occurring in crustaceans and cell wall of fungi. The removal of acetyl groups from chitin leads to the formation of chitosan, which is chemically 2-deoxy-β-D-glucopyranose. Till date, the major source of chitosan production is chitin, obtained from the shell of crustaceans, which is a waste from the seafood industry. The annual chitin production is estimated to be around 10^{10}-10^{11} ton (Gortari and Hours, 2013; Nair and Dufresne, 2003). Extraction of chitin from crustacean wastes involves mechanical pre-treatment for size reduction by grinding, followed by demineralization followed by deproteinization and removal of pigments (decolouration). Moreover, seasonal availability of the marine source and the highly variable property of the chitin are the major drawbacks of crustacean wastes as the feedstock for chitin and chitosan production and their application.

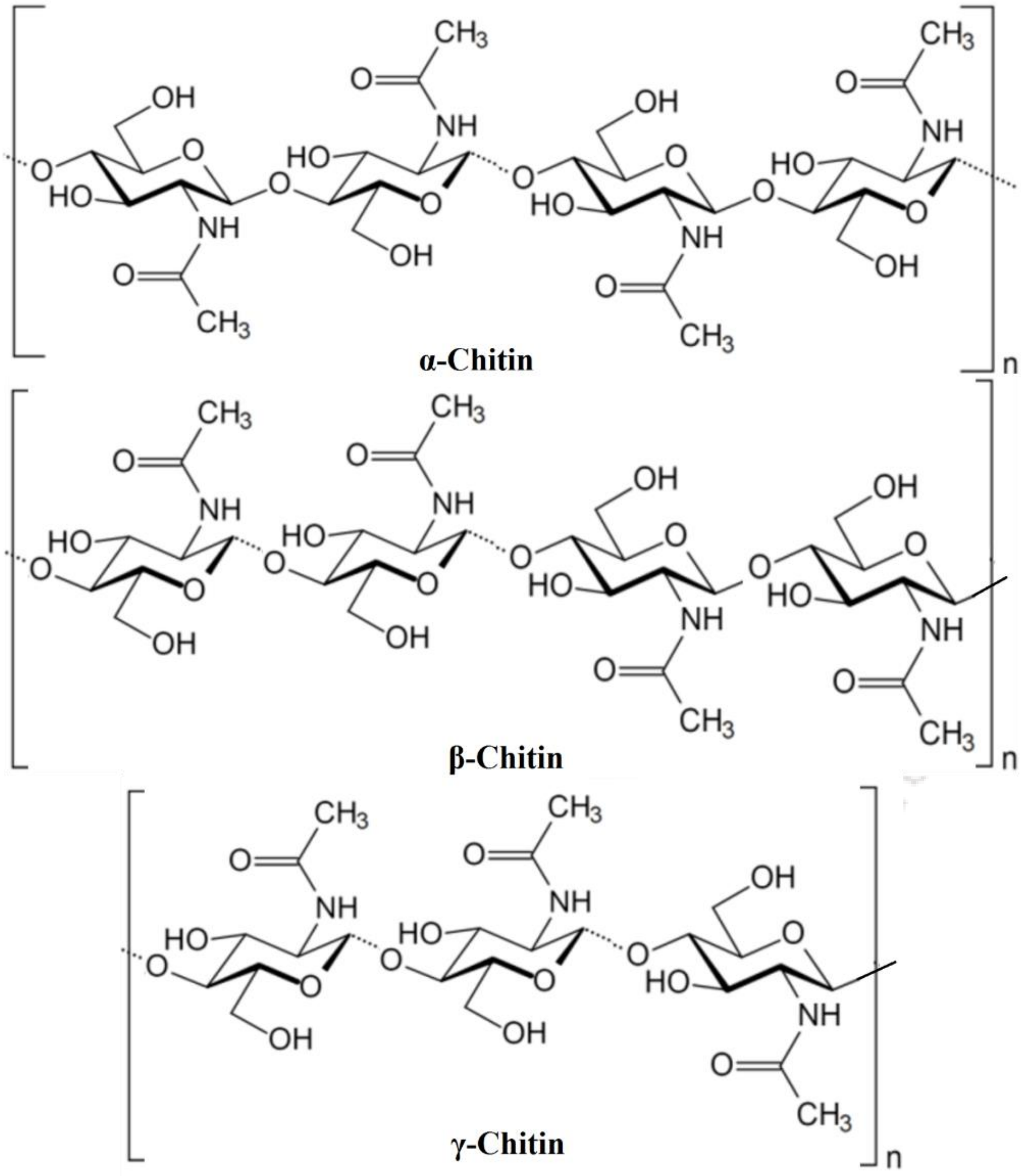


Fig. 2.2 Structure of different forms of chitin (Rufato et al., 2018)

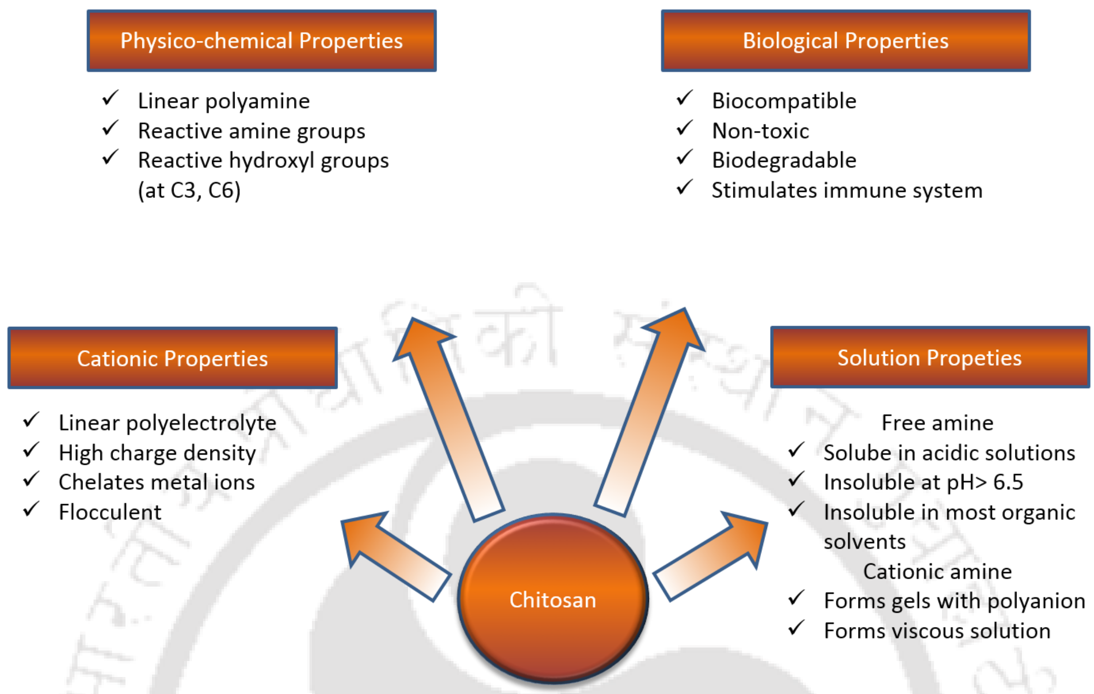
The cell wall of fungi contains chitin as one of its principal components, as it is highly essential for maintenance of the cell structure (Ruiz-Herrera, 2016). The zygomycetes class of fungi contain abundant chitosan than chitin in their cell walls. Along with chitin, glycoproteins and glucan make up the fungal cell wall. Chitin and glucans (both β-1,3 and β-1,6-glucans) form the structural component whereas the glycoproteins (glucoronoproteins, galactoproteins, mannoproteins, etc.) form the interstitial components. Thus, an intricately complex network is formed due to the cross-linking of chitin, glucan and gluconoproteins, thereby providing structural stability to the fungal cell wall (Bowman and Free, 2006).

Owing to its unique properties, chitosan is widely used in agriculture, environment and pharmaceutical sectors as discussed in the next section. Therefore, there is an increased interest toward developing an efficient and economical process of chitosan production to meet the market demands which were estimated to reach 40,465 metric tonnes per year by the end of 2018 (Philibert et al., 2017). This chapter reviews the literature on various sources and routes of chitin and chitosan production along with alternative technologies which could be employed to achieve a sustainable way of chitin and chitosan extraction.

2.2 Chitosan- properties

Due to the diverse properties of chitosan, it has been studied extensively for various applications as listed in Fig 2.3.

(a)



(b)

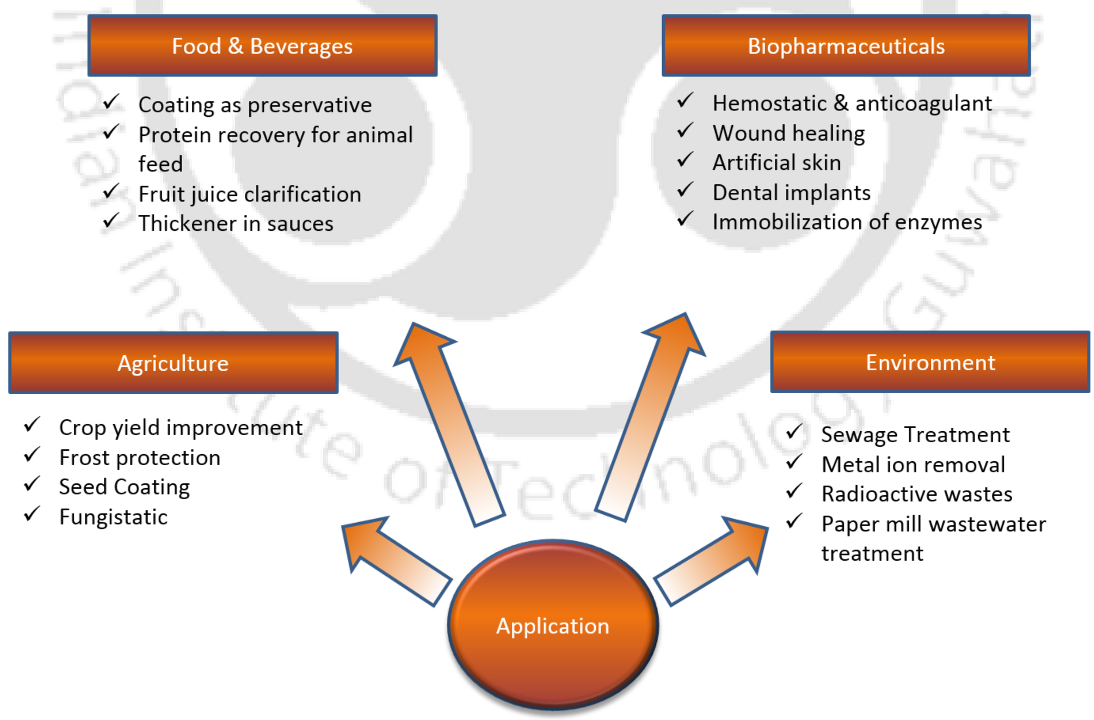


Fig. 2.3 Chitosan- (a) properties and (b) applications (Kumar, 2000; Rinaudo, 2006)

2.2.1 Physico-chemical

Chitosan is a linear polyamine with reactive amino and hydroxyl groups. The presence of these groups is responsible for its solubility in acids and its availability in various forms such as flakes and gels. Degree of deacetylation and molecular weight are other two important parameters which determine the application of chitosan in different fields. Both these parameters are influenced during the conversion of chitin to chitosan, resulting in changes in charge distribution, which in turn affects the agglomeration. The average molecular weight of chitin and chitosan are $1 \times 10^6 - 2 \times 10^6$ and $1 \times 10^5 - 5 \times 10^5$, respectively (Kumar, 2000).

2.2.2 Bioactivity

The presence of reactive functional groups such as amino ($-NH_2$) and hydroxyl (-OH) groups renders it capable of being chemically or enzymatically modified to form several chitosan derivatives. Carboxymethylchitosan, chitosan 6-O-sulfate, alkylated chitosan and carbohydrate branched chitosan are some of the derivatives of chitosan which can be produced by chemically or biologically mediated reactions (Fig. 2.4) (Rinaudo, 2006).

2.2.3 Biodegradability

In vivo digestion of chitosan in mammals produces oligosaccharides of varying length, which being non-toxic can be taken up by the several metabolic pathways for further conversions or be converted to glucoproteins (Aranaz et al., 2009). Chitosan biodegradability depends upon number of acetyl groups, their distribution and its molecular weight (chain length) (Huang et al., 2004). Chitosan biodegradability decreases with an increase in the degree of acetylation as well as length of the polymer (Hirano, 1988; Tomihata and Ikada, 1997; Zhang and Neau, 2001).

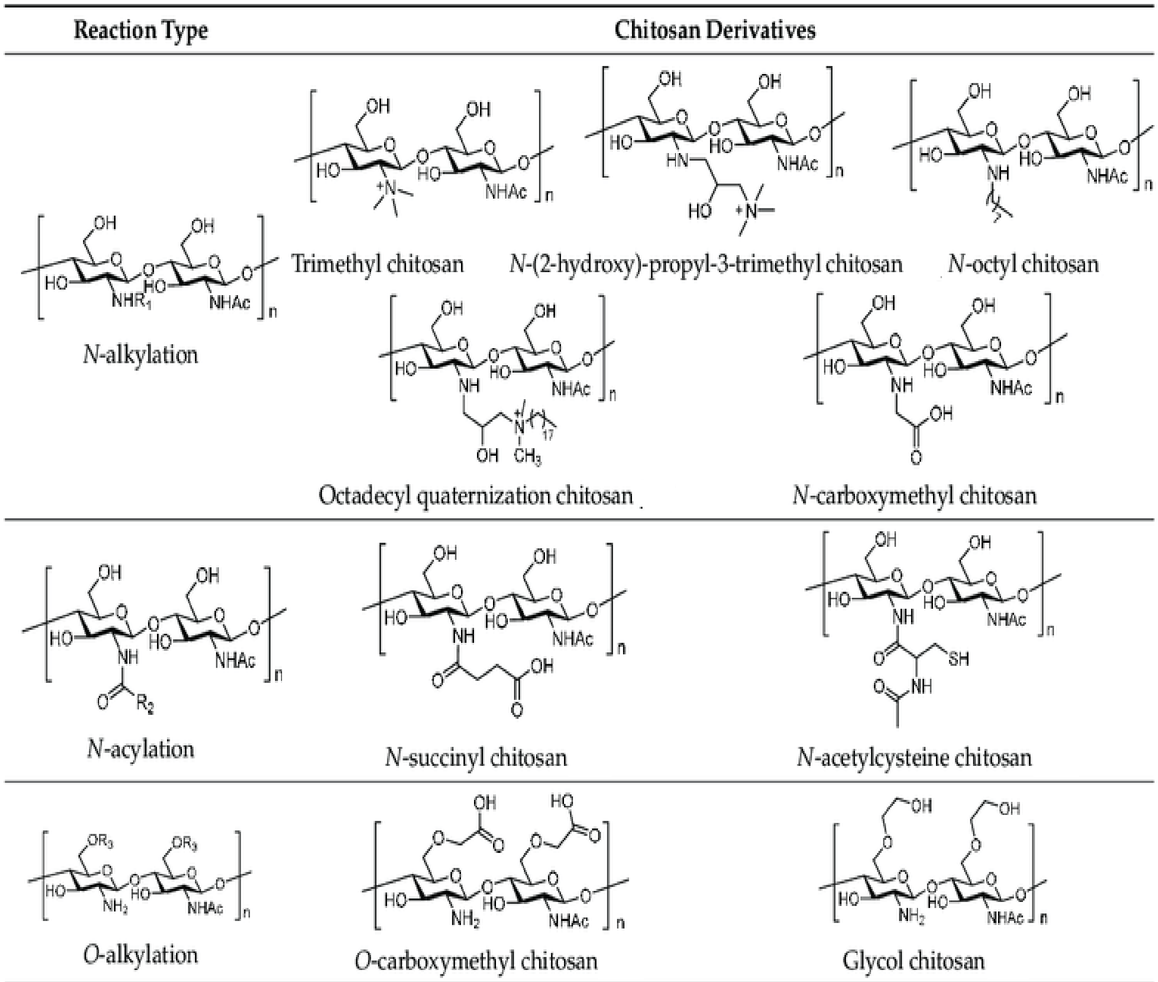


Fig. 2.4 Chitosan derivatives obtained by different modification reactions. (Li et al., 2018)

2.2.4 Analgesic and anticholestrolemic

Chitosan has been reported to show analgesic properties. Chitosan being polycationic in nature, under low pH conditions the amine groups in chitosan protonate. The peptide, Bradykinin which is one of the reasons of pain was reported to be absorbed by the protonated chitosan, thus exhibiting the analgesic effect (Okamoto et al., 2002).

Chitosan has been found to have anticholestrolemic properties. It can bind to negatively charged fatty acids and lipids through their reactive amino and hydroxyl groups, and due to hydrophobic interactions (Muzzarelli et al., 2006; Thongngam and McClements, 2005).

2.2.5 Chelation and adsorption

Owing to its chelation and adsorption properties, chitosan has been reported to bind to metal ions, and adsorb phenolics. This property has been utilised for the removal of metal ions, phenols and other contaminants from water and wastewater for environmental applications (Hadi, 2013; Ngah et al., 2011).

2.2.6 Immobilization

Chitosan can be moulded into different forms like membranes, beads and gels. They are selectively permeable to various molecules and, therefore, can be used for various unit operations and enzyme immobilization (Ghanem and Ghaly, 2004; Muzzarelli, 1980; Taqieddin and Amiji, 2004).

2.3 Chitosan- application

Owing to these unique properties, chitosan is widely used in agriculture, food, and environment sectors as discussed in this section.

2.3.1 Agriculture

Crop productivity in agriculture is an important factor nowadays due to the rising world population and the pressure it exerts on agriculture. Incessant fertilizer and pesticide use has led to soil quality deterioration and various other hazardous effects such as soil and water pollution due to the run offs from the agricultural fields (Malerba and Cerana, 2018). Several studies have extensively reported the positive effects of chitosan application on the productivity of plants. For example, the productivity of strawberry plants to produce fruits increased to 56% after the application of chitosan through pre flowering to post flowering stage (Akter Mukta et al., 2017). The application of chitosan to sweet basil plants under drought stress conditions led to an increase in growth parameters viz. plant height, root length, leaf area and chlorophyll and carotenoid pigments as compared to the control plant (Ghasemi Pirbalouti et al., 2017). Similar increase in the growth parameters of *Zea mays* L was observed when chitosan based copper nanoparticles were applied to the seeds and transferred to fields. These chitosan-Cu nanoparticles also led to enhanced production of antioxidants and plant defense enzymes when sprayed on to the soil every day during the cultivation (Choudhary et al., 2017).

Antimicrobial property of the chitosan has also led to its use as a prevention mechanism against fungal infections. Chitosan based nanoparticles when applied to wheat induced resistance against *Fusarium graminearum*, preventing it from Fusarium head blight disease (Kheiri et al., 2017). Resistance to *Phytophtora infestans* and *Alternaria solani* infections was observed in tomato plants when treated every day with low molecular weight chitosan sprays on the leaves (Kiprushkina et al., 2017). Chitosan solutions (0.5-2 g/L) were found to be effective in controlling *Ralstonia solanacearum* infection which is root cause of wilt disease in potatoes (Farag et al., 2017).

2.3.2 Food and beverages

Owing to its biodegradability and antimicrobial properties, chitosan is an attractive option for improving the shelf life of fruits, vegetables and other agricultural products (Rocha et al., 2017). Coating the agricultural produce with chitosan or chitosan derivative films results in reduced gas exchange, respiration and water loss, thereby helping in post-harvest preservation of these products (Malerba and Cerana, 2018; Palou et al., 2016). Chitosan-g-salicylic acid treatment of grapes before harvesting, led to increased shelf life and protection from *Botrytis cinerea* attack (Shen and Yang, 2017). In vivo and in vitro experiments showed that chitosan treatment of pomegranate resulted in preventing spoilage by infection of different fungal species (*Botrytis* spp., *Penicillium* spp. and *Pilidiella granati*) (Munhuweyi et al., 2017). Ethylene production in large amounts in mango leads to rapid ripening of the fruit which leads to post harvest decay during storage and transport. Chitosan solution (1%) was applied as a coating on mango, which resulted in a delayed ripening of the fruit and with no signs of spoilage due to fungal infections (Jongsri et al., 2016). Fungal infections by different *Colletotrichum* species on mango were inhibited by the addition of chitosan and chitosan-essential oil conjugates (de Oliveira et al., 2017). Several studies have been reported where chitosan based edible coatings due to its mechanical properties and transparent appearance have been employed to reduce food spoilage; and improve the quality and appearance (Bourbon et al., 2011; Park et al., 2014).

Chitosan (73% DA) in low concentrations (<1%) led to efficient clarification of fruit juices (Ghorbel-Bellaaj et al., 2012a). Fungal chitosan derived from *Absidia glauca* when employed for clarification of apple juice gave better results as compared to chitosan derived from shrimp shells (Rungsardthong et al., 2006). The effect of different molecular weights of chitosan on the preservation of juices and milk was investigated by Fernandes et al. (2008). Lower molecular weight of chitosan acted efficiently against the gram-negative bacteria whereas higher molecular weight showed better antimicrobial properties against gram-positive bacteria. The clarification efficiency of chitosan was higher for juices when compared with milk, which could be due to interferences by the protein present in milk. Addition of chitosan resulted in delayed spoilage of apple juice by the yeasts (Kisko et al., 2005). Chitosan addition (< 1 g/L) to orange juices helped in overcoming the necessity of thermal treatment to preserve its quality without affecting the sensory properties of the juice (Martín-Diana et al., 2009).

2.3.3 Environment

Owing to its chelation properties and the presence of different functional groups, chitosan is suitable for cross-linking with other compounds to enhance its adsorption capacity as well as improve its resistance to extreme environments. Chitosan and chitosan derivatives have been utilized for dye and heavy metal removal from wastewaters. For example, chitosan-clay composite was used for methylene blue (MB) dye removal in batch and fixed batch systems with a maximum adsorption capacity of 142 mg/g (Auta and Hameed, 2014). Brilliant blue dye removal of 2.505 mmol/g was achieved by ammonium derivatives of chitosan-gluteraldehyde resin (Elwakeel et al., 2012). Various studies have reported efficient dye removal by chitosan and chitosan derivatives (Gao et al., 2014; Jiang et al., 2014; Peng et al., 2013; Zhu et al., 2010).

Chitosan beads cross linked with diepoxyoctane were employed for removal of Cr (VI) ions from aqueous solution with a maximum adsorption capacity of 325.2 mg/g (Vakili et al., 2018). Chitosan with graphene oxide resulted in a maximum adsorption capacity of 1076 mg/g for Au(III) and 216 mg/g for Pd (II), following Langmuir isotherm model for sorption (Liu et al., 2012). Hexavalent state of uranium, toxic radioactive element usually found in the environment was adsorbed on to chitosan-epichlorohydrin cross-linked compounds. Adsorption kinetic studies revealed that chemisorption was the possible route of adsorption as it followed pseudo second order kinetics. The adsorption data showed best fit with Langmuir isotherm model (Wang et al., 2009).

Chitosan has been coated on to magnetite and maghemite nano-sized particles for investigating its efficiency of metal removal from aqueous solutions and wastewaters. Magnetite particles are non-toxic, cost effective and easily modified to improve its surface characteristics. Adsorption capacity of magnetic chitosan beads for Cu^{2+} and Hg^{2+}adsorption was reported to be 138.12 and 193.83 mg g^{-1}, respectively (Fan et al., 2018). The efficiency of chitosan magnetic beads were evaluated by adsorption recovery index (ARI) and effect vector data visualization. High ARI values indicate a better performance of the magnetic bead. Pb (II) and Ni (II) adsorption were also carried out using chitosan coated nanoparticles and a maximum adsorption capacity of 63.33 and 52.55 mg/g respectively (Tran et al., 2010).

2.4 Chitin and chitosan biosynthesis pathway

Chitin synthesis pathway is highly conserved and follows the same set of reactions for crustaceans, insects and fungi (Fig. 2.5). Chitin, a polymer of N-acetyl glucosamine (GlcNAc) is synthesised from different sugars or its storage compounds such as glycogen and trehalose (Becker et al., 1996; Francois and Parrou, 2001). The formation of glucosamine-6-phosphate from fructose-6-phosphate is the first specific step towards chitin synthesis. Glucosamine-6-phosphate is subsequently converted to N-acetyl glucosamine-6-phosphate by the action of an acetylase enzyme, in the presence of Acetyl-CoA. A mutase enzyme is responsible for conversion of N-acetyl glucosamine-6-phosphate to N-acetyl glucosamine-1-phosphate, which subsequently reacts with UTP following a variant of the Leloir pathway yielding the activated amino sugar Uridine-diphospho-N-acetyl glucosamine (UDP-GlcNAc). Finally chitin polymerization reaction is carried out by chitin synthetase using UDP-GlcNAc as the activated sugar donor (Merzendorfer, 2011).

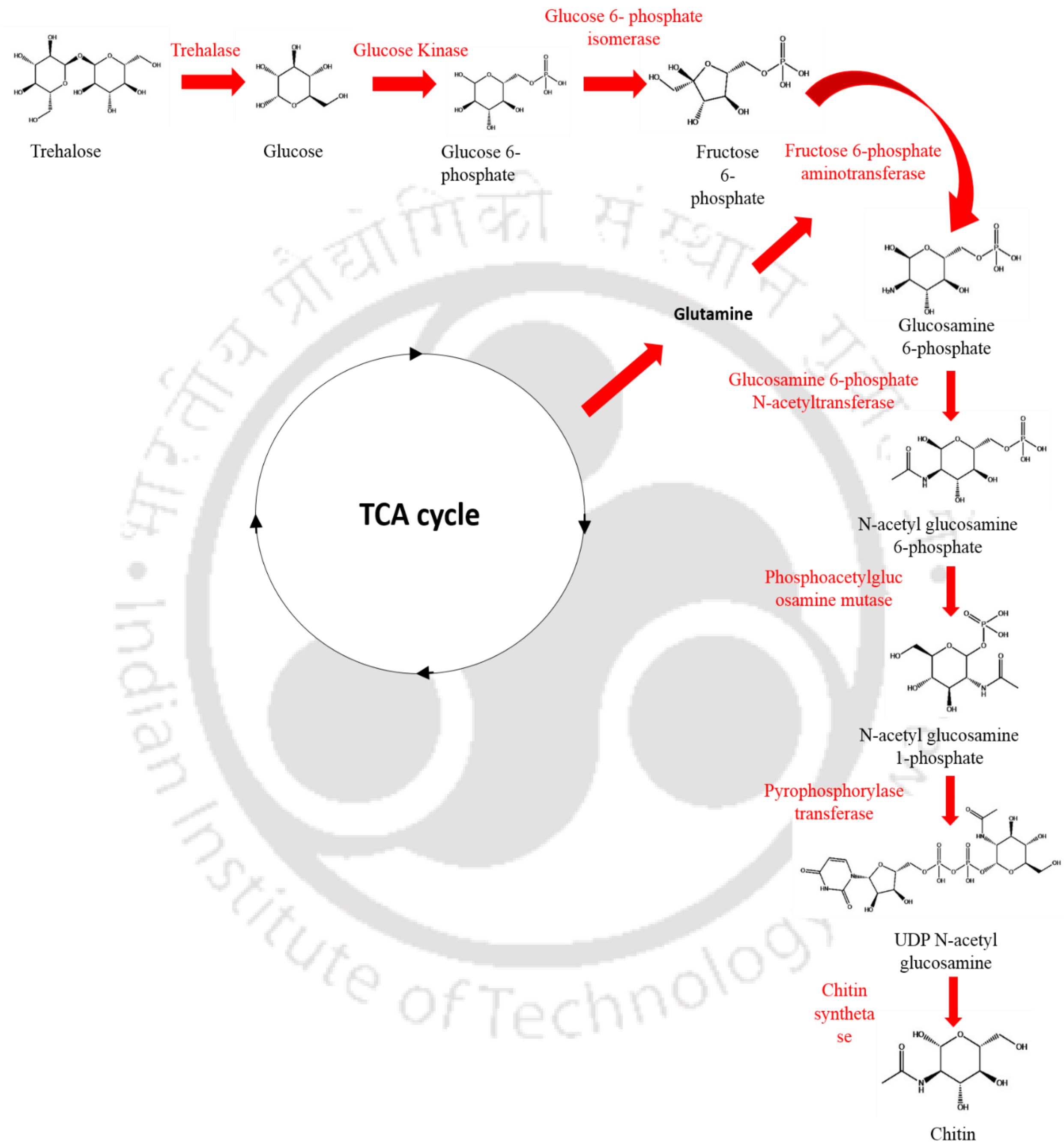


Fig. 2.5 Chitin biosynthesis pathway.

2.5 Sources of chitin and chitosan

2.5.1 Crustaceans

Shrimp, lobster, crab, krill, oyster and squid are the most studied sources that have been applied for commercial extraction of chitin/chitosan. Table 2.1 provides information on chitin content in some crustaceans (Cauchie et al., 1997; Synowiecki and Al-Khateeb, 2003). The shells of these crustaceans are abundant in chitin with a range of 13 to 42% of shell mass. However, due to the intricate organisation of these shells, the extraction of chitin from the other fractions of the shell requires very harsh chemical treatments (Fig. 2.6). These shells also contain proteins (30-40%) and mineral salts (30-50%) as their other major components (Johnson and Peniston, 1979).

The major sources of chitin and chitosan are crustaceans. According to the FAO report, 7862 thousand tonnes of crustaceans was produced and harvested in 2016 (*The State of World Fisheries and Aquaculture 2018*, 2018). Different shellfish like crabs and shrimps are harvested for human consumption, but only 40-50% of its weight is utilised for food processing. This production route leads to a large amount of waste generation, which is directly dumped into the seas and oceans. Low biodegradability of these wastes often results in major pollution of the coastal areas. Utilisation of these wastes for the production of some value added compound is a promising way for addressing these environmental concerns (Handayani et al., 2008; Xuemei and Hawkins, 2002). Chitin, a major component of the waste shells, can be extracted along with other products such as protein and mineral salts. Due to the presence of protein content in the waste, it has been used as chicken feed (Xu et al., 2008).

Table 2.1 Biological extraction of chitin from crustacean wastes

Waste Source	Microbial Strains	Substrate/Incubation	Chitin content (%)	Process efficiency (%) (DP/DM)	References
Callinectes bellicosus	*Lactobacillus* sp. B2	Crab waste/ Sugarcane Molasses 35 °C/200 rpm/120 h	34	56/88	(Flores-Albino et al., 2012)
Metapeneaus monoceros	*Bacillus cereusSV1,*	Shrimp shell waste/5% glucose (w/v) 37 °C/200 rpm/5 d	25.2	96/67	(Ghorbel-Bellaaj et al., 2012b)
	Bacillus subtilis A26,			92/37	
	Bacillus mojavencis A21,			90/38	
	Bacillus pumilus A1,			88/37	
	Bacillus licheniformis RP1			94/59	
	Bacillus amyloliquefaciens An6			83/42	
Metapenaeus monoceros	*B. mojavensis A21*	Shell waste homogenate 60 °C/6 h	18.5	88/-	(Younes et al., 2012)
	Bacillus subtilis A26			76/-	
	Bacillus licheniformis NH1			65/-	

	Bacillus licheniformis MP1			76/-	
	Vibrio metschnikovii J1			76/-	
	Aspergillus clavatusES1			59/-	
Metapeneaus monoceros	*B. pumilus A1*	70 g/L shrimp shells/50 g/L glucose 35 °C/150 rpm/6 d	29	94/88	(Ghorbel-Bellaaj et al., 2013)
Cancer pagurus	*Exiguobacterium spp.* *Bacillus licheniformis* *Bacillus subtilis* + *Lactobacillus spp* *Bacillus cereus* + *Pseudomonas spp.* *Pseudomonas spp* *Pseudomonas migulae* *Enterococcus sp*	Brown crab shell/10% glucose solution 30 °C/175rpm/7 d	14-16	-/99 (Maximum)	(Harkin et al., 2015)

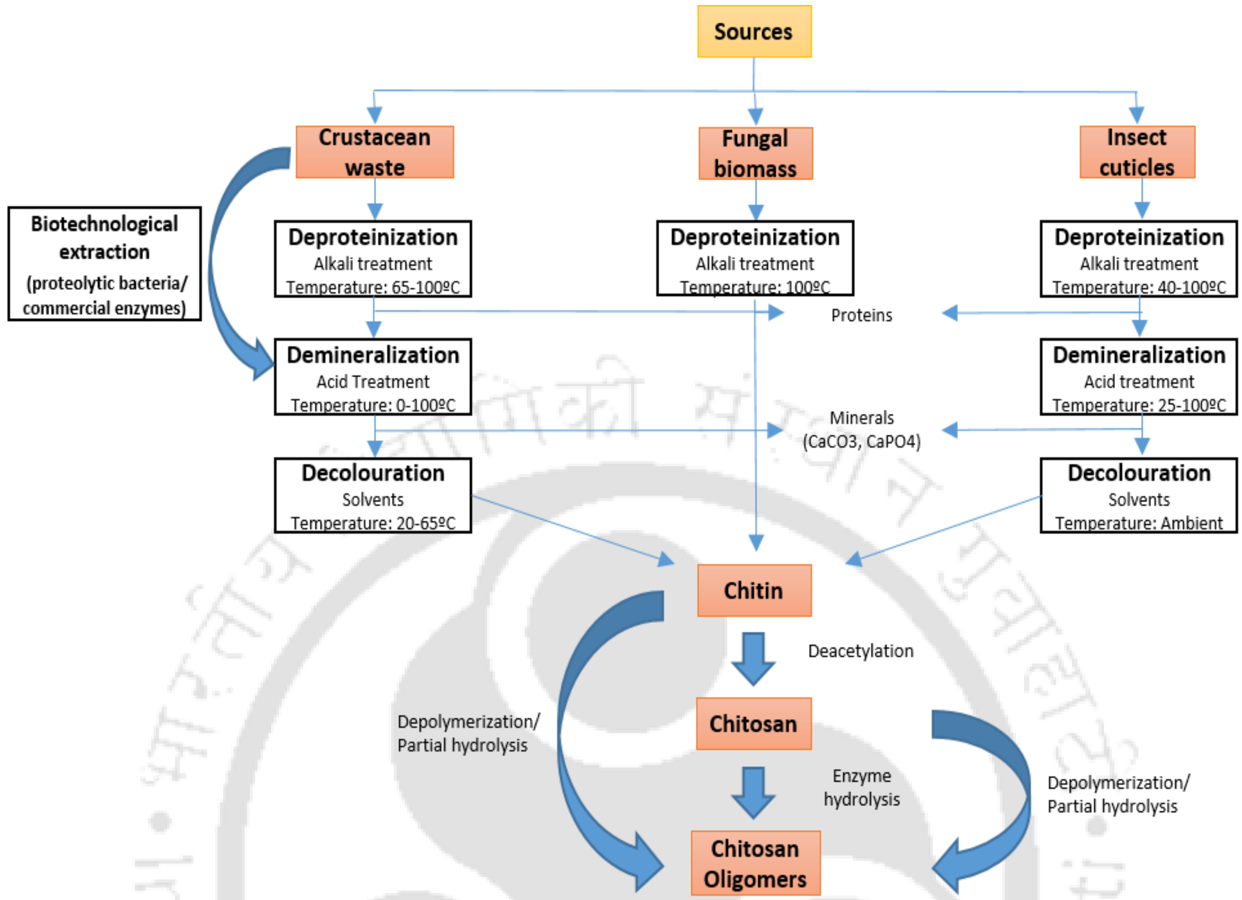


Fig. 2.6 Methods of extraction of chitin and chitosan from crustaceans, fungi and insects

2.5.1.1 Chemical Extraction

The industrial scale production of chitosan involves deproteinization, demineralisation, and removal of pigments followed by deacetylation of chitin to form chitosan. Alkaline treatment by sodium hydroxide, potassium hydroxide, sodium carbonate, sodium bicarbonate etc., or enzymatic treatment by proteases like trypsin, pepsin, etc., could be applied for deproteinization of the ground shells. Chitin is cross-linked with proteins inside the crustacean shells, and, hence, depolymerization of the biopolymer is essential prior to its deproteinization. NaOH is the most widely used reagent for this purpose. NaOH at different concentrations and varying temperature and time regimes have been applied for efficient deproteinization of the shells (Kurita, 2006; No and Meyers, 1995; Percot et al., 2003). Demineralisation of the calcified shells is carried out using strong acids such as hydrochloric acid, sulphuric acid and nitric acid. HCl is the preferred acid for the removal of minerals, primarily calcium carbonate (Johnson and Peniston, 1979; No and Hur, 1998; Teng et al., 2001). Water soluble salts are formed due to the reaction between the minerals and the acids, for example, decomposition of calcium carbonate results in carbon dioxide and calcium chloride, which are soluble in water (Palpandi et al., 2009; Tolaimate et al., 2003). Melanin and carotenoid pigments can be removed by treating with potassium permanganate, hydrogen peroxide or sodium hypochlorite. The chitin obtained following these treatment steps is then converted to chitosan by deacetylation using a combination of alkali and acid treatments. Alkali treatment using NaOH at mild to high temperatures, followed by acid treatment to remove impurities, leads to the formation of chitosan (Fig. 2.7).

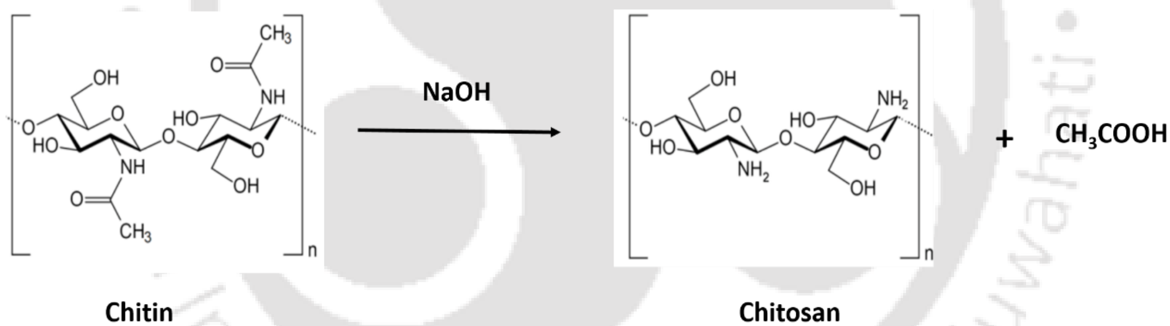


Fig. 2.7 Deacetylation of chitin to form chitosan under alkali treatment

The use of harsh chemicals is extremely detrimental to the properties of chitosan, as they lead to depolymerisation and, thus, affecting the molecular weight and viscosity. During this process, the waste effluent generated contains harmful chemicals, including unutilized mineral acid and other reagents (Manni et al., 2010; Pacheco et al., 2011). Due to the threat of environmental pollution, the waste generated needs to be properly treated before it is released into the nearby environment. Besides, the protein constituents of the shell are not available for application as animal feed (Al Sagheer et al., 2009; Xu et al., 2008). The use of chemicals further results in high production costs. Due to these various limitations, milder extraction methods are of interest. Biological extraction of chitin using microorganisms and utilisation of enzymes for extraction procedure are emerging to be a promising option (Giyose et al., 2010; Sini et al., 2007; Sorokulova et al., 2009).

2.5.1.2 Biological extraction

Bio-based products are widely accepted worldwide and are being favoured against chemically synthesised ones. Drawbacks associated with of the chemical extraction methods can be overcome by using enzymes or microorganisms (MOs). Biological extraction of chitin can be carried out either by using microbes directly for the extraction process, which is known as fermentation mediated extraction or by using the enzymes obtained from the microbes for deproteinization and demineralization. Researchers have found that lactic acid fermentation when coupled with the traditional method of extraction, resulted in lower amounts of alkali and acid required along with high chitin yields (Kaur and Dhillon, 2015).

Deproteinization of the waste shells could be carried out by the action of enzymes such as proteases. MOs could also be applied for this step after demineralisation of the shells (Jung et al., 2007). Proteolytic ability of the MOs leads to the formation of a protein and mineral rich liquid fraction which could be used as an animal feed and even for human consumption, along with a solid chitin fraction (Arbia et al., 2013; Rao et al., 2010). Khanafari et al. (2008) carried out a comparative study between biological and chemical extraction methods for chitin. This study observed that the structure of chitin was preserved in case of the biological extraction process, which also yielded a high molecular weight of chitin, when the deproteinization was carried out by MOs with proteolytic activity (Bustos and Healy, 1994). Deproteinization by enzymes or demineralisation and deproteinization by fermentation, which makes use of several MOs have been applied for biological extraction of chitin retrieval.

Proteases are used extensively for the deproteinization step, and crude proteases are preferred over purified enzymes, primarily because of the high cost of purified proteases. Bacteria and fish are the major sources of crude proteases, the most common among them being the bacterial proteases. Various bacterial and marine proteases have been reported for effective deproteinization of the shrimp waste for chitin recovery (Gildberg and Stenberg, 2001; Manni et al., 2010; Synowiecki and Al-Khateeb, 2003; Younes et al., 2012, 2014).

Fermentation, which utilises MOs for production of various value added products by the uptake of a wide range of substrates, has long played a significant role in the lives of human beings. Organic acid producing bacteria have found wide application in the extraction of chitin and chitosan. These bacteria utilise glucose and release various organic acids which lowers the pH of the media, thereby preventing the growth of other contaminating microbes. Calcium carbonate present in shrimp shell wastes is removed by reaction with lactic acid resulting in calcium lactate that is easily precipitated out. Proteases produced by these bacteria act upon the proteins present in shrimp waste and mediates the deproteinization steps. Table 2.1 enlists the various studies that have been carried out on biological extraction of chitin and chitosan using different microorganisms.

2.5.2 Insects

Insects have been widely explored as a major source of medicines, food and pesticides across the world ranging from ancient traditional civilizations to the industrial age (Dossey, 2010). However, due to the high protein, fat and biopolymer content in their body, they have emerged as a major source of biomass in recent times. Moreover, the high dry matter percentage along with their ability to grow on organic wastes prove the potential of insects as the source of biomass (Philibert et al., 2017). One of the major components of the exoskeleton of insects is chitin. The extraction process of chitin from insects, slightly differs from the crustacean sources, where a harsher demineralization step is required in case of chitin extraction from the insect cuticle (Fig. 2.6). The chitin content of several insect species viz., *H. piceus, R. linearis, A. bipustulatus, A. imperator* and *N. glauca* investigated were found to vary in the range 10-20% w/w. Thermal stability and crystalline index of chitin were also found to vary in the different species. These insects could be a promising source of chitin, especially *Hydrophilus piceus* which showed highest chitin (20%) yield (Kaya et al., 2014a). *Rhinolophus hipposideros*, bat guano was estimated to have 28% chitin content. Fourier-transform infrared spectroscopy revealed that the chitin present in the bat guano was in α-chitin form. Around 79% chitosan was retrieved from the chitin. Scanning electron microscopy and X-ray diffraction experiments showed that the chitin and chitosan were present in the form of nanofibers (Kaya et al., 2014b). Several studies have reported grasshoppers as a very good source of chitin. The structures of chitin obtained from seven different *Orthoptera* species were compared and it was observed that the chitin content from these various grasshopper species varied between 5.3 and 8.9%; the molecular weight of the chitin obtained was found to be low (Kaya et al., 2015a). *Celes variabilis, Decticus verrucivorus, Melanogryllus desertus, Paracyptera labiate* were investigated for the differences in chitin content in the male and female grasshoppers of the same species (Kaya et al., 2015b). The chitin content was found to be higher in the males than the female and the highest chitin yield was found to be 11.84% of its dry weight in the case of *Decticus verrucivorus*. Marei et al. (2016) estimated the chitin content from three different insects, viz. *Schistocerca gregaria* (locust), *Apis mellifera* (honey bee), and *Calosoma rugosa* (beetles) to be 12.2%, 5 and 2.5%, respectively. The chitin obtained was further deacetylated to obtained chitosan. The degree of deacetylation (DD) derived were 98%, 96% and 95%, respectively. These studies suggest that insects are a very promising source for chitin extraction with high yields (Table 2.2). Arthropods including the insects is such a vast and biodiverse genera that their complete potential is yet to be realised. They are the most unexplored species with regards to their contribution to various areas of application and as a bioresource (Philibert et al., 2017).

Table 2.2 Extraction of chitin from insects

Insects	Chitin content (%)	Chitosan productivity	Extraction	References
Agabus bipustulatus	14-15	71	1M HCl/ 90 °C/1 h	(Kaya et al., 2014a)
Anax imperator	11-12	67	1M NaOH /110 °C/18 h	
Hydrophilus piceus	19-20	74	Choloroform:methanol:water (1:2:4)	
Notonecta glauca	10-11	69		
Ranatra linearis	15-16	70		
Rhinolophus hipposideros	28	79	4M HCl/ 50 °C/24 h 4M NaOH /140 °C/24 h Choloroform:methanol:water (1:2:4)/4 h	(Kaya et al., 2014b)
Aiolopus simulatrix	5.3	-	4M HCl/ 75 °C/1 h	

Aiolopus strepens	7.4	-	2M NaOH /175 °C/18 h	(Kaya et al., 2015a)
Duroniella fracta	5.7	-	Choloroform:methanol:water (1:2:4)	
Duroniella laticornis	6.5	-		
Oedipoda caerulescens	8.9	-		
Oedipoda miniata	8.1	-		
Pyrgomorpha cognata	6.6	-		
Celes variabilis	9.93	-	4M HCl/ 75 °C/2 h	(Kaya et al., 2015b)
Decticus verrucivorus	11.84	-	2M NaOH /150 °C/20 h	
Melanogryllus desertus	7.35	-	water	
Paracyptera labiate	7.60	-		

2.5.3 Fungi

Chitin, a simple polysaccharide is, present in the cell wall of almost all fungi known till date. A high chitosan and chitin content is found to be present in fungi belonging to the zygomycetes family as with the other classes (Hu et al., 2004; Pochanavanich and Suntornsuk, 2002; Sousa Andrade et al., 2003).

Chitin in the cell wall of fungi folds back on itself to form anti-parallel chains and form intra-chain hydrogen bonds that stiffen into immensely strong fibrous microfibrils structures tougher than any other molecule in nature. β(1,3)- glucan, which is another structural polysaccharide present in most fungal cell walls, is attached covalently to this chitin microfibril network. The composition of the cell wall is subject to change and may vary within a single fungal isolate depending upon the conditions and growth stage. The glycoprotein, glucan and chitin components are extensively cross-linked together to form a complex network, which forms the structural basis of the cell wall.

Zygomycetes are found to have a higher percentage of chitosan than chitin. Chitosan is synthesised by a deacetylase enzyme that can convert the chitin produced by the previously mentioned pathway to chitosan (Fig. 2.5). It is believed to be synthesized by the tandem action of chitin synthase and chitin deacetylase enzymes. Uridine-diphospho-N-acetyl glucosamine (UDP-GlcNAc) is added to the chitin chain by the action of the chitin synthases and directed to the cell wall simultaneously. These chitin synthases are localized in sub-cellular organelles called chitosomes. The chitosomes on reaching the cell surface may produce a tightly linked complex of long microfibrils or be present in a dispersed form. Chitin deacetylase present in the cell wall act upon this newly synthesized chitin to convert it into chitosan. However, only the chitin present in the dispersed form is susceptible to the action of this enzyme, and it is ineffective on crystalline chitin. As chitosan is reported to be more abundant than chitin in zygomycetes fungi, it could be inferred that the chitin synthase sub units are majorly present in a dissociate form in these fungi. The motive of this N-deacetylation of carbohydrate moeities is to offer resistance to lysozyme action, specifically from chitinases (Muzzarelli et al., 1999).

Zygomycetes group of fungi have non-septate thalli and form dark thick walled sexual spores which are called zygospores. Entomopthorales and Mucorales make up most of the zygomyectes class of the fungi. Among these, Mucorales produce both chitin and chitosan in significant amounts in their cell wall. Mucorales are saprophytes which mainly target plants vertebrates as they are endoparasites (Guarro et al., 1999). Fungal chitosan production has received increased attention recently due to a number of significant advantages. Whereas the supply of crustacean wastes are limited by the sites of fishing industry as well as seasons, fungal fermentation processes are devoid of any geographical or seasonal limitation. Moreover, mycelia do not require a demineralisation step due to its low inorganic material content, consistent properties of chitin and chitosan due to controlled fermentation conditions. Chitosan production in the cell wall of zygomycetes fungi eliminates the expensive chemical deacetylation step presently used in the conventional process of chitosan production from crustacean wastes. Additionally, zygomycetes can grow on a variety of waste materials, which makes it environmentally acceptable.

Absidia glauca, Aspergillus niger, Aspergillus awamori, Mucor rouxii, Gongronella butleri, Absidia glauca, Cunninghaella elegans, Penicillium citrinum Rhizopus oryzae and Lentinus elodes have been investigated for chitin and chitosan production (Arcidiacono and Kaplan, 1992; Cardoso et al., 2012; Chatterjee et al., 2005; Namboodiri and Pakshirajan, 2019; Rane and Hoover, 1993a; Teng et al., 2001). Among these species, *Mucor rouxii* is the most researched species as it contains chitosan up to 30-35% of the dry weight of cell wall of the mycelia (Synowiecki and Al-Khateeb, 2003). Chitosan production by various fungi along with their extraction procedure and degree of deacetylation are presented in detail in Table 2.3 (Dhillon et al., 2013).

Chitosan extraction from fungal mycelia involves a mild alkali treatment using 1M NaOH at 120°C for 15-20 min Chitin and chitosan are insoluble in alkali whereas majority of the carbohydrates, lipids and proteins are dissolved in the supernatant. The alkali insoluble material (AIM) is washed thoroughly and subjected to an acid treatment using 2% v/v acetic acid at 90ºC for 6-7 h. Chitosan can be recovered after this treatment by increasing the pH of the acid solution to 8.0-9.0 because chitosan precipitates out in alkaline environment. The precipitated chitosan is washed with water, ethanol and acetone and finally dried at 65°C for 12-24 h. A detailed schematic of the extraction procedure is shown in Fig. 2.8 (Chatterjee et al., 2005).

Fig. 2.8 Schematic showing chitosan extraction from fungal biomass

2.5.3.1 Fungal chitosan production from waste resources

For an economical production of chitosan from fungi, it is imperative to use alternate cheap carbon sources as substrate for the fungal growth. Several studies are being conducted with the focus on developing a viable process for chitosan production from fungi at a large scale. Fungal species belonging to *Aspergillus, Cunninghamella, Mucor, Penicillium, Rhizopus, etc.* have been studied for chitosan production from inexpensive carbon sources (Table 2.3).

Rice and corn were used as substrates for growing *R. oryzae* by Hang (1990) for chitosan production. 600 mg/L chitosan was produced after a cultivation period of 48 h at 30ºC. Rice straw supplemented with minerals for fungal chitosan was investigated by Khalaf (2004) using *R. oryzae*, *P. citrinium*, *A. niger* and *F. oxysporium* under SSF conditions. *R. oryzae* gave the highest chitosan yield of 5.63 g/kg biomass followed by *A. niger* after 12 days of incubation. Soybean and mung bean residues were studied for chitosan production by four fungal strains and among these strains, *R. oryzae* gave the highest chitosan yield of 4.3 g/kg of substrate when grown on soybean in plastic bags with cotton plugs at 30 °C after 16 days of cultivation (Pochanavanich and Suntornsuk, 2002).

Cunninghamella bertholletiae was reported to produce chitosan by Amorim et al. (2006a) from sugarcane juice and molasses as non-commercial substrates. A chitosan yield of 128 mg/g of dry mycelia was achieved under the optimal conditions of pH 4.5, 150 rpm agitation, 28 °C for 7 days. A newly isolated strain of *Syncephalastrum racemosum* was used for chitosan production in media comprising molasses and sugarcane juice as carbon source. Highest chitosan yield of 74 mg/g dry mycelia was observed with the sugarcane juice based media when cultivated at 28°C, 400 rpm for 60 h (Amorim et al., 2006b).

Buckwheat/barley and sweet potato were used for chitosan production by *G. butleri* resulting in a yield of 730 mg/L chitosan using a sweet potato based medium obtained from shochu distillery wastewater at pH 5.0 and 30 °C temperature after 5 days of fermentation (Yokoi et al., 1998). Solid state fermentation was applied for *G. butleri* using sweet potato as the substrate and the influence of different nitrogen sources was analysed to obtain a chitosan yield of 11% dry cell weight (Nwe and Stevens, 2004). *A. niger* produced highest amount of chitosan (17-18 g/kg DS) on soya bean residues, when compared with corn seed and canola residues, which were used as the naturally available non-commercial carbon sources (Maghsoodi et al., 2008). *Penicillium* waste obtained from pharmaceutical industries served as a zero cost source for chitosan extraction. A chitosan yield of 5.7% from *P. chrysogenum*, with an 86% degree of deacetylation was reported by Wang et al. (2007). Namboodiri and Pakshirajan (2019) reported fungal chitosan production using paper mill wastewater by *Penicillium citrinum* biomass at 28°C temperature and pH 4.5. A maximum chitosan yield of 13.8 % dry fungal biomass was achieved after acetic acid addition at low levels.

Table 2.3 Fungal chitosan from alternate carbon sources

Fungi	Fermentation	Culture medium	Chitosan Extraction	Chitosan yield	Degree of deacetylation	References
Aspergillus awamori	Submerged Fermentation pH-3.7/ 150 rpm/ 30 °C/ 96 h	Raw distillery thin stillage	0.5 N NaOH/ 121 °C/20 min 1% (v/v)H_2SO_4 / 121 °C/20 min	7-9%	68.89%	(Ray and Ghangrekar, 2016)
Aspergillus niger	Solid state fermentation pH-5.9-6.4/ static/ 30 °C/ 16 d	Soya bean, canola and corn seed residues	1N NaOH/ 120 °C/20 min 2% (v/v) acetic acid/ 95°C/6 h	17.05 ±0.95 g/kg ds soya bean 12.73±1.22 g/kg canola residue	-	(Maghsoodi et al., 2008)

Cunninghamella berthollettae	Submerged Fermentation pH-4.5/ 150 rpm/ 28 °C/ 7 d	Yeast peptone dextrose medium, Sugarcane Juice	NaOH/ Acetic acid	55 mg/g dcw (YPD) 128 mg/g dcw (sugarcane)	88.20% 89.70%	(Amorim et al., 2006a)
Cunninghamella elegans	Submerged Fermentation pH-5.6/ 150 rpm/ 28 °C/ 72 h	Corn steep liquor and molasses	-	33.13 mg/g dcw		(Berger et al., 2014)
Cunninghamella elegans UCP-542	Submerged Fermentation	Yam bean medium	2% (w/v) NaOH/ 90 °C/2 h 10% (v/v)acetic acid/ 60 °C/6 h	66 mg/g dcw	85%	(Stamford et al., 2007)

	pH-7.0/ 150 rpm/ 28 °C/ 96 h					
Gongronella butleri	Submerged Fermentation pH-4.5/ 150 rpm/ 30 °C/ 110 h	Apple pomace	2% NaOH/90 °C/2h (200 rpm) 10% (v/v) acetic acid/ 60 °C/6 h (150 rpm)	1.19 g/L 20% dcw	-	(Streit et al., 2009)
Gongronella butleri CCT 4274	Submerged Fermentation					
Lentinus edodes	Solid state fermentation	Wheat straw	1N NaOH/ 120 °C/1.5 h	6.18 g/kg biomass (SSF)	88-90%	(Crestini et al., 1996)

	28 °C/ 19 d Submerged Fermentation 400 rpm/ 28 °C/ 16 d		2% (v/v) acetic acid/ 95 °C/14 h	120 mg/L (SmF)		
Mucor hiemalis	Submerged Fermentation pH-5.5/ 150 rpm/ 32 °C/ 72 h	Wheat hydrolysate	1% NaOH/ 120 °C/20 min 2% (v/v) acetic acid/ 95 °C/8 h	0.46 g/g AIM	-	(Vinche et al., 2013)
Penicillium chrysogenum	-	Waste mycelia of pharmaceutical industry	1N NaOH/ 95 °C/1.5 h	5.7%	86%	(Wang et al., 2007)

			1 M acetic acid/ 45 °C/1.5 h			
Penicillium citrinum	Submerged Fermentation pH-4.5/ 200 rpm/ 28 °C/ 72 h	Paper mill wastewater	1M NaOH/ 120 °C/20 min 2% (v/v) acetic acid/ 95 °C/8 h	13.8%	81%	(Namboodiri and Pakshirajan, 2019)
Rhizomucor pusillus	Submerged Fermentation pH-5.7-6.2/ / 36-38 °C/ 4 d	Xylose-rich wastewater from ethanol plant	1N NaOH/ 90 °C/2 h 2% H_2SO_4/ 95-121 °C/20 min	45.7% AIM	97.5%	(Zamani et al., 2007)
Rhizopus arrhizus	Submerged Fermentation	Corn steep liquor and honey	1N NaOH/ 121 °C/15 min	29.3 mg/g dcw	86%	(Cardoso et al., 2012)

	pH-5.6/ 150 rpm/ 28 °C/ 96 h		2% (v/v) acetic acid/ 100 °C/15 min			
Rhizopus oryzae	Submerged Fermentation 200 rpm/ 35 °C	Hemicellulose hydrolysate of corn straw after H_2SO_4 hydrolysis	1N NaOH/ 121 °C/15 min 2% (v/v) acetic acid/ 95 °C/24 h	0.58 g/L	89-90%	(Tai et al., 2010)
	Solid state fermentation	Rice straw	1N NaOH/ 121 °C/20 min 2% (v/v) acetic acid/ 95 °C/8 h	5.63 g/kg of fermented medium	73-90%	(Khalaf, 2004)

	Solid state fermentation 30 °C/ 16 d	Soybean and mung bean residues	1N NaOH/ 121 °C/20 min 2% (v/v) acetic acid/ 95 °C/8 h	4.3g/kg soybean residues 1.6 g/kg mung bean residues	-	(Pochanavanich and Suntornsuk, 2002)

2.5.3.2 Bioreactor considerations

As discussed previously fungal chitosan production has been the focus of research over the last two decades owing to the various advantages fungi possess as a source of chitin and chitosan. However, the recent focus is to scale-up this route of chitosan production to an industrial level. A number of researchers have successfully demonstrated strategies of fungal chitosan production by applying lab scale bioreactors.

Solid-state fermentation

Solid-state fermentation has been widely used for cultivating fungi for the production of various metabolites, particularly enzymes. This mode of fermentation is highly suited to the growth characteristics as well as the metabolism of fungi. Low water demand, high product volume and absence of catabolite repression are the major advantages of employing solid-state fermentation. Various bioreactor designs have been developed for fungal fermentation of solid substrates depending upon the need of aeration (diffusion/ forced), agitation (static/ mixing), fungal morphology, substrate characteristics and moisture content. Solid-state fermentation can be carried out using simple trays or sealed plastic bags with cotton plugs at small scale; Koji bioreactors, horizontal and rotating drum bioreactors are commonly used (Fig. 2.9).

Perforated tray and rotating drum fermenters are the most widely applied bioreactor configurations for metabolite production by fungi. The substrate is placed on trays that are perforated for better air transfer. These trays are placed in temperature and humidity controlled environment such as a chamber or room. Although this set up is easy to scale up but it suffers from a number of serious drawbacks. Substrate layer thickness along with the changes in porosity of the substrate due to the growth of fungi pose serious oxygen transfer limitations. Moreover, scaling up of this process is labour intensive and requires large operational area.

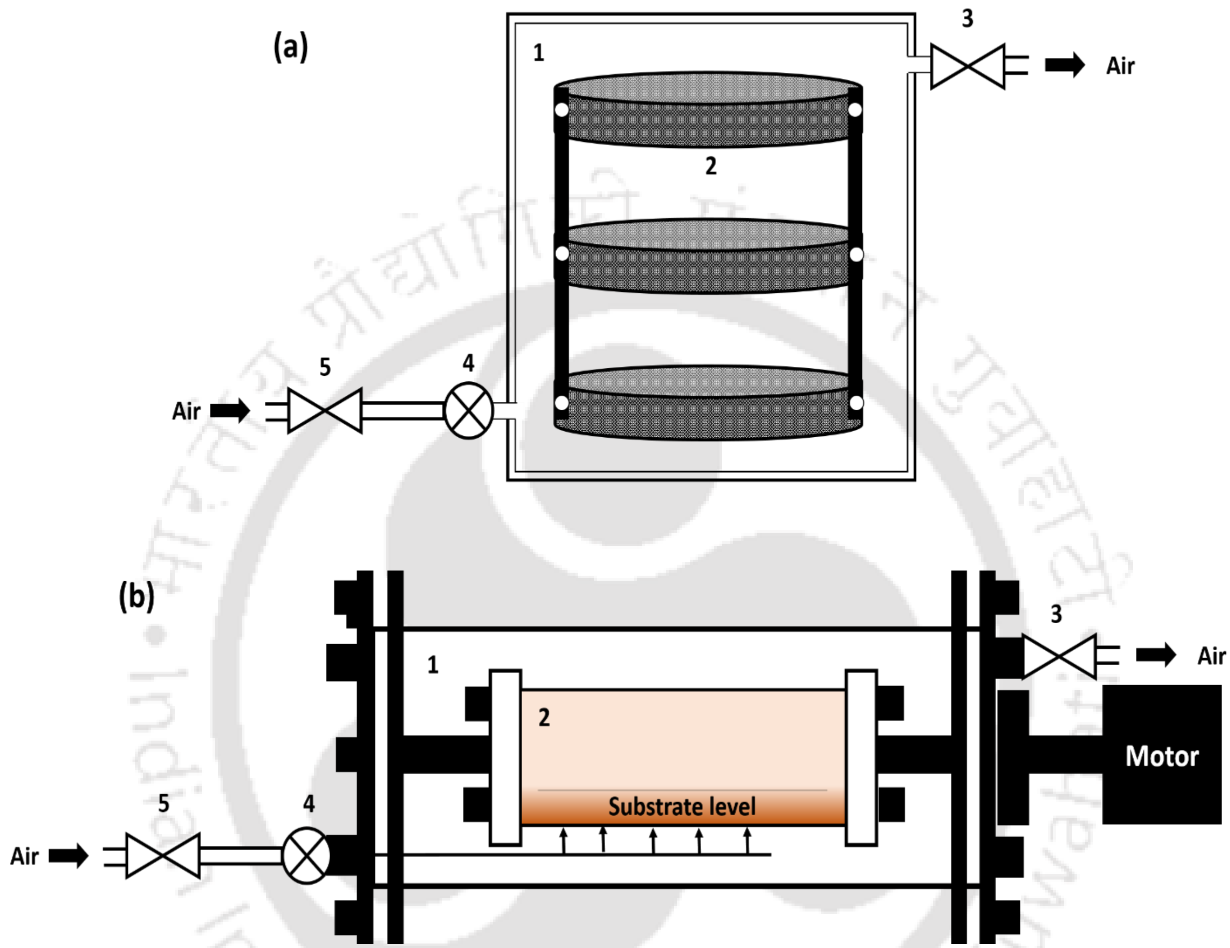


Fig. 2.9 (a) Tray fermenter: (1) Reactor vessel, (2) Trays, (3,5) Air filters, (4) Humidifier, and (b) Rotating Drum filter: (1) Reactor vessel, (2) Rotating drum, (3,5) Air filters, (4) Humidifier.

Rotary drum fermenters make use of horizontal cylindrical vessel which is partially filled with the solid substrate. Aeration is done by passing the air through the headspace, which then diffuses into the solid phase. These cylinders or drums are rotated and therefore mixing is achieved due to the tumbling motion of the substrates inside. The mixing is carried out in continuous or intermittent modes. Continuous agitation is found to be detrimental for the fungal growth due to damage caused by the shear stress. The advantage of using a rotary drum fermenter over the static fermenters is that sufficient air transfer is achieved along with the dissipation of heat produced due to the fungal metabolism (Durand, 2003; Manan and Webb, 2017). The major issues associated with scale-up of such solid state reactors are the heterogeneous nature of the system along with the intense metabolic heat generation. Hence, several bioreactor designs with certain modifications have come up which attempt to mitigate these issues but only a few of these have been applied at a large scale. Several studies have reported to employ solid state fermentation for fungal chitosan production that have been presented in Table 2.3.

Submerged Fermentation

Filamentous fungi have been widely explored for primary and secondary metabolite production and biomass. Various factors such as oxygen demand and rheology of the culture medium govern the success of submerged fermentation using fungal biomass. However, they are highly susceptible to shear stress imparted due to agitation in the reactors. Moreover, viscosity of the broth increases with the growth of fungi and the fungal morphology is a crucial parameter that leads to highly viscous culture medium. The fungi grow as pellets or as free mycelia depending upon the agitation speed in the reactors. The formation of pellets is desirable and has been shown to enhance the overall efficiency of a fermentation process. Therefore, optimization of various process parameters along with modifications in bioreactor configurations is necessity.

The most commonly used reactor configurations for the production of fungal chitosan under submerged fermentation conditions are stirred tank and airlift fermenters (Fig. 2.10), with the former being the most widely used. Rushton impellers with radial flow are the default agitation system that is attached to the reactors. While impellers with alternate designs and radial flow have shown to be better than Rushton turbines for bulk mixing, they have been used in the case of exopolysaccharide production where high viscosity of the broth lead to acute mixing problems. Although, axial flow impellers provide better mixing than radial flow impellers, the shear stress imparted to the fungal mycelia is higher which is detrimental to the process. With the increase in scale however, the axial flow impellers provided superior agitation and the shear damage imparted to the mycelia decreased (de Carvalho, 2016; Gibbs et al., 2000).

Four different configurations of the stirred tank reactor was studied to observe the difference in the morphology of the chitosan producing strain *Absidia coerula* at 25ºC and pH 4.5. These configurations differed in the working volume (1-10 L) and arrangement of baffles and aeration setups. The changes in stirring speed and aeration led to varied sizes of pellets and accretion of mycelium on the impeller, baffles, probes, etc. This study showed the importance of impeller design as an important factor in the cultivation of fungi in bioreactors (Davoust and Hansson, 1992). Rane and Hoover (1993b) carried out submerged fermentation using airlift and stirred tank reactors (STR) for chitosan production. The efficiency of the both these reactors were hampered due to mycelial agglomeration. Small pellets were obtained after the addition of an antifoam 289, which led to better results. Another important factor governing the design of the reactor and impellers is the medium composition.

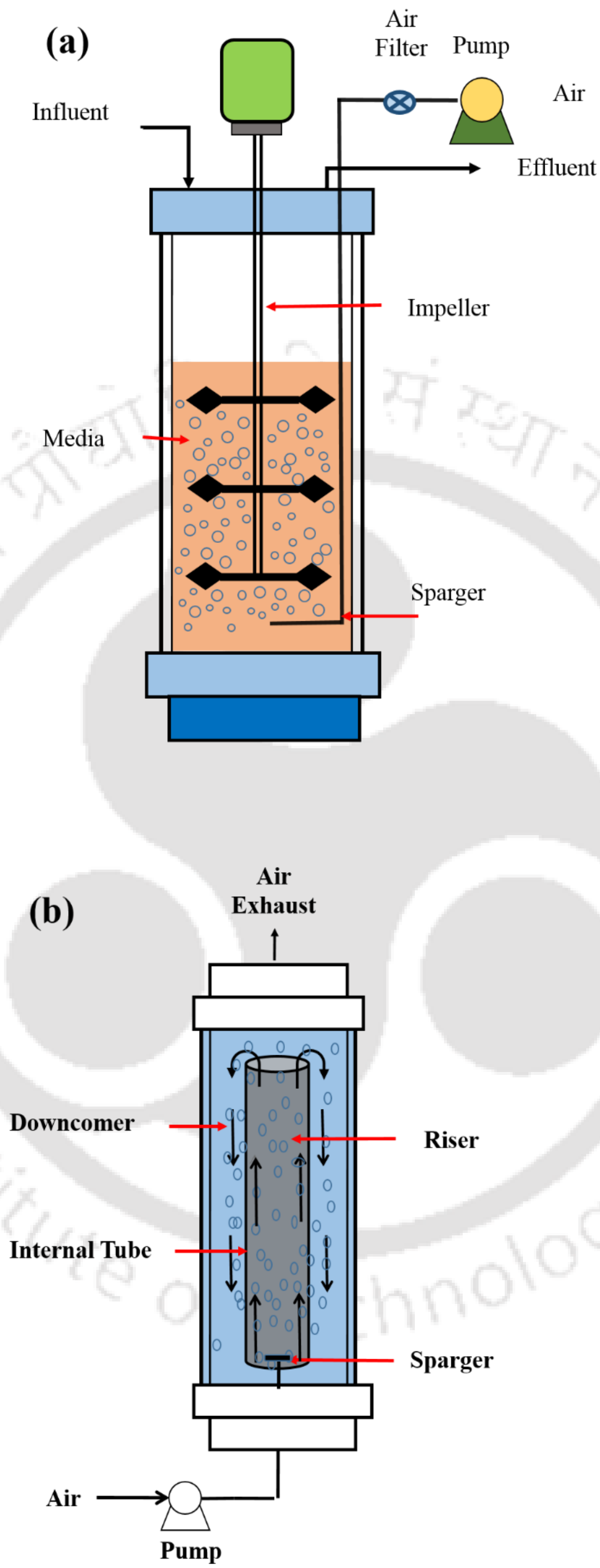


Fig. 2.10 Schematic of (a) Stirred Tank Reactor and (b) Airlift Reactor

The issues of shear damage and mixing associated with STRs have led to airlift fermenters being the choice of fungal fermentation involving fragile fungal mycelia and highly viscous media. Airlift reactors are specialised bubble column reactors that are divided into two sections: riser and downcomer. Circulation is achieved due to the difference in hydrostatic pressure and fluid density depending upon the point of sparging. Low shear damage, better mixing, reduced energy costs and low contamination risks due to the absence of impeller shaft have led to the use of airlift fermenters in industrial applications. Advances have been made where micro-bubbles are generated using fluidic oscillator which result in increased contact time between the gas and liquid, better mixing as well as less energy dissipation (AL-Mashhadani et al., 2015). Different synthetic and alternate media have been employed for carrying out scale-up studies for fungal chitosan production.

Chemical extraction methods to derive chitin and chitosan from marine sources is the widely used and accepted route of their production. However, harsh chemical treatments for demineralization and deproteinization result in hazardous wastes and undesired by-products. This in turn leads to high production costs of chitin and chitosan. Moreover, the variation in the properties of the derived chitosan is a serious drawback for applying the chemical mode of chitin extraction. The biological route of chitin and chitosan production is a very efficient and potent alternative. The use of microorganisms such as bacteria and fungi and utilization of their efficient enzyme systems would lead to a better and economically viable option to obtain chitin and chitosan from the marine wastes. Insects, alongside crustacean waste, are also an important source of chitin. High chitin content and the biodiversity of this group opens up a lot of options for a viable route of chitin and chitosan production. On the other hand, the economics of insect cultivation, time required along with the seasonal availability and inconsistent properties of the chitin and chitosan seem to be some of the serious drawbacks of using insects and crustaceans for chitosan production. Fungi are a promising option as a primary source of chitin and chitosan. Fungi contain both chitin and chitosan within their cell wall, which, therefore, negates the harsh demineralisation step required for marine sources. Fungi are known to utilise industrial and agricultural wastes as substrates for their growth, which can be applied to reduce the costs incurred for chitosan production. Moreover, properties of the chitosan are consistent and can be controlled by changing the different fermentation parameters. However, the techno-economic feasibility of fungal based chitosan production process need to be evaluated for establishing the process as an economically viable and sustainable green technology for chitin and chitosan production.

CHAPTER THREE

MATERIALS & METHODS

3. Materials and methods

3.1 Fungal strains, media and culture conditions

Two different fungal strains, *Cunninghamella elegans* and *Penicillium citrinum,* were evaluated for their ability to utilize organics present in different wastewater and produce chitosan. *C. elegans* was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh; *P. citrinum* was isolated from an infected bamboo plant, which was available locally. In the case of *P. citrinum,* infected bamboo shoot was retrieved from its whole plant, and a small piece was placed on a potato dextrose agar plate before incubating the plate at 30ºC. A single colony of the culture grown on the plate was sub cultured by transferring to freshly prepared plates. The isolated colony was stained with Lactophenol Cotton Blue (Leck, 1999) and observed under light microscope. Both the strains were grown on potato dextrose agar plates (PDA) for 5-6 days at 28 °C and pH 5.6 to form densely sporulated colonies. Spore suspension of the respective fungal cultures was then prepared using 0.1% Tween 20 solution which served as the inoculum for fungal chitosan production in this study.

3.1.1 Identification of fungi

Identification of the fungal isolate from the infected bamboo plant was carried out by morphological and molecular analysis of the strains.

3.1.1.2 Morphological identification

Morphological identification of the isolated fungi was done by the tease mount method, which involves transferring a small amount of sporulated fungal mycelia from PDA plate to a glass slide. The fungal mycelia dispersed sparsely with the help of teasing needles prior to staining with lactophenol cotton blue (LPCB). Cover slip is then placed over the slide before viewing under light microscope at 40X magnification. Morphological characteristics such as sporangiophore, spore shape and mycelia structure were then studied for its identification.

3.1.1.3 Molecular identification

Molecular identification involved extraction of fungal genomic DNA, PCR amplification using universal ITS (Internal transcribed spacer region) primers, DNA sequencing and applying bioinformatics tools to identify the organism.

Fungal genomic DNA extraction was carried out by Cetyl trimethylammonium bromide (CTAB) method with slight modification (van Burik et al., 1998) . Freshly grown mycelia (50 mg) of the pure culture was ground under liquid N_2 in mortar and pestle and added with pre-warmed (60°C) TES buffer for its DNA extraction as reported by Moller et al. (1992). Purity and content of isolated genomic DNA extracted from the fungi were analyzed by performing 0.7 % agarose gel electrophoresis and by spectrophotometry.

PCR amplification of the DNA was carried out using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR amplification step was carried out under the following conditions: initial denaturation at 98 °C for 5 min, followed by 30 cycles of (45 s of denaturation at 98 °C, 30 s of annealing at 61.1 °C and 1 min of extension at 72 °C) and a final extension at 72 °C for 5 minutes. 10 µl of total sample volume was prepared with the components provided in Phusion™ amplification kit. Purity and content of the PCR amplified product were examined and sent to Europhins Scientific, India, for sequencing of the DNA based on Sanger dideoxy sequencing method. The fungal isolate was identified based on the sequence variation present in 18s rRNA.

3.2 Fungal chitosan production from wastewater

Different wastewaters were screened and optimized for fungal chitosan production. The effect of acetic acid as an inducer was studied in detail followed by bioreactor studies were carried out under batch, fed-batch and continuous modes.

3.2.1 Batch shake flask experiments

All the preliminary screening and optimization studies were carried out in 250 mL Erlenmeyer flasks in an orbital shaker maintained at controlled temperature environment.

3.2.1.1 Wastewater characterization

Three different wastewaters, viz., domestic wastewater, paper mill wastewater and dairy wastewater, were examined as substrates for chitosan production by the novel fungal isolate *P. citrinum* and the results were compared with those obtained using *C. elegans*, which is well reported in the literature for chitosan production. Whereas domestic wastewater and dairy wastewater were collected from nearby locations in and around Guwahati, India, paper mill wastewater was collected from Nagaon, India. Raw/untreated wastewater samples were collected from these locations in airtight containers and stored at 4ºC for further use.

These wastewaters were characterised for their COD (chemical oxygen demand), TSS (total suspended solids), nitrogen content and pH as per American Public Health Association (1998) (Table 3.1). According to American Public Health Association (1998), total phenolic content was measured using Folin Ciocalteu reagent (Singleton and Rossi, 1965). Wastewater decolourization assay was performed by measuring the absorbance of effluent at 465nm and pH 7.6. A colour measurement of 3774 on Pt/CO scale shows absorbance of one at 465 nm. Standard ascorbic method was applied to measure the total phosphate content; Composition of the reagent is as follows: 5N H_2SO_4 (50 mL), 0.1 M ascorbic acid (30 mL), 4% ammonium molybdate solution (15 mL) and antimony molybdate solution (5 mL). About 4 mL of this reagent was mixed with 2.5 mL of the sample in a 50 mL volumetric flask and distilled water was added to make up the volume to 30 mL. The reaction was allowed to proceed for 10 min at ambient temperature prior to measuring its absorbance at 880 nm using a UV visible spectrophotometer (Cary 100, Varian, Australia).

Table 3.1 Characterization of raw domestic, dairy and paper mill wastewaters used in this study

Parameters	Domestic Wastewater	Dairy wastewater	Paper mill wastewater
COD (mg/L)	571	2104	3038-3250
TSS (mg/L)	188	5.46	256
Nitrogen (mg/L)	13.4	289	28
Total P (mg/L)	12.1	5.3	3
pH	7.8	6.3	8.4
Colour	Black to dark grey	Dark grey	Dark brown
Smell	Pungent	Pungent	Pungent

3.2.1.2 Screening and optimization of wastewater for fungal chitosan production

For batch fungal fermentation using the different wastewaters as the substrate, initial experiments were carried out using Erlenmeyer flasks containing wastewater supplemented with mineral salt media (MSM). Composition of MSM (g/L) is as follows: NH_4Cl (1.34), K_2HPO_4 (1.74), $MgCl_2{\cdot}6H_2O$ (0.2), $CaCl_2{\cdot}2H_2O$ (0.07), K_2SO_4 (0.174), NaCl (0.058) and trace elements. The medium was added to the respective wastewaters in 1:9, 1:3, 1:2 and 1:1 ratios, and labelled as MSM (9:1), MSM (3:1) MSM (2:1) and MSM (1:1), respectively. This addition was done to supplement the wastewaters with N-source and minerals required for maximum COD uptake and chitosan production by the fungal biomass. Different N-sources were employed to investigate their effect on overall growth and chitosan production by the fungi. Physical parameters such as pH, temperature and agitation rates were optimized for the batch experiments. All the batch experiments were carried out by incubating the flasks at optimised conditions of 180 rpm and 28 °C for 3 days, and samples were collected at every 12 h intervals. Following batch fermentation, the fungal biomass was harvested by filtration, washed thoroughly and dried overnight. Dry weight of the biomass was estimated prior to chitosan extraction.

3.2.1.3 Chitosan extraction

Chitosan extraction from the fungi consisted of two major steps (Chatterjee et al., 2005): the first step involved recovery of chitin/chitosan rich part of the fungal mycelia from the biomass by a mild alkali treatment using 1M NaOH at 120 °C for 20 min and during which alkali soluble carbohydrates, lipids and proteins in the biomass were dissolved in the alkali solution. The second step involved an acid treatment for the extraction of acid soluble chitosan from the alkali insoluble material (AIM) and during which chitosan is selectively dissolved in the acid, which is then recovered by increasing the pH of the solution to 8-9. The precipitated chitosan is then washed with water, ethanol and acetone, in a step wise manner, and finally dried at 65 °C for 12-24 h.

3.2.1.4 Effect of acetic acid on fungal chitosan production

Acid hydrolysate has been reported to enhance chitosan yield from *Rhizopus sp* (Tai et al., 2010). Hence, in order to assess its effect on chitosan production by *P. citrinum*, acetic acid, which is a main component of such hydrolysates, at a concentration in the range 20-150 mg/L was added to paper mill wastewater that showed maximum chitosan production compared with the other wastewaters. Biokinetics of *P. citrinum* biomass growth on paper mill wastewater in the presence of acetic acid as an inducer was later studied by fitting the experimental data on substrate utilization, product formation and growth of *P. citrinum* biomass to the following kinetic models (equations 3.1-3.3) reported in the literature for a better understanding on the effect of acetic acid in this study (Table 3.2).

$$\mu = \frac{1}{b}\frac{db}{dt} \tag{3.1}$$

$$V = \frac{1}{c}\frac{dc}{dt} \tag{3.2}$$

$$P = \frac{1}{z}\frac{dz}{dt} \tag{3.3}$$

In the above equations μ is the specific growth rate (1/h), V is the Substrate consumption rate (g/L·h), P is the Chitosan production rate (mg/L·h), b is the biomass concentration (g/L), c is the substrate concentration (g/L) and z is the chitosan concentration (mg/L) (Arun et al., 2017; Gopinath et al., 2011; Naveena et al., 2016).

Table 3.2 Various biokinetic models considered to study the effect of acetic acid concentration on: (a) specific growth rate, (b) substrate uptake rate and (c) chitosan production rate (Vergara-Fernández et al., 2018).

S. No.		Biokinetic models	Eq. nos.
1	Monod	$\left[\mu\,(or)\,V\,(or)\,P\right]=\mu_{\max}\,(or)\,V_{\max}\,(or)\,P_{\max}\left[\frac{x}{x+K_S}\right]$	3.4
2	Haldane	$\left[\mu\,(or)\,V\,(or)\,P\right]=\mu_{\max}\,(or)\,V_{\max}\,(or)\,P_{\max}\left[\frac{x}{x+K_S+\frac{x^2}{K_I}}\right]$	3.5
3	Andrews	$\left[\mu\,(or)\,V\,(or)\,P\right]=\mu_{\max}\,(or)\,V_{\max}\,(or)\,P_{\max}\left[\frac{x}{\left(x+K_S\right)\left(1+\frac{x}{K_I}\right)}\right]$	3.6
4	Webb	$\left[\mu\,(or)\,V\,(or)\,P\right]=\mu_{\max}\,(or)\,V_{\max}\,(or)\,P_{\max}\left[\frac{x\left(1+\frac{x}{K}\right)}{x+K_S+\frac{x^2}{K_I}}\right]$	3.7
5	Yano	$\left[\mu\,(or)\,V\,(or)\,P\right]=\mu_{\max}\,(or)\,V_{\max}\,(or)\,P_{\max}\left[\frac{x}{x+K_S+\frac{x^2}{K_I\left(1+\frac{x}{K}\right)}}\right]$	3.8

*Where, μ_{max}, V_{max}, P_{max}, x, K_S, K_I and K in these equations represent, maximum specific growth rate (1/h), maximum consumption uptake rate (g/L·h), maximum chitosan production rate (mg/L·h), acetic acid concentration (mg/L), Half saturation constant (mg/L), acetic acid inhibition constant (mg L^{-1}) and kinetic parameter (mg/L), respectively.

For xylose reductase (XR) and xylitol dehydrogenase (XDH) activity, a sample of biomass taken during the experiment was subjected to lysis and the homogenate was centrifuged at 7000×g for 10 min. The supernatant was collected and further used for the assay. Xylose reductase (XR) and xylitol dehydrogenase activity (XDH) were estimated spectrophotometrically (340 nm) at 25±1 ºC (Lima et al., 2004). The reactant mixture for determining XR activity consisted of NADPH (3 µM), xylose (5mM), phosphate buffer (0.1 M) at pH 7.2 and β-mercaptoethanol (0.1 M). For XDH activity determination, the assay mixture consisted of NAD^+ (2.5 µM), xylitol (5 mM), Tris-buffer (0.5 M) at pH 8.6 and β-mercaptoethanol (0.1 M). Specific activities of the enzymes were determined by measuring its total protein content (Kruger, 2009).

3.2.2 Batch bioreactor experiments

Batch bioreactor experiments to study fungal chitosan production using paper mill wastewater as the substrate was carried out using a 3L fermenter (Bio Console ADI 1025, Applikon Biotechnology, Holland) (Fig 3.1). Paper mill wastewater was supplemented with MSM (composition provided in section 3.2.1.2) containing ammonium chloride as the N-source. The effect of agitation and aeration rates on the biomass and chitosan yields were investigated along with the changes in morphology of the fungi in the medium. The bioreactor experiments were performed at 28 °C temperature, 200 rpm agitation speed, 2 vvm aeration rate, 1 L working volume and pH 4.5. Spore suspension containing 2×10^6 spores/ml was used as the inoculum in these bioreactor experiments. Samples were taken from the bioreactor at 12-h intervals for the analyses of COD, biomass and chitosan. Chitosan extracted from the fungal biomass was expressed as dry weight (Chatterjee et al., 2005).

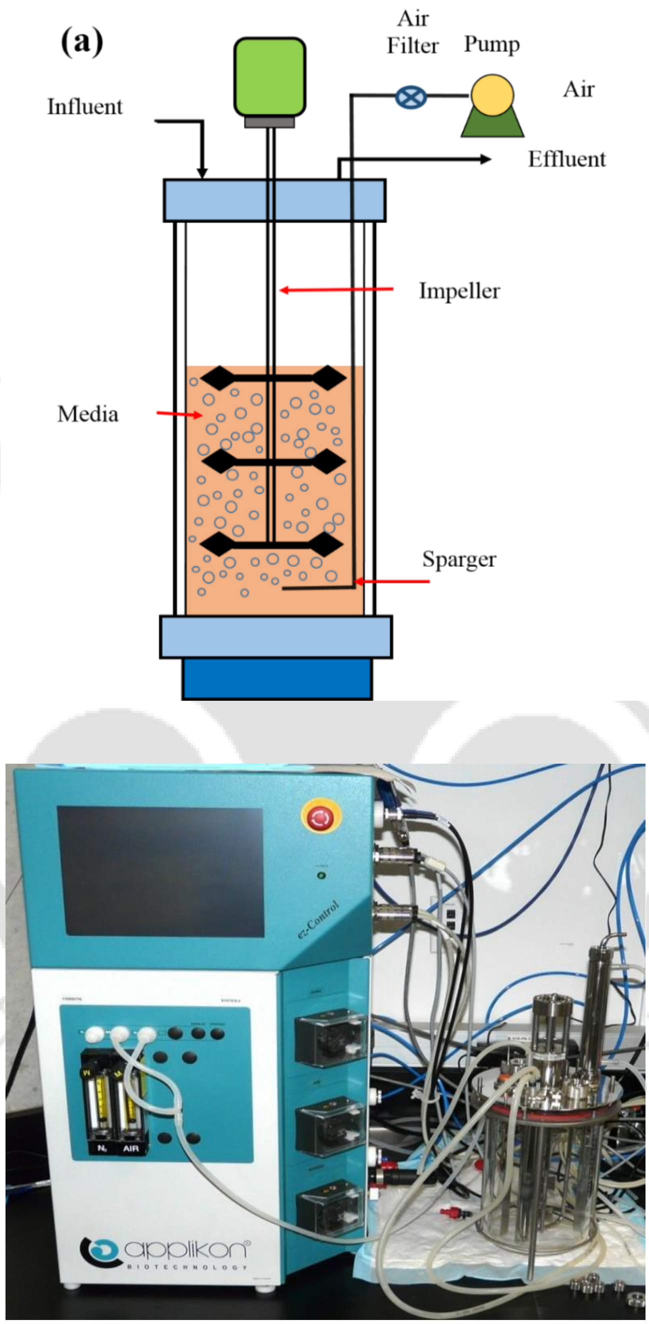


Fig. 3.1 (a) Schematic and (b) image of the stirred tank fermenter with control for fungal chitosan production for paper mill wastewater using *P. citrinum.*

Biokinetic constants, viz. specific growth rate (μ) and biomass yield ($Y_{X/S}$) were calculated from the batch growth profile and, accordingly, the feed flow rate for operating the bioreactor under fed-batch mode was estimated using the following equation (3.9). All the continuous experiments were carried out until three consecutive steady state COD values were obtained. Sample analyses were performed in triplicate and the results reported are average of three values. Chitosan extraction from the fungi was carried out as discussed in section 3.2.1.3.

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{\frac{X}{S}} S_0} \tag{3.9}$$

3.3 Fungal chitosan production from hemicellulose hydrolysate

Hemicellulose hydrolysate, which predominantly consists of xylose, was investigated as substrate for chitosan production by *P. citrinum*, such that external addition of acetic acid is not required. Fermentations were carried out in bioreactors operated under batch and fed-batch mode.

3.3.1 Batch shake flask experiments

From the previous experiments on screening of different industrial wastewater for fungal chitosan production, rapid utilization of xylose along with enhanced chitosan production by *Penicillium citrinum* were the direct result of adding acetic acid to paper mill wastewater. Hence, hemicellulose hydrolysate was chosen as an ideal substrates for chitosan production due to the fact that ~70% of hemicellulose hydrolysate is made up of xylose, which is the preferred sugar for *P. citrinum* biomass. Moreover, external addition of acetic acid is not required as it is the major by-product of acid hydrolysis of almost all lignocellulosic feedstocks.

Assam, being one of the leading rice producing states in India generates of rice straw as waste in large amounts, and most of which is incinerated in the open, contributing to the already high levels of air pollution. Rice straw was, therefore, chosen as a suitable feedstock for chitosan production by *P. citrinum* biomass in this study. Rice straw was collected from paddy fields nearby a village in North Guwahati, Assam, India. The straw was cut to 1-2 cm pieces prior to hydrolysis. Acid hydrolysis was then carried out by adding dilute sulphuric acid (2% v/v) to rice straw at 10% (w/v) solid loading rate, followed by autoclaving the mixture at 120 ºC for 1 h and the supernatant (hydrolysate) was collected by vacuum filtration and stored at 4 ºC until further use. The hydrolysate was detoxified by overliming through the addition of calcium hydroxide, which increased the initial pH to 10. The pre-treated mixture was agitated at 60 ºC for 1 h prior to filtration and the liquid fraction obtained after filtration of the sludge was neutralised by adding H_2SO_4 to set the pH to 4.5 for fungal fermentation.

Xylose, glucose, arabinose, acetic acid and furfurals in the rice straw hydrolysate (RSH) was measured by high performance liquid chromatography (Shimadzu, Tokyo, Japan) with Bio-Rad Aminex HPX-87H column (Bio-Rad, USA) coupled with UV and IR detectors. 5 mmol H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml/min for the HPLC analysis. Table 3.3 presents the values of the various components in the RSH before and after detoxification with calcium hydroxide.

Table 3.3 Composition of RSH before and after detoxification

Components (g/L)	Before detoxification	After detoxification
Xylose	24.6	21.3
Glucose	4.3	3.7
Arabinose	3.9	3.0
Acetic acid	1.2	1.1
Furfural	0.45	0.19

3.3.2 Bioreactor studies

Batch bioreactor experiments to study fungal chitosan production using rice straw hydrolysate as the substrate was carried out using a 3L fermenter (Bio Console ADI 1025, Applikon Biotechnology, Holland). The effect of agitation and aeration rates on the biomass and chitosan yields were investigated. The bioreactor experiments were performed at 28°C temperature, 220 rpm agitation speed, 2.5 vvm aeration rate, 1 L working volume and pH 4.5. Spore suspension containing 2×10^6 spores/ml was used as the inoculum in these bioreactor experiments. Samples were taken from the bioreactor at 12-h intervals for the analyses of COD, biomass and chitosan. Chitosan extracted from the fungal biomass was expressed as dry weight (Chatterjee et al., 2005). Sample analyses were performed in triplicate and the results reported are average of three values.

3.4 Fungal chitosan production using agricultural and industrial residues

Rice straw, paper mill sludge and citrus peels were investigated for their suitability for fungal chitosan production by *P. citrinum*. Optimization of various parameters such as particle size and moisture content were done using Erlenmeyer shake flasks sealed with cotton plugs prior to its scale up in a lab scale tray fermenter.

3.4.1 Screening of agricultural and industrial residues for fungal chitosan production

Three agricultural and industrial wastes viz., rice straw (RS), sweet lemon peels (CP) and paper mill sludge (PMS) were examined for chitosan production by *P. citrinum*. All the substrates were collected locally from Guwahati, India. Cellulose, hemicellulose and lignin were measured by the methods described in National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008). 1 g biomass was mixed with 27 N H_2SO_4 and stirred for 1 h at 30 ºC followed by the addition of distilled water to reduce its concentration to 1.5 N. The mixture was then autoclaved and subsequently filtered at room temperature. The residual filtrate obtained was neutralised with 1 M calcium carbonate to estimate the glucose content. Cellulose content was calculated from the glucose content as 1 g of glucose corresponds to 1.1 g of cellulose. Hemicellulose content corresponded to the remaining amount present in the filtrate (Sluiter et al., 2008). The composition of the solid substrates are summarised in Table 3.4.

Table 3.4 Composition of different solid substrates

Parameter	Rice straw	Paper mill Sludge	Citrus peel
Cellulose	34.1	17.9	19.7
Hemicellulose	27.3	4.3	18.4
Lignin	11.7	26.3	8.6
Pectin	2.1	-	13.2
TKN	0.45	5	0.67
Ash	12.1	32	3.4

For screening of the solid substrates for fungal chitosan production, the solid substrates were cut into 1-2 cm pieces and placed in 250 mL sterilised Erlenmeyer flasks. Mineral salt solution containing a spore suspension of 2×10^6 spores/mL was then added to these substrates in a 3:1 ratio to achieve an initial moisture content of 60%. Fermentation was carried out under static and acidic pH (4.5) conditions. Samples were collected everyday for the period of fermentation to estimate the profile of chitosan production. These experiments were performed in triplicates and the values reported are average of three sample analyses.

3.4.2 Fungal chitosan production using rice straw

Rice straw is made up of cellulose (~34%), hemicellulose (~27%) and lignin (~11.7%) which are bound together by covalent and hydrogen bonds. The amount of lignin present in rice straw is the lowest among all the lignocellulosic residues. *P. citrinum* is shown to utilise lignocellulosic substrates efficiently, therefore, rice straw was investigated for an economically viable route of fungal chitosan production.

3.4.2.1 Shake flask studies

Effect of pre-treatment, particle size, moisture content and acetic addition were investigated using sterilised 250 mL Erlenmeyer flasks followed by fermentation in lab scale fermenters.

Effect of pre-treatment

Different pre-treatment techniques have been employed on lignocellulosics to disintegrate the crystalline structure formed by lignin, cellulose and hemicellulose polymers. However, among these components, alkaline and acid pre-treatment have been very well reported to yield better results for solid-state fermentation of lignocellulosic substrates by fungi. Alkaline treatment in this study was carried out using 2% NaOH at room temperature for 24h. Acid treatment included 2% H_2SO_4 at 60 °C for 3 h. These substrates were washed thoroughly to achieve a neutral pH and weighed prior to addition of MSM followed by inoculation with the spore suspension (2×10^6 spores/ml). The flasks were incubated at 28ºC for 9-10 days for proper growth. Composition of MSM (g/L) is as follows: NH_4Cl (1.34), K_2HPO_4 (1.74), $MgCl_2 \cdot 6H_2O$ (0.2), $CaCl_2 \cdot 2H_2O$ (0.07), K_2SO_4 (0.174), NaCl (0.058) and trace elements.

Effect of particle size and moisture content

Particle size is an important parameter which directly influences success of a solid-state fermentation process. Mass transfer limitations are a serious drawback in SSF, which depend on the thickness and porosity of the substrate layer. Rice straw was chopped to obtain different size ranges 1-2 cm and 4-5 mm and 1-2 mm, and denoted by PS1, PS2 and PS3, respectively. A powdered form (<1 mm) of the straw was obtained by grinding it in a mixer grinder, which was denoted as PS4.

Moisture content is another vital parameter of concern as the fungi requires water for its growth and metabolism. Chitosan production by *P. citrinum* was investigated by varying the moisture content from 50-80%.

Effect of acetic acid on chitosan production

The effect of acetic acid on the biomass growth and chitosan production by the fungal isolate *P. citrinum* on rice straw was investigated at different concentrations in the range 50-120 mg/L. Acetic acid was added to the MSM solution to adjust its pH to 4.5 before its addition to the rice straw.

3.4.2.2 Bioreactor experiments

A lab scale airtight tray fermenter with three trays (12cm×6.5cm) was used for chitosan production by *P. citrinum* biomass as shown in Fig. 3.2. The trays were perforated by making 1mm diameter holes for achieving efficient diffusion of air through the substrate bed. Volume of each tray was 6.8×10^2 cm^3 with a working volume of 2×10^2 cm^3. The fermenter was sterilized by autoclaving prior to the addition of the substrate to the trays, spread evenly. The fermentation was carried out at 28 °C temperature by supplying filtered and humidified air at a flow rate of 0.6 L/min outside the trays. The fermentation was carried out for 8-9 days followed by chitosan extraction from the biomass.

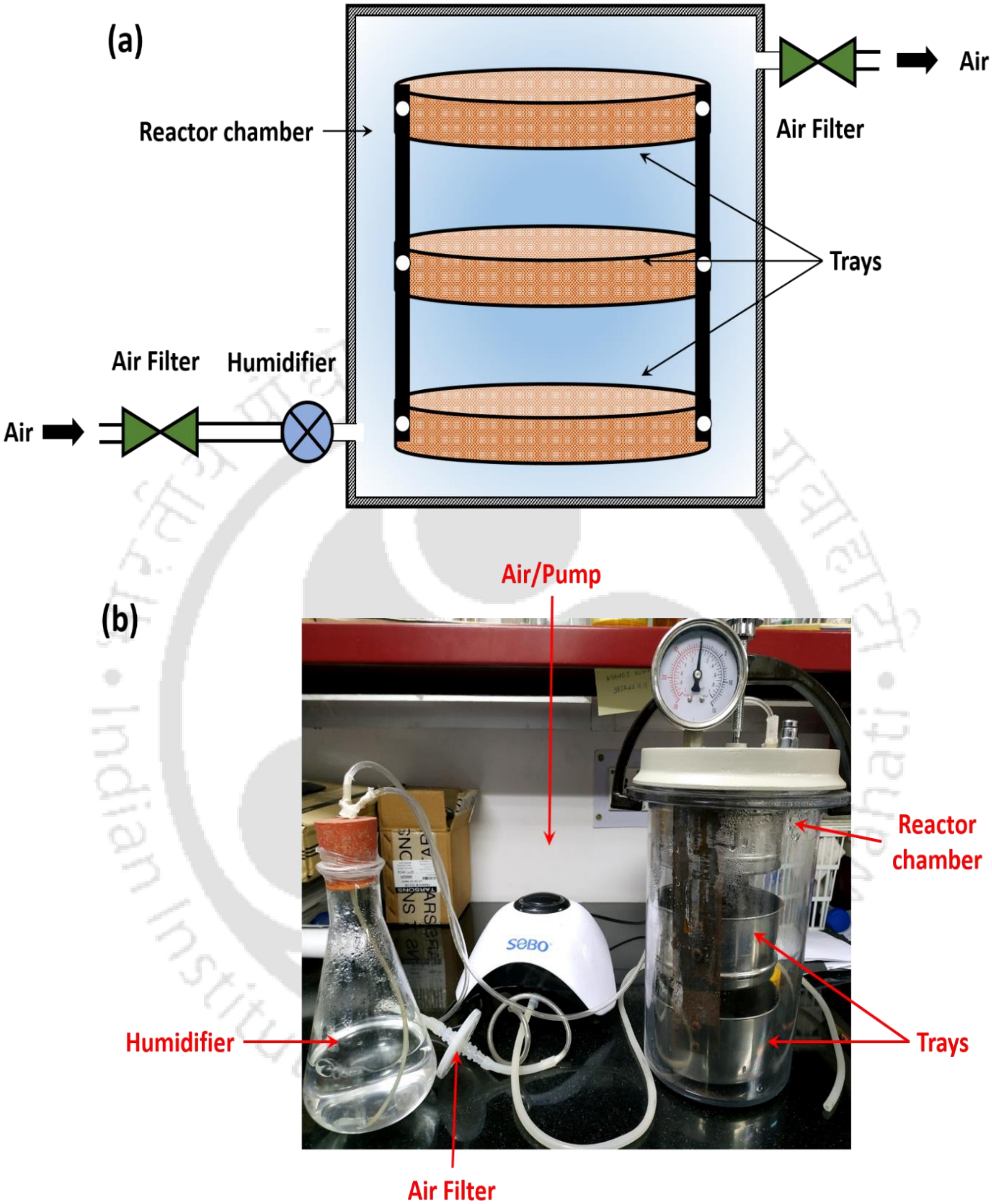


Fig. 3.2 (a) Schematic and (b) image of lab scale tray fermenter used for chitosan production by *P. citrinum* using rice straw as the substrate

3.4.3 Fungal chitosan production using paper mill sludge and paper mill wastewater

Penicillium citrinum showed promising results for chitosan production when grown on paper mill sludge. Results obtained from the previous screening experiments showed that the chitosan yield values are similar for both rice straw and paper mill sludge. Although paper mill sludge contain a large amount of cellulose, it could not be efficiently utilised was not able to utilise it efficiently even after alkali pre-treatment, which could be attributed to the fact that lignin was the major component of the sludge (Table 3.3). In order to overcome this problem, microwave assisted alkali treatment was applied to release the sugars present. Other parameters such as moisture content and particle size were also optimized as described earlier in section 3.4.2.1. As *P. citrinum* biomass was able to utilize paper mill wastewater efficiently under submerged fermentation conditions, the effect of adding of paper mill wastewater supplemented with MSM, to the paper mill sludge for maintaining the initial moisture content of the substrate was further investigated. Different concentrations of the wastewater and MSM (5%, 10%, 15%, 20% and 25%) were examined for achieving maximum biomass growth and chitosan production.

3.4.4 Chitosan extraction

Chitosan extraction from the fungal biomass grown on solid substrate was carried out according to previous reports with some minor modifications (Kleekayai and Suntornsuk, 2011). Fermented cakes in the fermenter were collected and mixed properly. NaOH (1M) was then added to the fermented cakes in a 1:25 solid to liquid ratio and autoclaved at 120 °C for 20 min for removal of alkali soluble components such as proteins and glucans. The insoluble residues obtained after the treatment was separated by filtration and washed with distilled water to set the pH to neutral. The alkali insoluble material (AIM) was subjected to an acid treatment using 2%

acetic acid (v/v) in a 1:40 solid to liquid ratio at 95 °C for 8 h. The acid insoluble fraction was discarded after filtration and the pH of the filtrate was adjusted to 10. Centrifugation of the filtrate was carried out at 12,000×*g* for 15 min to collect the precipitated chitosan. Chitosan in the pelleted form was washed with distilled water, ethanol (95% v/v) and acetone in a stepwise manner and by maintaining a solid to liquid ratio of 1:20 during the washing steps. Chitosan yield was reported by measuring its dry weight after drying at 60 °C overnight.

3.5 Chitosan characterization

Fungal chitosan obtained in this study was characterized by estimating its degree of deacetylation and its molecular weight. Fourier transform infrared spectrometer (Shimazdu, IR Affinity-1) was used for infrared spectroscopy analysis. Degree of deacetylation (DD) was calculated by using the following equation (Baskar and Kumar, 2009; Pal et al., 2016)

$$DD\ \% = 97.67 - \left(26.486\frac{A_{1655}}{A_{3450}}\right) \qquad (3.10)$$

where A_{1655} and A_{3450} are absorbance values at wavelengths 1655 cm^{-1} and 3450 cm^{-1}, respectively.

Ubbelohde viscometer was used to measure the intrinsic viscosity [η] of chitosan at 25±1 °C. A mixture containing 0.02 M acetic acid and 0.1 M sodium chloride was used as the solvent to dissolve chitosan at varying concentrations. The following Huggins equation (3.11) was used to calculate the chitosan intrinsic viscosity (ml/gm) (de Moura et al., 2011).

$$\frac{\eta_{sp}}{C} = [\eta] + K[\eta]^2 C \qquad (3.11)$$

where $\frac{\eta_{sp}}{C}$ is the reduced viscosity in ml/gm, η_{sp} is the relative viscosity of the polymer in solution and the solvent, which is dimensionless, C is the concentration of the solution in gm/ml and K is a dimensionless constant. The intrinsic viscosity values were used in the following Mark-Houwink-Sakurada equation (3.12) to estimate the viscosity average molecular weight (M_v) of chitosan in the study (de Moura et al., 2011).

$$[\eta] = KM_v{}^{\alpha} \tag{3.12}$$

where K = 1.81 × 10^{-3} ml/g and α = 0.93 are constants.

3.6 Synthesis, characterization and application of chitosan based nano biosorbents for heavy metal removal

In this study, magnetic nanoparticles coated with chitosan and carboxymethyl chitosan were synthesised for improving the biosorption efficiency and easy separation of the biosorbent for reuse.

3.6.1 Carboxymethyl chitosan synthesis

Carboxymethyl chitosan (CMC) was synthesised by following the method described by previously reported protocol by Kalliola et al. (2018) with some minor modifications. Fungal chitosan (2 g) and NaOH (2.7 g) were added to a mixture of water (12 mL) and 2-propanol (48 ml) and stirred continuously for 1 h at 60 ºC. After 1 h, chloroacetic acid (3 g) dissolved in 4 ml isopropanol, was added to the mixture dropwise and stirred continuously for 4 h at 60 ºC. The solid fraction was retrieved by centrifuging the mixture at 10,000×g for 10 min and washed several times with different concentrations of ethanol in the range 70-90%. The solid product was dried overnight at room temperature and purified by dissolving it in 100 ml of distilled water and discarding the insoluble fraction. Pure carboxymethyl chitosan precipitated out by the addition of absolute ethanol (800 mL) to the supernatant was subsequently washed with 70% and then with 100% ethanol. The final product was dried overnight at room temperature prior to grinding to obtain carboxymethyl chitosan in fine powder form.

3.6.2 Synthesis of magnetic iron nanoparticles

Co-precipitation method was used for the preparation of magnetic iron nanoparticle synthesis in which Fe^{3+} and Fe^{2+} ions co-precipitate under alkaline conditions (Rajput et al., 2016). Distilled water was first degassed and used for dissolving $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ in a 2:1 molar ratio by continuous stirring at 60 ºC under N_2. Addition of ammonium hydroxide dropwise to the mixture turned the solution to a black colour at a pH above 8.0. The black coloured Fe_3O_4 nanoparticles formed due to the co-precipitation reaction were separated by applying a magnetic field using a strong magnet, washed with deionised water 3-4 times and stored after drying in airtight containers.

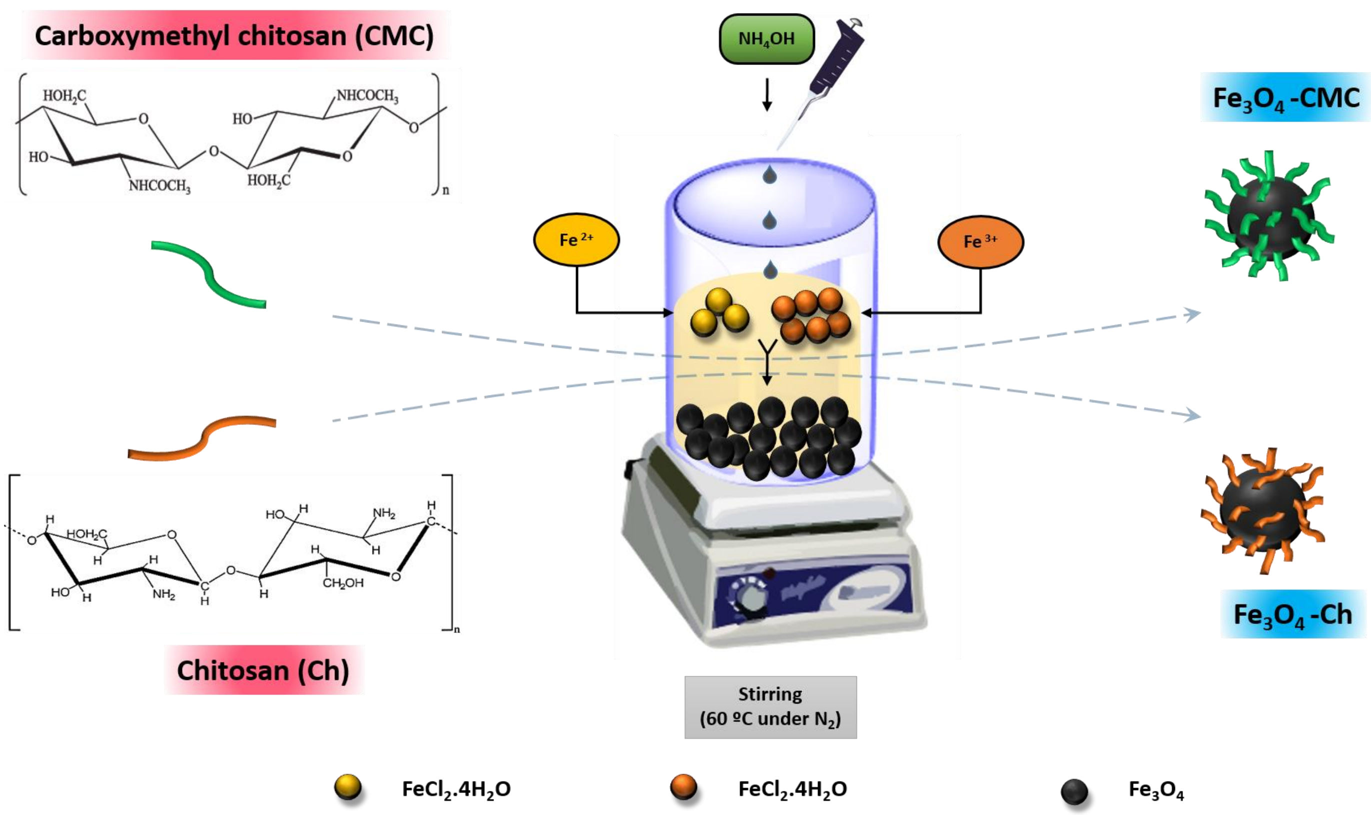


Fig. 3.3 Schematic showing synthesis of chitosan and carboxymethyl chitosan nano-biosorbents.

3.6.3 Synthesis of chitosan and CMC iron nanoparticles

The previously synthesised iron nanoparticles (100 mg) were dispersed in 100 mL of acetic acid (2% v/v) containing chitosan (0.2 mg/ml) and incubated at room temperature for 12 h under vigorous stirring to ensure even coating of chitosan on the nanoparticles (Zhu et al., 2008). The resulting chitosan iron nanoparticles (CNP) were washed and dispersed repeatedly in water to remove the uncoated chitosan following which the biosorbent was dried and stored in air-tight containers.

Carboxymethyl chitosan coated iron nanoparticles (CMCNP) were prepared using the method described by Zhou et al. (2006). Iron nanoparticles were dispersed in phosphate buffer A (pH 6) containing phosphate (0.003 mol L^{-1}) and NaCl (0.1 mol L^{-1}) followed by addition of 1 mL of carbodiimide solution and further ultrasonicated for 5 min. Carboxymethyl chitosan solution (50 g/L) was then prepared by dissolving CMC in buffer A and 5 mL of this solution was added to the previous mixture and mixed vigorously for 1 h. The CMCNP thus formed were separated by using a magnet and then washed with a mixture of ethanol and water repeatedly before drying. Figure 3.3 shows schematic of steps followed for the synthesis of CNP and CMCNP in this study.

3.6.4 Characterization of nanoparticles

Physical properties

Size and morphology of the synthesised nanoparticles was examined by field emission scanning electron microscope (FESEM) and field emission transmission electron microscope (FETEM) analyses. For FESEM analysis, the CNPs and CMCNPs were washed with sterile milliQ water and diluted 20 times. A small drop of this sample was then loaded onto aluminium stubs prepared by using double sided carbon tape and kept for drying at room temperature for 12 h. Double gold coating of the sample was done by a coating instrument (JEOL JFC-1300) prior to its observation under FESEM (Zeiss, Sigma,, Germany).

For FETEM analysis, a diluted nanoparticles sample was loaded on a copper grid coated with carbon and dried at room temperature overnight prior to its observation under FETEM (JOEL, 2100F, Japan)

Chemical properties

In order to confirm the successful coating of chitosan and carboxymethyl chitosan on to the magnetic iron nanoparticle surface, functional group analysis of the CNPs and CMCNPs was carried out by Fourier transform infrared spectroscopy (Nicolet, U.S., Thermo Scientific, USA) in the range of 4000-500 cm^{-1}.

X-ray diffractometer (XRD) (d8Advance, Bruker AXS, Germany) was used to analyse the nanoparticle structure and phase. The samples were scanned for $2\theta = 5 - 80^{o}$ with a scan rate of 0.05^{o} per 0.5 s and the profile was recorded at 45 kV and 4 mA using CuKα radiation.

Magnetic properties

Vibrating sample magnetometer (VSM-7410, Lake Shore Cryotronics, USA) was used to determine the magnetic properties of the Fe_3O_4 nanoparticles coated with chitosan and carboxymethyl chitosan. Hysteresis loop was plotted to determine the saturation magnetization of iron nanoparticles, CNPs and CMCNPs.

3.6.5 Adsorption kinetic studies

The adsorption experiments were done in batch method under the effect of some physicochemical parameters viz. variable pH of initial solution (2-10), initial heavy metal ion concentration (50-250 mg/L) and dosage of adsorbents (0.2-2 mg/ml). All the adsorption experiments were carried out in 250 mL Erlenmeyer flasks, agitated on a thermostatic shaking incubator at 120 rpm for a period of 4-6 hours. After the incubation period is over, the adsorbents are filtered out and the concentration of un-adsorbed Cr (VI) was determined by UV-Vis spectrophotometry at λmax. = 540 nm using diphenyl carbazide as a complexing agent in acidic medium. The concentrations of the Pb (II) ions were measured by atomic emission spectroscopy (AES) (Agilent, 4210 MP-AES, US).

Adsorption capacities of chitosan and carboxymethyl chitosan nanoparticles were calculated by the Eq (3.13).

$$q = \frac{(C_0 - C_t) \times V}{w} \tag{3.13}$$

Where q (mg/g) is the adsorption capacity, C_0 and C_t are the initial and final concentrations (mg/L) of metal ions in the solution, V is the volume (L) of the heavy metal solution and w is the mass of the nanoparticle (g) added. Pseudo-first order, pseudo-second order and intra-particle diffusion models were evaluated to evaluate the kinetic parameters of adsorption (Table 3.4).

Table 3.5 Models applied to evaluate the heavy metal sorption kinetics in the study

S. No.	Kinetic models*		Eq. nos.
1	Pseudo-first order	$\frac{dq_t}{dt} = k_1(q_e - q_t)$	3.14
2	Pseudo-second order	$\frac{dq_t}{dt} = k_2(q_e - q_t$	3.15
3	Intra-particle diffusion	$q_t = k_{id}t^{1/2} + c$	3.16

*Where, q_t (mg/g) and q_e (mg/g) = extent of heavy metal adsorbed by a biosorbent at time ‘t’ and in equilibrium respectively, k_1 (min^{-1}) = pseudo-first order rate constant, k_2 (g/mg min) = pseudo-second order rate constant, k_{id} (mg/g $min^{1/2}$) = rate constant for intra-particle diffusion and c (mg/g) = boundary layer thickness constant.

3.6.6 Sorption isotherm studies

Sorption isotherm studies of Cr (VI) and Pb (II) on both CNP and CMCNP were carried out by adding 0.1 g of biosorbents to individual heavy metal containing solutions in the range 25-200 mg/L. The mixture was then shaken at 250 rpm for 12 h equilibrium time and samples were collected for determining the residual heavy metal concentration in solution. The experimental data was fitted to various sorption isotherm models for understanding the heavy metal removal mechanism involved.

Table 3.6 Sorption isotherm models applied to study heavy metal removal by CNP and CMCNP

S. No.	Isotherm model		Eq. nos.
1	Langmuir	$q_e = \frac{Q_{LM} L C_e}{1 + L C_e}$	3.17
2	Freundlich	$q_e = F C_e^{1/n}$	3.18
3	Dubinin-Radushkevich	$q_e = Q_{DR} \exp^{-K_{DR}\varepsilon^2}$	3.19
4	Frumkin	$\frac{\theta}{1-\theta} e^{-2\alpha_{FK}\theta} = K_{FK} C_e$	3.20
5	Redlich-Peterson	$q_e = \frac{Q_{RP} K_{RP} C_e}{1 + K_{RP} C_e^g}$	3.21

*Where, q_e (mg/g) = amount of Cr(VI) adsorbed per unit adsorbent weight at equilibrium, Q_{LM} (mg/g) = maximum adsorption capacity, C_e (mg/L) = equilibrium concentration of the adsorbate, L (L/mg) = Langmuir isotherm constant, F (mg/g) = Freundlich constant for bio-sorption capacity; 1/n = empirical parameter that relates to bio-sorption intensity, Q_m (mg/g) = Dubinin-Radushkevich maximum bio-sorption capacity, K_{DR} (mol^2/J^2) = the mean free energy of bio-sorption, ε= the Polanyi potential in the equation $\varepsilon = RTln(1 + \frac{1}{C_e})$; R = universal gas constant i.e. 8.314 J/mol/K and T= temperature in Kelvin (K), K_{FK} = Frumkin constant, θ = fractional coverage of sorption sites, ($1-C_e/C_o$) and α_{FK} = interaction parameter for sorbate-sorbent interaction, Q_{RP}, K_{RP} and g = Redlich-Peterson constants.

3.6.7 Biosorbent reuse experiments

Desorption refers to the reversed adsorption process which involves the removal of the adsorbate from the adsorbents surface using a suitable desorption medium. The recyclability of

CNP and CMCNP was evaluated by continuous adsorption-desorption studies. For this study, CNPs and CMCNPs following heavy metal removal from solution, the heavy metal laden biosorbents (10 mg each) were washed gently with water and added to EDTA solution (10 mL) of concentration of 0.01 mol L^{-1} incubating at an ambient room temperature, for 24 h at 200 rpm on an orbital shaker. The CNPs and CMCNPs were separated by using a magnet and the ion concentration in the solution was measured as mentioned before. Once washed the treated biosorbents are dried and again used for next cycle of adsorption. All the adsorbents were tested for desorption studies for up to 4 cycles.

CHAPTER FOUR

RESULTS & DISCUSSIONS

4. Results and Discussions

Results obtained for fungal chitosan production from different wastewater and hemicellulose hydrolysates, particularly paper mill wastewater, mechanism of induction by acetic acid with kinetics and bioreactor studies were discussed in this chapter. Solid state fermentation of agro-industrial wastes such as rice straw and paper mill sludge were also discussed in this section along with the application of fungal chitosan nanoparticle as a nano-biosorbent for heavy metal ions

4.1 Identification of novel fungal isolate

Fungal chitosan production has received increased attention recently due to a number of significant advantages. Whereas the supply of crustacean wastes are limited by the sites of fishing industry as well as seasons, fungal fermentation processes are devoid of any geographical or seasonal limitation. Additionally, fungi can grow on a variety of waste materials, which makes it environmentally acceptable. Therefore, the aim was to isolate fungal strains, which could easily utilise waste substrates for enhanced chitosan production.

4.1.1 Morphological identification

The isolated fungi, grown on PDA showed a dark green colour on the surface and was pale yellowish on the reverse, with a powder like granular surface. Microscopic analyses revealed that the isolate had symmetric biverticillate conidiophore with smooth stipes and small, globose conidia. Fig. 4.1 shows the fungal mycelia along with spheroidal spore showing a septate hyphae and sporangiophore attached to it. These features revealed that the fungal isolate belonged to *Penicillium* spp., which was further confirmed by molecular analysis.

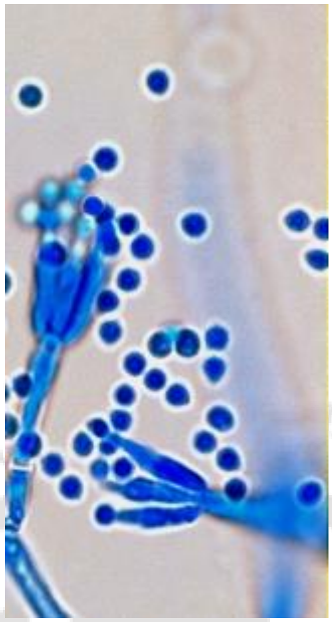


Fig. 4.1 Microscopic characteristics of *Penicillium citrinum*

4.1.2 Molecular Identification

For molecular analysis of the fungal isolate, its genomic DNA was extracted and amplified by PCR. Fig. 4.2 show the extracted genomic DNA and the PCR amplified product which was later sent for sequencing based on Sanger dideoxy sequencing method. Based on the sequence variation present in 18s rRNA of the fungal isolate by carrying out BLAST nucleotide analysis, the isolate was found to be phylogenetically related to members of the genus *Penicillium citrinum*. Phylogenetic tree was assembled using the neighbour joining method and the isolated strain has a close relation to *Penicillium citrinum* strain ZAS3 (99%) (Fig. 4.3). The gene sequence has been submitted to the NCBI gene database with the accession number MH392275.

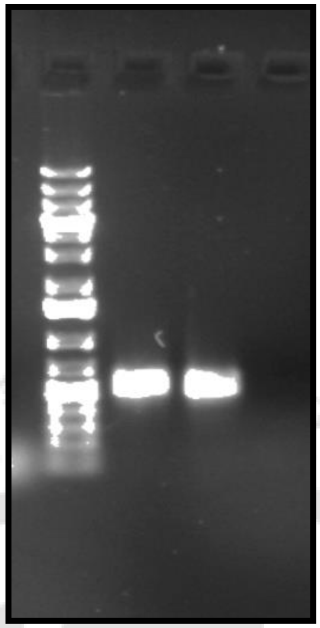


Fig. 4.2 Electropherogram showing the genomic DNA extracted from *Penicillium citrinum* and the PCR amplified DNA of the ITS regions.

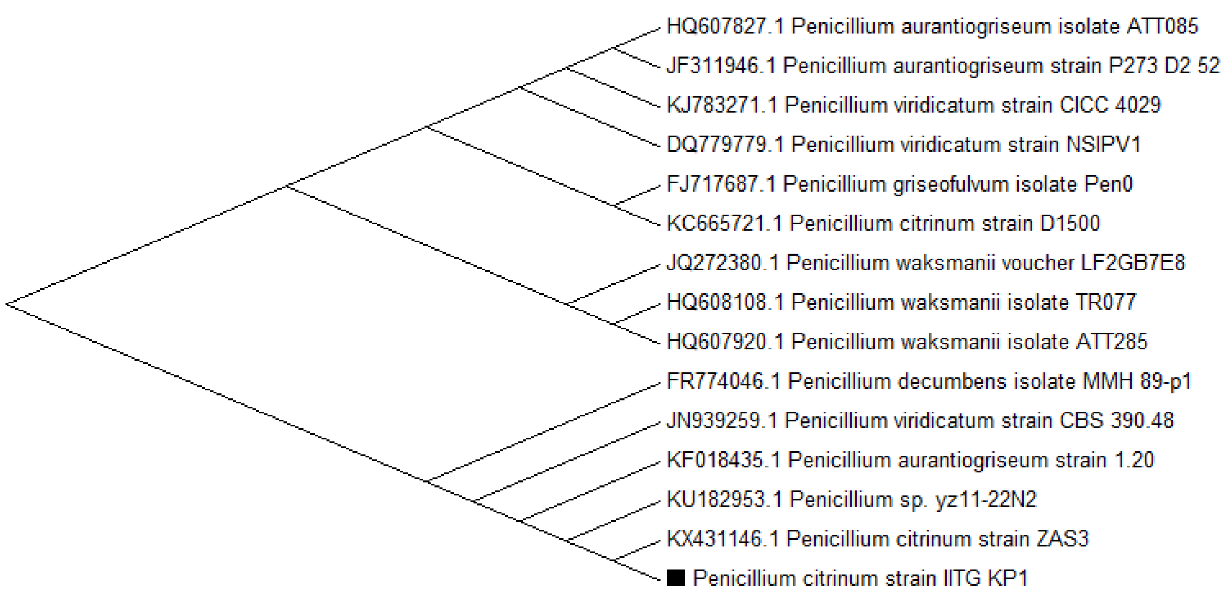


Fig. 4.3 Phylogenetic relation between the fungal isolate identified as *Penicillium citrinum* IITG_KP1 strain and the various *Penicillium* species reported for chitosan production.

4.2 Influence of N-sources and physical parameters on biomass growth and chitosan production by *P. citrinum*

Optimum nutrient and culture conditions are vital for fungal growth and chitosan production was discussed. For this, the different N-sources, pH, temperature and agitation rates were investigated for fungal growth and chitosan production.

4.2.1 Effect of different N-source

Nitrogen is an essential component for proper fungal growth and chitosan production. Different N-sources were added to the mineral salt media with glucose (4 g/L) as the carbon source to investigate its effect on the biomass growth and chitosan production by *P. citrinum* biomass. Among the various N-sources tested, ammonium chloride, yeast extract and peptone yielded similar results in terms of biomass growth and chitosan production (Fig. 4.4). A maximum biomass production of 1.48 g/L was achieved by using yeast extract as the N-source, followed by 1.46 g/L with ammonium chloride. Maximum chitosan production of 176 mg/L and 177 mg/L was obtained when the media was supplemented with ammonium chloride and peptone, respectively. However, compared with peptone or yeast extract, ammonium chloride is a simple inorganic substrate that is easily assimilated by the microorganism. Hence, it was selected for further experiments.

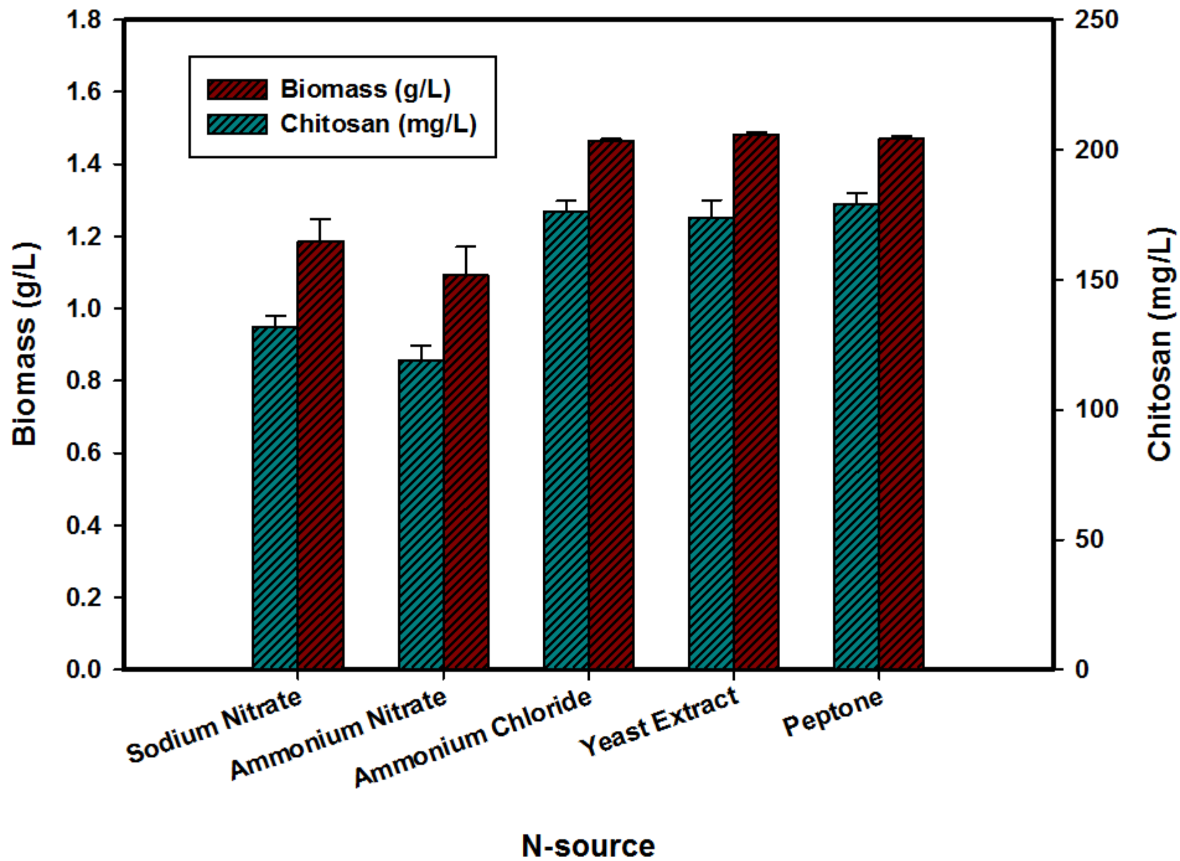


Fig. 4.4 Effect of different N-source on biomass growth and chitosan production by *P. citrinum*

4.2.2 Effect of pH

Optimum pH for the growth of filamentous fungi is generally in the acidic range. However, several reports have shown that maximum growth of filamentous fungi is achieved in the pH range 3.0-8.0 (Pitt and Hocking, 2009; Wheeler et al., 1991). Fig. 4.5 shows that *P. citrinum* has an optimal pH in the range 4.0-5.5; maximum biomass and chitosan production of 1.78 g/L and 210 mg/L are obtained at pH 4.5. These results are consistent with the literature on the effect of pH on growth of various *Penicillium* species (Thompson et al., 1993). All further experiments were therefore carried at pH 4.5 using *P. citrinum* for chitosan production.

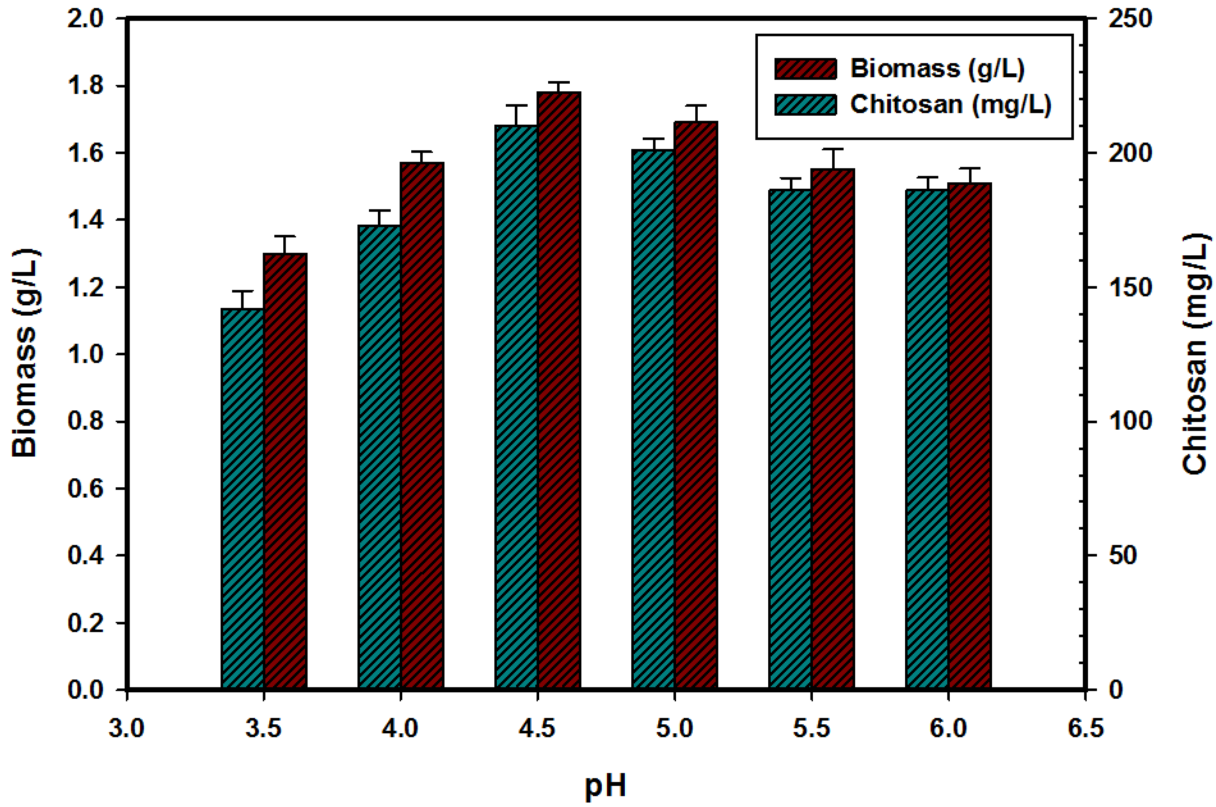


Fig. 4.5 Effect of pH on biomass growth and chitosan production by *P. citrinum*

4.2.3 Effect of temperature

From Fig. 4.6, which shows the effect of temperature on fungal growth and chitosan production by *P. citrinum*, the optimum temperature is in the range 25-30 °C. This is in agreement with the fact that most of the filamentous fungi grow on food wastes and non-refrigerated organic matter. Maximum biomass growth of 1.87 g/L was achieved at 28 °C, which also corresponded well with the maximum chitosan production of 214 mg/L. In some studies, the optimum temperature for *P. citrinum* biomass growth is 30 °C (González et al., 1988; Montani et al., 1988), which is very close to the findings in this study.

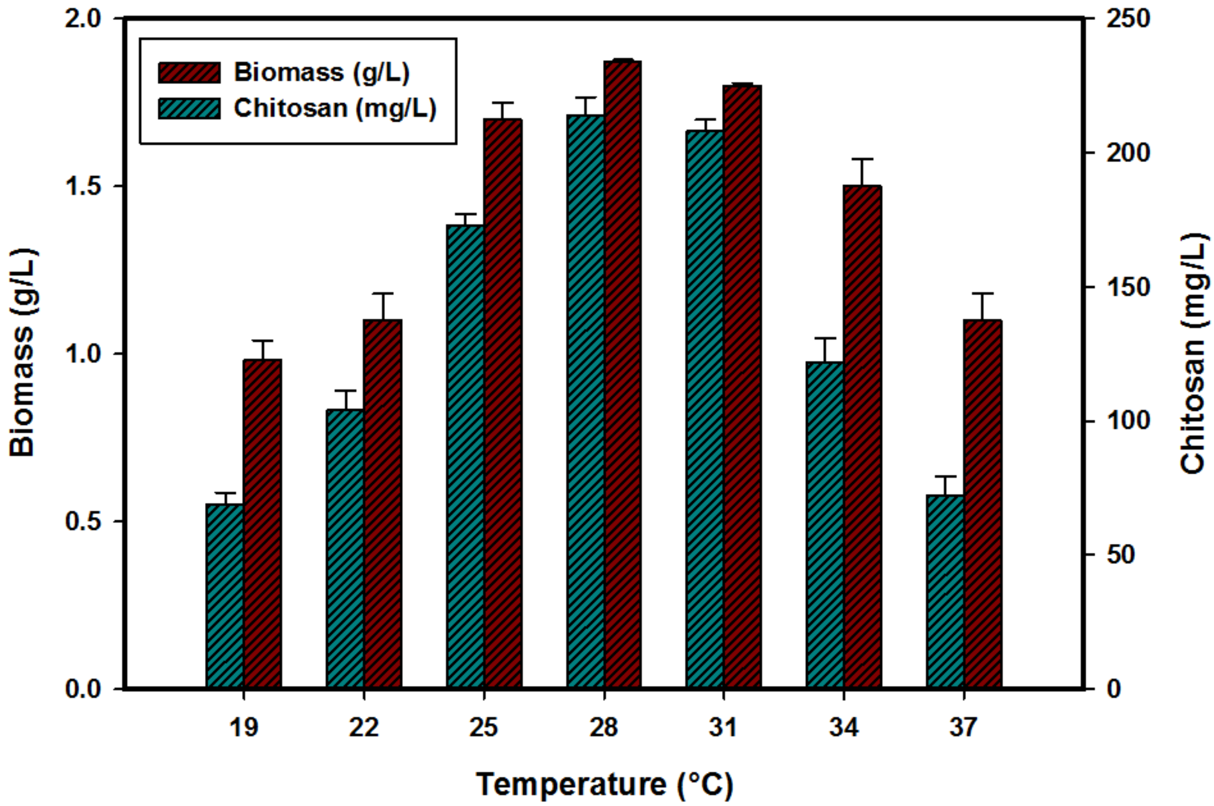


Fig. 4.6 Effect temperature on biomass growth and chitosan production by *P. citrinum*

4.3 Fungal chitosan production using wastewater

Fungal chitosan production has been predominantly been carried out in nutrient rich media which although results in high chitosan yields but renders the process economically unviable. Utilization of organic wastes for fungal fermentation is a promising option for a sustainable route for chitosan production. High organic loading in wastewaters results in low nutritional requirements for the fungi to grow on. In addition, a net positive energy gain along with the above advantages makes wastewaters an attractive option for a clean and sustainable process for fungal chitosan production. For this, different wastewaters were screened and optimized for fungal chitosan production. The effect of acetic acid as an inducer was studied in detail followed by bioreactor studies were carried out under batch, fed-batch and continuous modes.

4.3.1 Screening of wastewaters for fungal chitosan production

Domestic, dairy and paper mill wastewaters were evaluated as alternate cheap carbon source for chitosan production by *P. citrinum.* All the experiments were carried out using Erlenmeyer flasks for 72 h by pre-adjusting the solution pH to 4.5.

Among the different wastewater evaluated in this study, a high COD removal by *P. citrinum* was observed with domestic wastewater as the substrate, which could be because domestic wastewater, in general, contains simple compounds that are easily assimilated by the fungi (Fig. 4.7). However, a low chitosan production is attributed to a low COD content of the wastewater (Table 3.1), which limited the fungal biomass growth. Although the biomass increased even after 36 h of culture, the chitosan content was very less, suggesting the presence of other contaminating microorganisms in the wastewater mainly because it was not subjected to any kind of pre-treatment prior to the fungal fermentation. This was further confirmed by determining the value of the most probable number (MPN) (1200) of the raw domestic wastewater. Thus, using domestic wastewater as the substrate 85-90 % COD removal was achieved along with a chitosan production of 9 mg/L and 13 mg/L for *C. elegans* and *P. citrinum*, respectively.

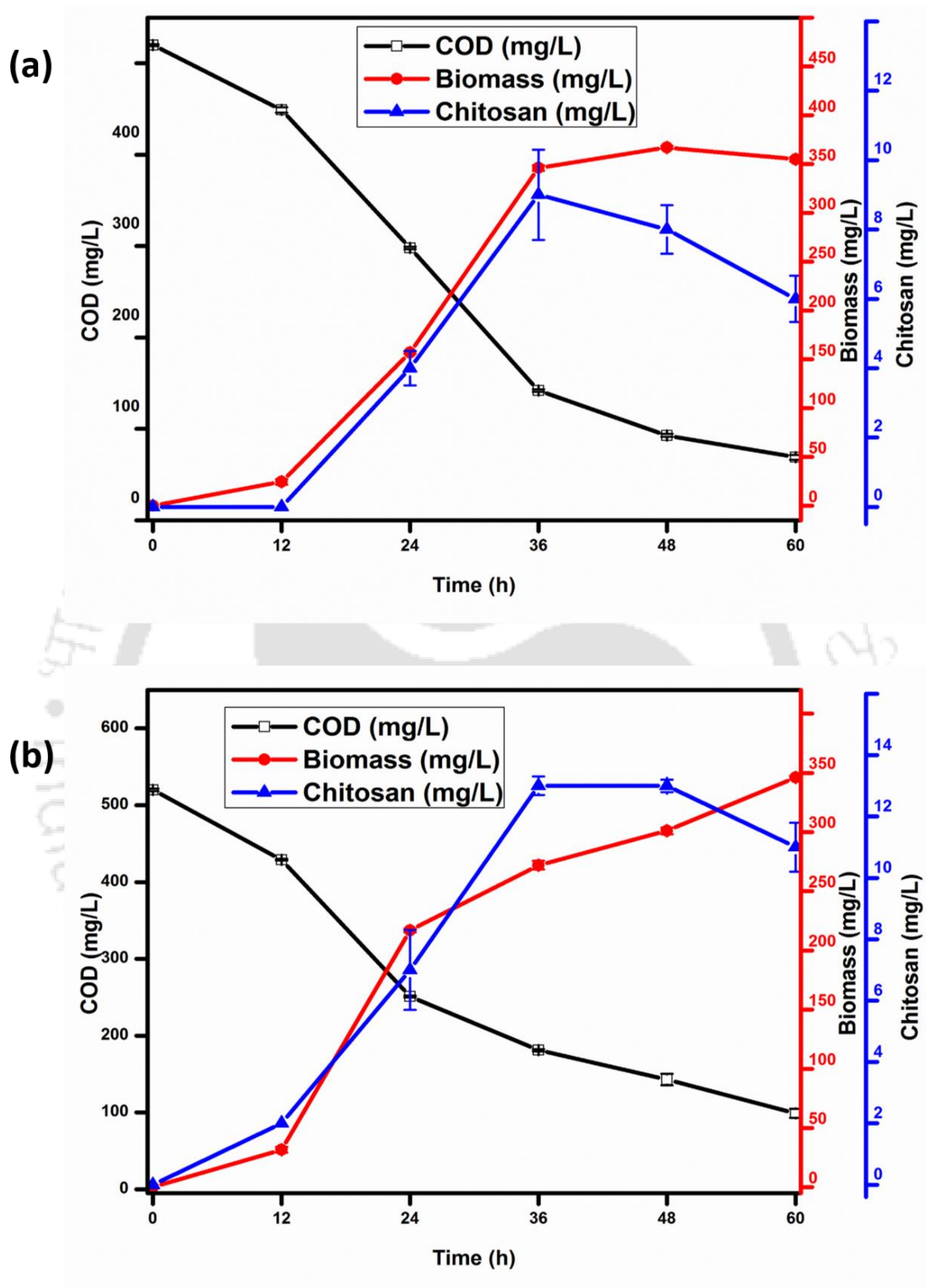


Fig. 4.7 Time profile of biomass growth, chitosan production and COD removal by (a) *C. elegans* and (b) *P. citrinum* using domestic wastewater as substrate.

Fungal fermentation of dairy wastewater showed a maximum chitosan production of 50 mg/L with a COD removal efficiency of 53%, suggesting that the COD present in the wastewater was not easily utilisable by the fungi (Fig. 4.8). Maximum chitosan production of 50 and 41 mg/L were obtained by *P. citrinum* and *C. elegans*, respectively. Thus, *P. citrinum* showed better results in terms of chitosan production as well as COD removal than *C. elegans* with dairy wastewater as the substrate. Dairy wastewater is known to contain complex sugars and fats which are not easily assimilated by the fungi for its growth. Additionally, the low COD removal and low chitosan production towards the end of the fermentation is attributed to microbes other than the fungi initially present in the wastewater (Oragui et al., 1993); the MPN value of this wastewater was 1100.

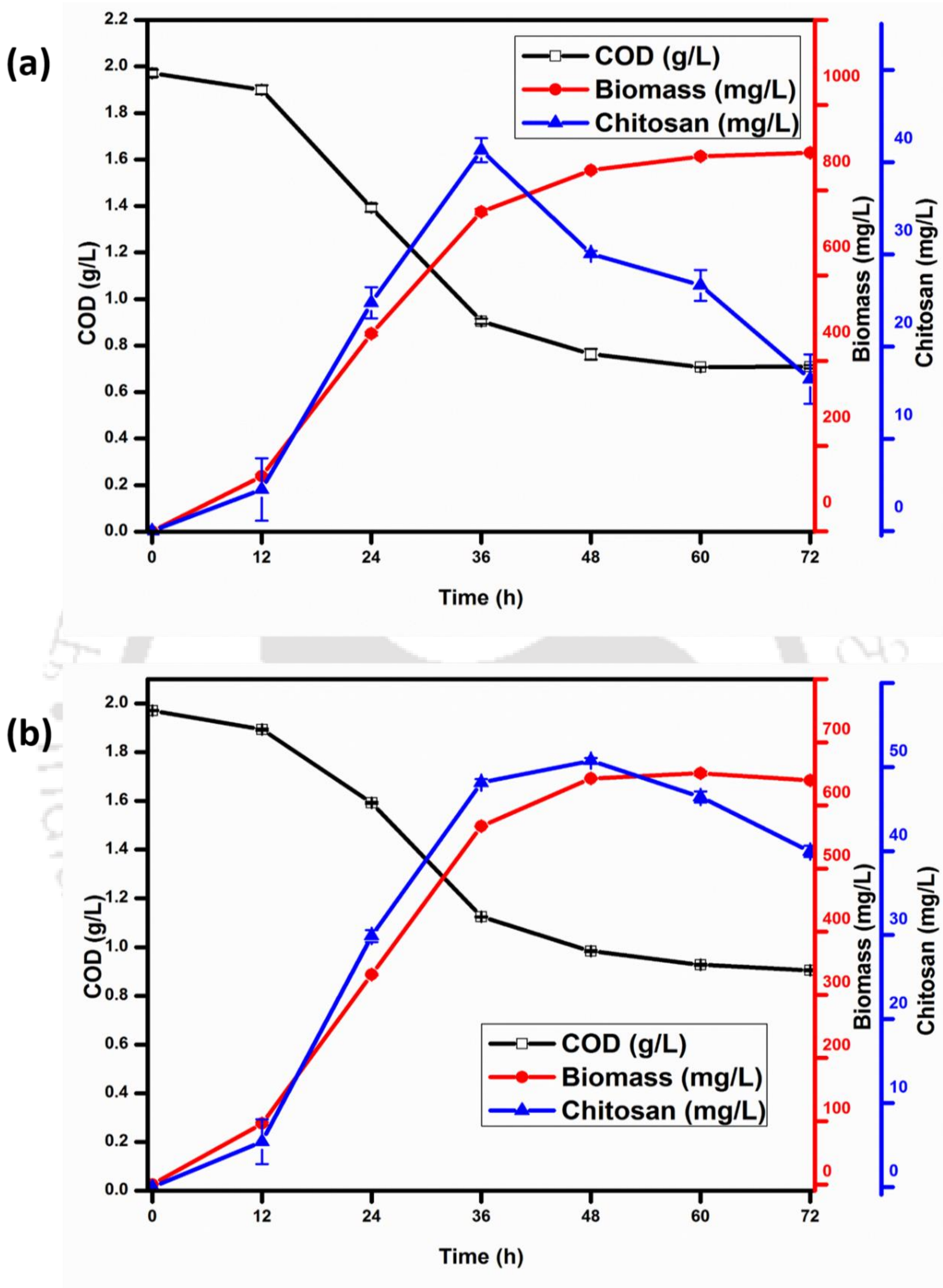


Fig. 4.8 Time profile of biomass growth, chitosan production and COD removal by (a) *C. elegans* and (b) *P. citrinum* using dairy wastewater as substrate.

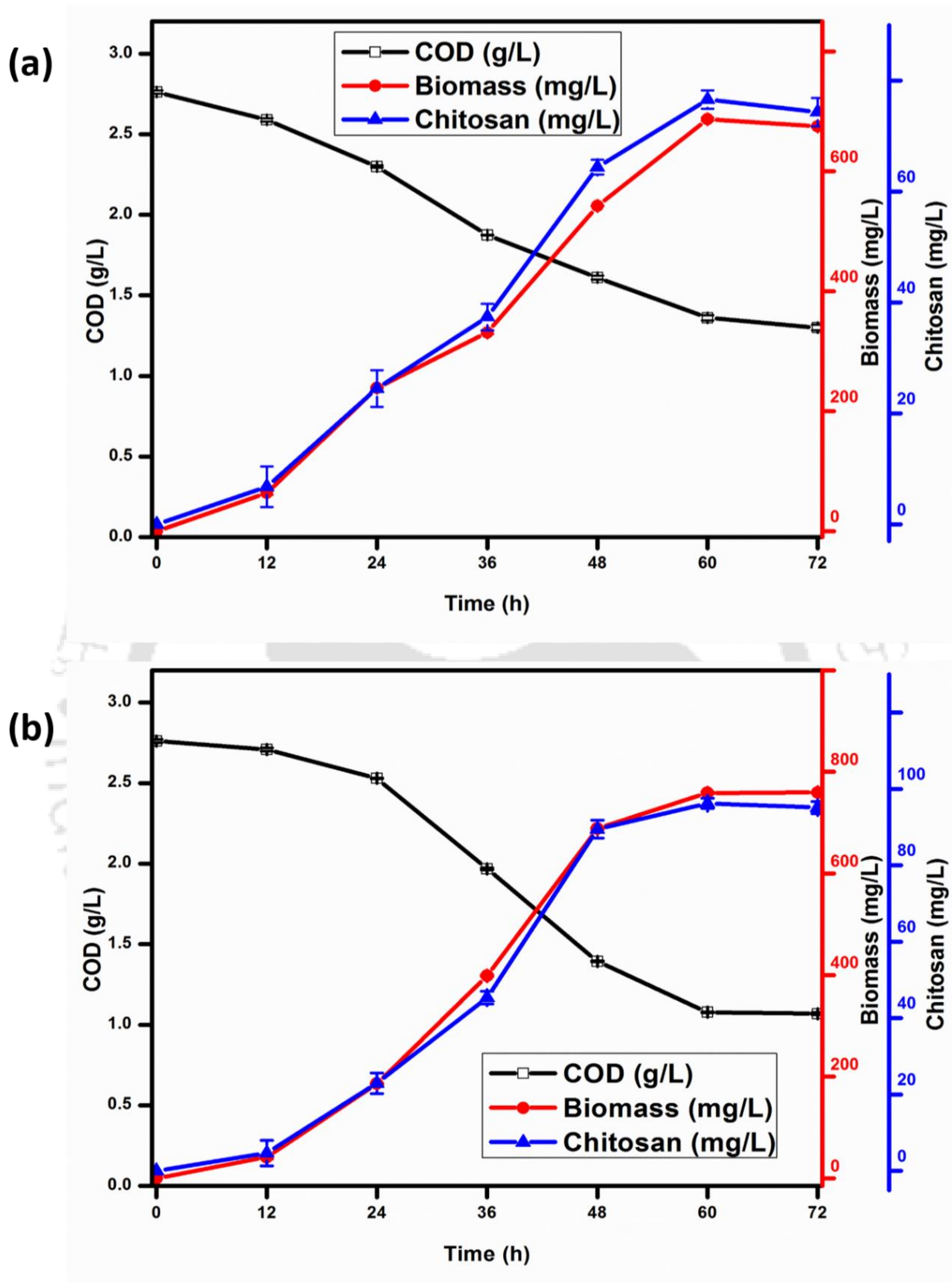


Fig. 4.9 Time profile of biomass growth, chitosan production and COD removal by (a) *C. elegans* and (b) *P. citrinum* using paper mill wastewater as substrate.

Fungal fermentation of paper mill wastewater by *P. citrinum* showed a high COD removal efficiency of 62% (Fig. 4.9). A maximum chitosan production of 96 mg/L was obtained along with a chitosan yield of 127 mg/g biomass with *P. citrinum*, whereas *C. elegans* was able to reduce only 53% COD present in the wastewater. A low chitosan titre (77 mg/L) and yield of 112 mg/g was achieved with *C. elegans* when compared with the values obtained using *P. citrinum*. Paper mill wastewater contains diverse compounds including the natural polymer lignin and phenolics, which are known to be degraded by the fungi. The very high COD removal along with high chitosan producing capability of *P. citrinum* using this wastewater suggests its potential for simultaneous fungal chitosan production and treatment of paper mill wastewater. From the results obtained it is clear that *Penicillium citrinum* is better than *C. elegans* at utilising dairy and paper mill wastewater for biomass growth and chitosan production. Hence, *P. citrinum* was used for further experiments.

4.3.1.1 Effect of mineral salt supplementation

Low nitrogen content in wastewater is a major hurdle to for achieving maximum biomass growth and efficient removal of COD. Moreover, wastewater is often deficient in essential trace elements and ions, which act as cofactors for various enzymes in the metabolic pathway of chitin/chitosan synthesis in fungi. For example, divalent cations such as Mg^{2+} and Co^{2+} are reported vital for the synthesis of chitin by polymerization of UDP-N-acetylglucosamine (Rane and Hoover, 1993; Ryder and Peberdy, 1977). Therefore, the effect of supplementing wastewater with mineral salt media containing N-source and essential trace elements on fungal growth and chitosan production was investigated (Fig 4.10 and Fig 4.11).

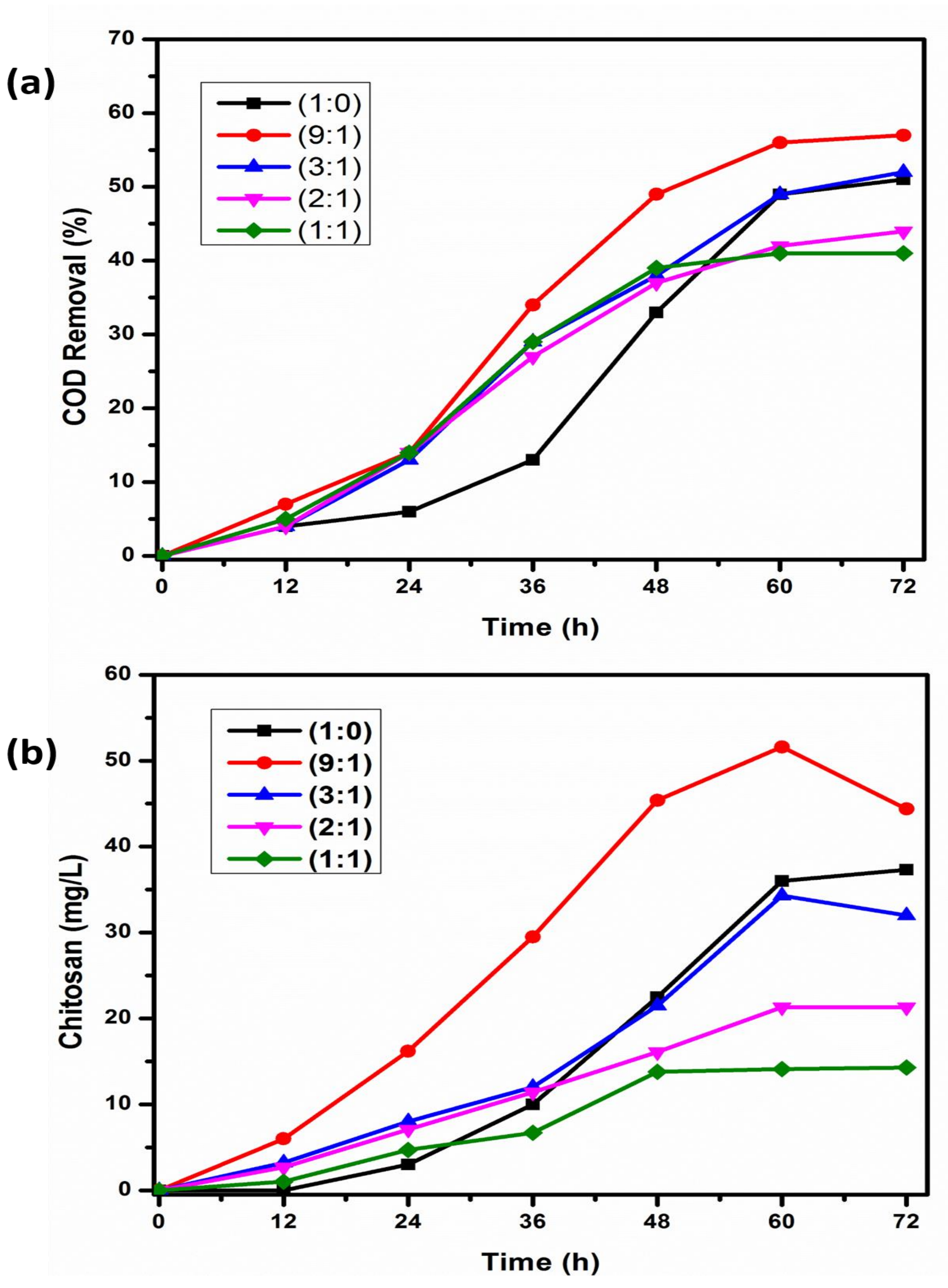


Fig. 4.10 Effect of MSM addition on (a) COD removal and (b) chitosan production by *P. citrinum* biomass using dairy wastewater as substrate.

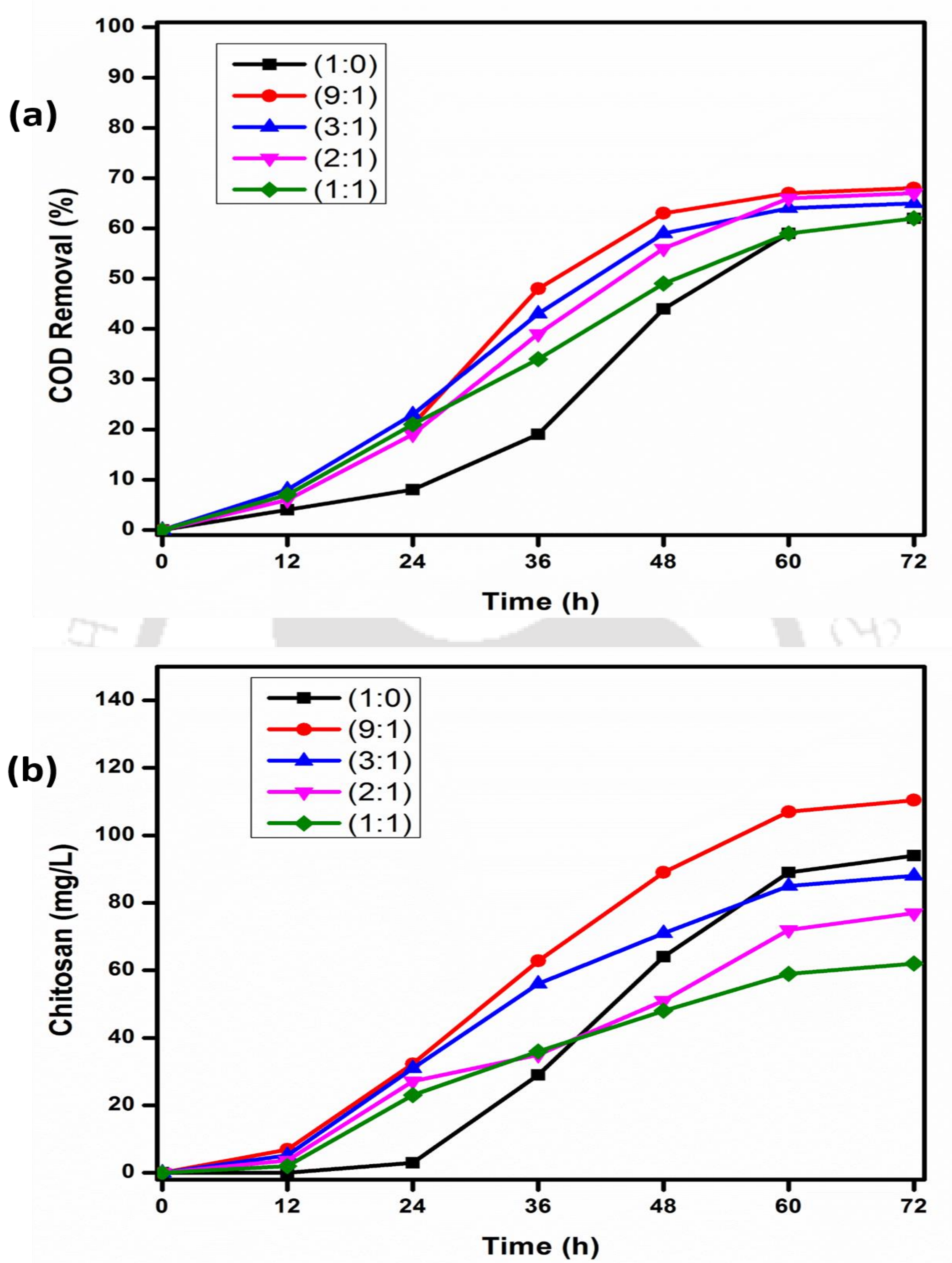


Fig. 4.11 Effect of MSM addition on (a) COD removal and (b) chitosan production by *P. citrinum* biomass using paper mill wastewater as substrate.

Fig. 4.10 shows that addition of mineral salt media to dairy wastewater led to a considerable increase in both COD removal as well as chitosan production. Moreover, the lag phase in COD removal or chitosan production was shorter in case of supplementing the wastewater with MSM than without the supplementation. MSM supplementation in the ratio 9:1 resulted in the maximum COD removal of 56% and a chitosan production of 51 mg/L. However, Fig. 4.10b revealed that the chitosan production decreased when MSM was added to the wastewater at a high quantity, which could be attributed to a reduction in total COD of the wastewater.

Fungal fermentation of paper mill wastewater with the addition of MSM enhanced the biomass growth, COD removal and chitosan production. The decrease in lag phase is evident from Fig. 4.11a and 4.11b. A considerable increase in COD uptake as well as chitosan production after 12 h of incubation is observed in all the flasks containing wastewater with MSM, whereas this trend was found only after 24 h in the flask containing wastewater without any added MSM. Maximum COD removal of 66% and maximum chitosan production of 110 mg/L was achieved following supplementation of the wastewater with MSM at 9:1 ratio. The initial and final TDS values of the paper mill wastewater were 3400 mg/L and 1219 mg/L, respectively, which is well within the wastewater discharge standard of <1500 mg/L (WHO, 2006). Hence, based on these results, wastewater supplemented with MSM in 9:1 ratio was used for further experiments.

4.3.2 Effect of agitation speed

Agitation in batch shake flask experiments is a vital parameter that helps in maintaining homogeneity of the system as well as in maintaining proper aeration. As chitosan is a growth-associated product, biomass growth is of utmost importance and this can be achieved by optimum oxygen transfer to the fermentation media by proper mixing. Effect of different agitation rates

(150, 180, 210, 240 and 270 rpm) on fungal growth and chitosan production was studied (Fig. 4.12).

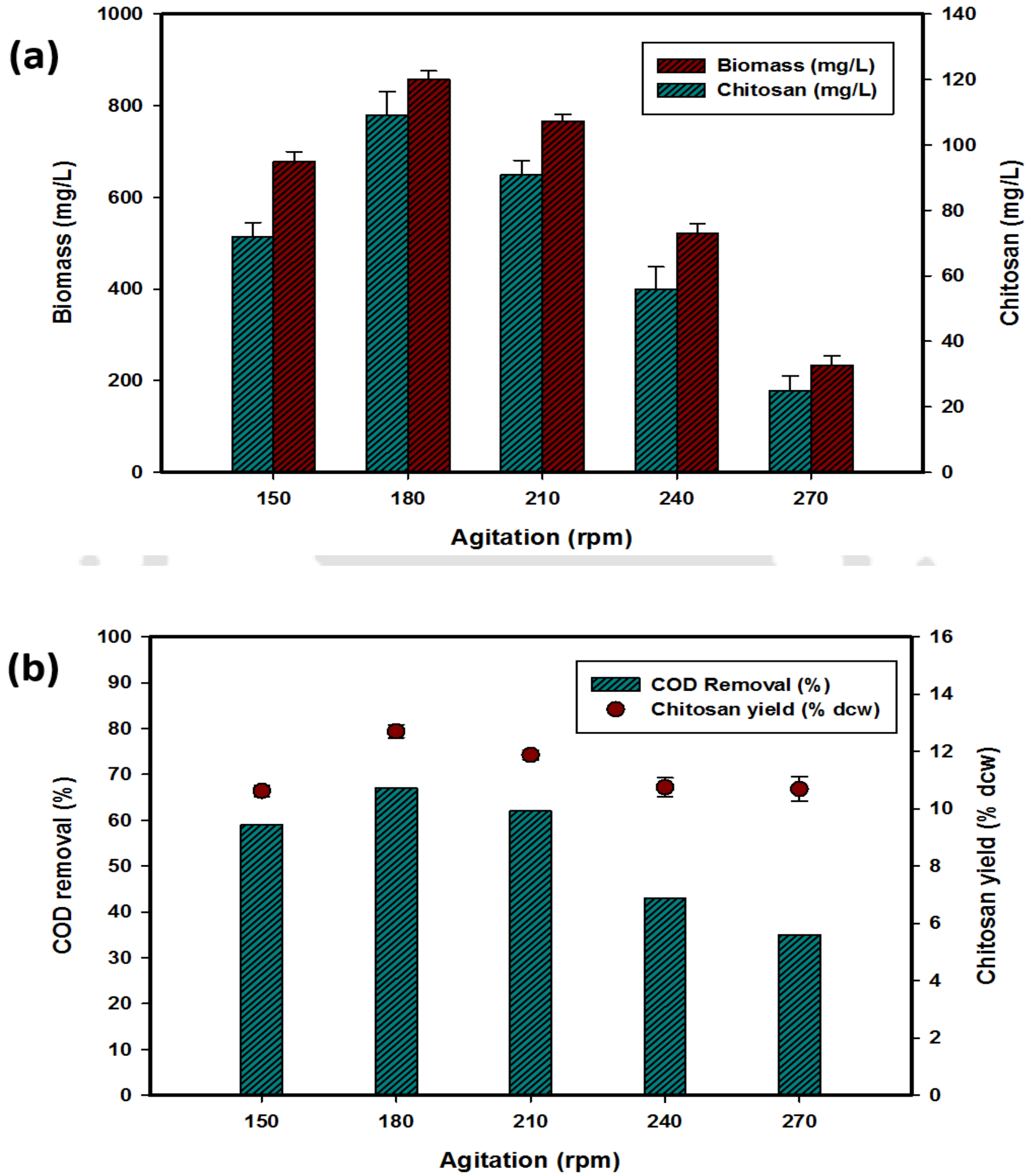


Fig. 4.12 Effect of agitation on (a) biomass and chitosan production, and (b) COD removal by *P. citrinum* biomass using paper mill wastewater as substrate.

Biomass growth and chitosan production increased with an increase in agitation speed from 150 to 180 rpm. However, further increase in the agitation rate beyond 210 rpm resulted in a sharp decline in biomass and chitosan values, which could be attributed to shear stress on the fungal mycelia, which led to decrease in fungal growth; COD removal and chitosan yields showed similar patterns. Maximum biomass concentration of 858 mg/L and a maximum chitosan titre of 109 mg/L was achieved when the experiments were carried out at an agitation rate of 180 rpm.

4.3.3 Effect of acetic acid

Acetic acid was added at varying concentrations of 20, 50, 80, 100 and 150 mg/L to the paper mill wastewater supplemented with MSM in order to study its effect on chitosan production by *P. citrinum*. The results showed that around 67% COD removal could be achieved with the addition of 50 mg/L acetic acid (Fig. 4.13) to the wastewater. Besides, there was a considerable increase in the chitosan yield (13.7%) was achieved due to the acetic acid addition (Fig. 4.14).

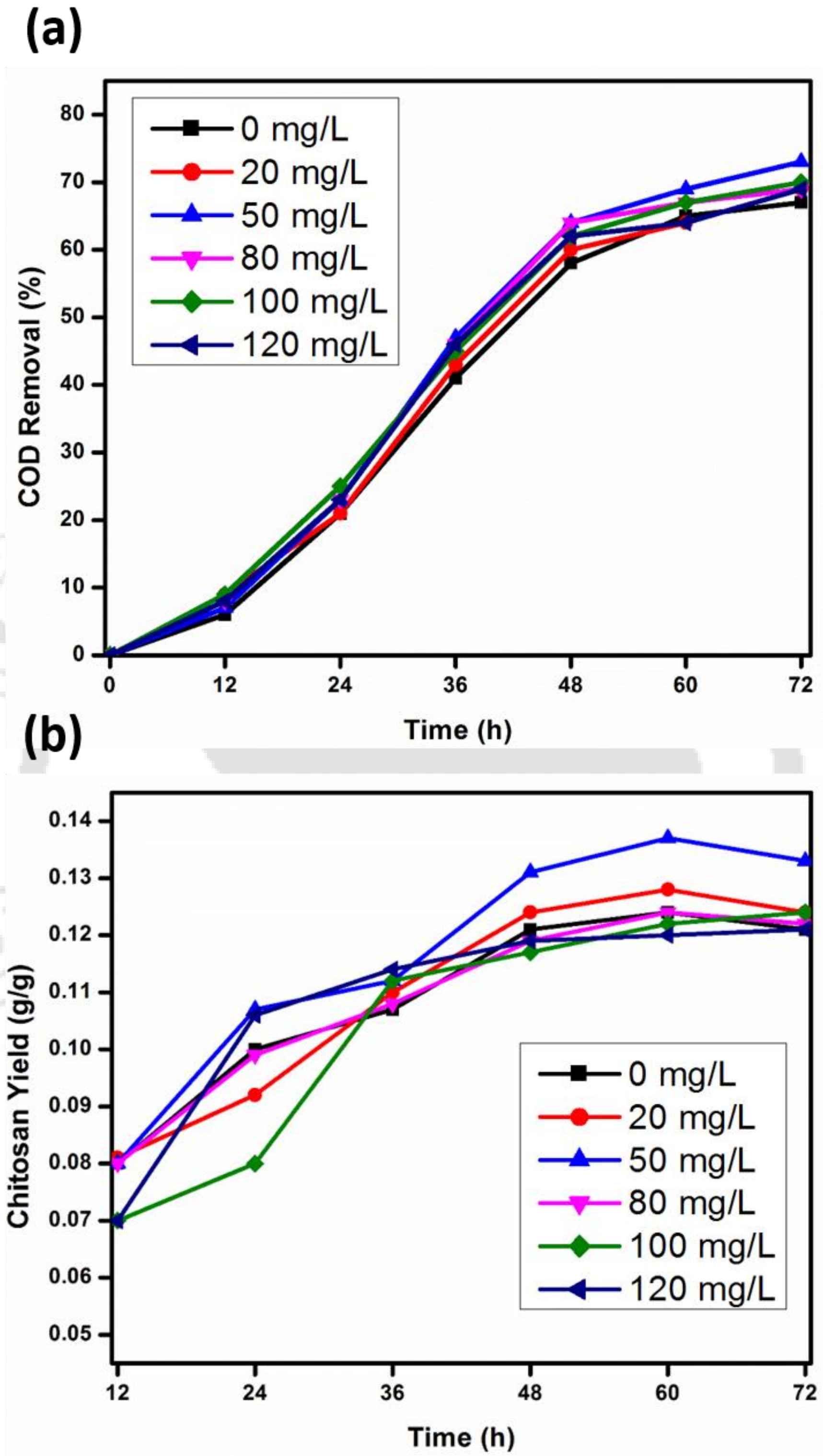


Fig. 4.13. Effect of acetic acid on (a) COD removal and (b) chitosan yield by *P. citrinum* biomass using paper mill wastewater as the substrate

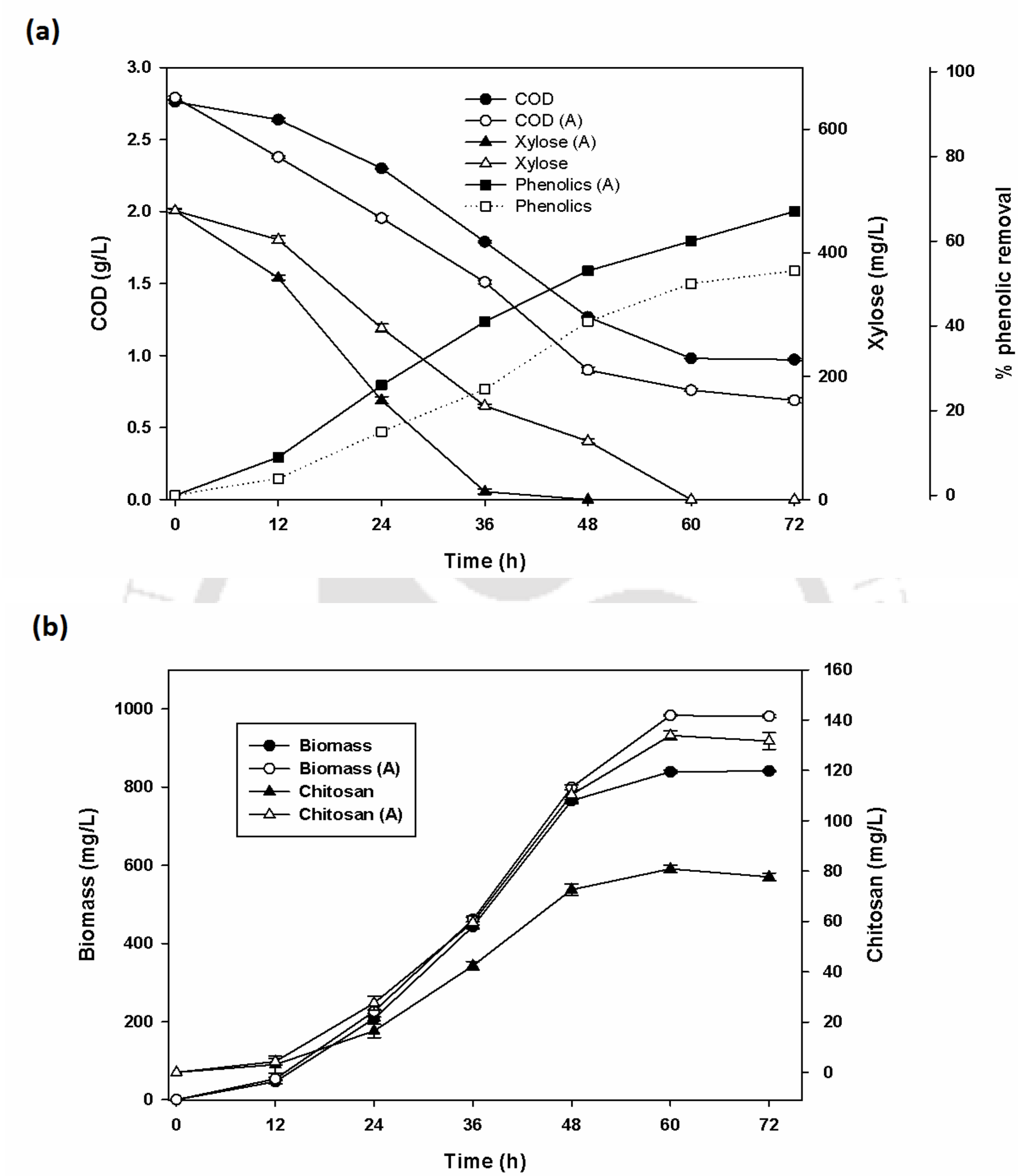


Fig. 4.14 Effect of acetic acid on (a) COD removal, xylose uptake and phenolics removal and (b) biomass and chitosan production by *P. citrinum* biomass using paper mill wastewater as the substrate.

Fig. 4.14 clearly shows that COD removal was drastically higher than that in absence of externally added acetic acid. A similar trend was observed for phenolics present in the wastewater; ~70% removal efficiency was achieved due to acetic acid addition. Fig. 4.14a also shows the effect of acetic acid on xylose uptake by *P. citrinum* biomass, which clearly indicate that in the presence of acetic acid, the xylose utilization rate increased drastically. The initial xylose concentration in the fermentation broth was 467.9 mg/L. In the absence of externally added acetic acid addition, the xylose level dropped to 278 mg/L and 94.7 mg/L at the end of 24 h and 48 h of fermentation, respectively. However, in the presence of acetic acid, the xylose concentration reduced quickly to 161 mg/L within 24 h and it was almost completely utilized by 36 h of fermentation. These results strongly support the positive effect of acetic acid on the capability of the fungi to utilise the wastewater components, thereby resulting in a very high COD removal efficiency and chitosan production by *P. citrinum*. Highest biomass (983 mg/L) and chitosan production (133 mg/L) were achieved due to acetic acid addition, which further establish the role of acetic acid for simultaneous chitosan production and COD removal by *P. citrinum.*

Over the few decades, various kinetic models have been developed to study the specific growth rate, substrate consumption rate and product formation rate. For instance, inhibitory effect of Cu (II) on nitrate uptake by cyanobacteria was studied by Arun et al. (2017); similarly, Gopinath et al. (2011) studied inhibitory effect of various heavy metals on dye consumption rate by *Bacillus sp.* and Naveena et al. (2016) investigated the effect of ultrasonic pulsation on biomass growth rate and ethanol production rate. These kinetic models were also used to describe the biomass growth and substrate uptake by various fungal species such as *Aspergillus awamori*, *Fusarium solani* and *Aspergillus niger* (Stoilova et al., 2006; Vergara-Fernández et al., 2018, 2006). The most widely used biokinetic models are Monod and Haldane model; whereas the former neglects the toxic/inhibitory effect, the latter considers the inhibitory effect due to substrate. Webb, Andrews and Yane are few other models that are closely related to Haldane model. In any biological system, the effect of media components is very important and kinetic modelling of biological reactions helps in predicting its performance. To our knowledge, kinetic modelling of fungal species are scant.

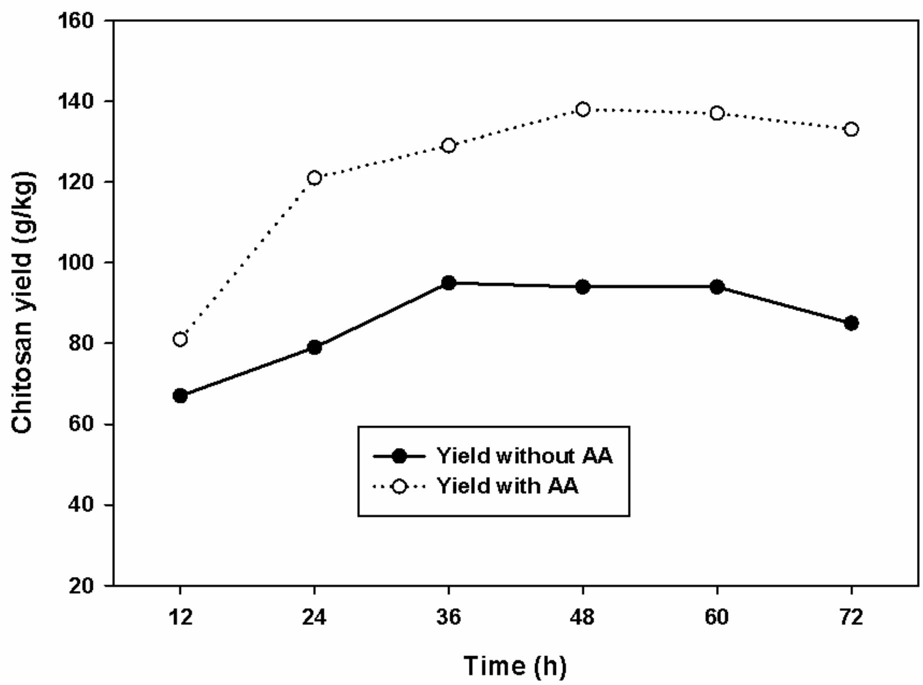


Fig. 4.15 Effect of acetic acid addition on chitosan yield from *P. citrinum* biomass

Acetic acid was added at different concentrations of 20, 50, 80, 100 and 150 mg/L to the paper mill wastewater supplemented with MSM in order to study its effect on chitosan production by *P. citrinum*. The biokinetic model parameters obtained in the study are presented in Table 4.1.

Table 4.1 Biokinetic model parameters on the effect of acetic acid on: (a) Specific growth rate, (b) substrate consumption rate and (c) chitosan production rate

(A)

S. No.	Model	μ_{max} (1/h)	K_s (mg/L)	K_I (mg/L)	K (mg/L)	R^2
1	Monod	0.067	0.449	-	-	0.67
2	Haldane	0.069	0.917	5237	-	0.92
3	Andrews	0.068	0.91	8240	-	0.84
4	Webb	0.068	30.84	0.193	0.2	0.72
5	Yano	0.065	0.91	163.7	-5.5	0.33

(B)

S. No.	Model	V_{max} (g/L·h)	K_s (mg/L)	K_I (mg/L)	K (mg/L)	R^2
1	Monod	0.023	0.72	-	-	0.23
2	Haldane	0.026	3.55	910	-	0.99
3	Andrews	0.026	3.069	890	-	0.84
4	Webb	0.022	19.59	0.801	0.778	0.36
5	Yano	0.026	0.7	0.863	0.003	0.23

(C)

S. No.	Model	P_{max} (mg/L·h)	K_s (mg/L)	K_I (mg/L)	K (mg/L)	R^2
1	Monod	2.57	10.95	-	-	0.94
2	Haldane	3.187	18.89	575	-	0.99
3	Andrews	3.30	19.56	556	-	0.99
4	Webb	2.86	18.01	550.12	941.6	0.92
5	Yano	2.75	12.47	550.12	58.69	0.95

Among the kinetic models analysed in the study, Haldane model fitted the data well with a high coefficient of determination value (R^2) greater than 0.9 for specific growth rate, substrate consumption rate and chitosan production rate by *P. citrinum*. In Fig. 4.16, a high specific growth rate (0.0671 1/h) at 50 mg/L of acetic acid concentration corroborated well with the observed high substrate consumption rate (0.0230 g/L·h). This shows that low concentrations of acetic acid favoured the utilization of substrates present in the paper mill wastewater, which as well led to a significant increase in the fungal growth rate. The specific growth rate increased up to 50 mg/L and after which it remained almost similar with a slight decrease beyond 150 mg/L of acetic acid, whereas, chitosan production rate increased with an increase in acetic acid concentration up to 100 mg/L (2.33 mg/L·h). However, only a slight increase in the rate of chitosan production was observed when acetic acid concentration was increased from 50 mg/L to 100 mg/L. Moreover, the maximum chitosan yield from biomass (13.8%) was obtained with the addition of acetic acid at 50 mg/L, which strongly indicates the positive effect of acetic acid on chitosan production. As the Haldane model considers the effect of inducers and inhibitors in the medium, it accurately fitted the experimental data on specific growth rate, substrate consumption rate and chitosan production rate due to acetic acid addition at all different concentrations. Andrews model, which is a slightly modified form of the Haldane model, also fitted the experimental data accurately. Haldane model has been commonly used to describe the growth of various fungal species with inhibitory substrates. For instance, Vergara-Fernández et al. (2006) found that the Haldane model accurately fitted the data on growth of *Fusarium solani* treating hexanol and hexane. Similarly, Iranmanesh et al. (2015) found that the Haldane model accurately fitted the experimental data on growth of fungal consortium treating n-hexane and toluene.

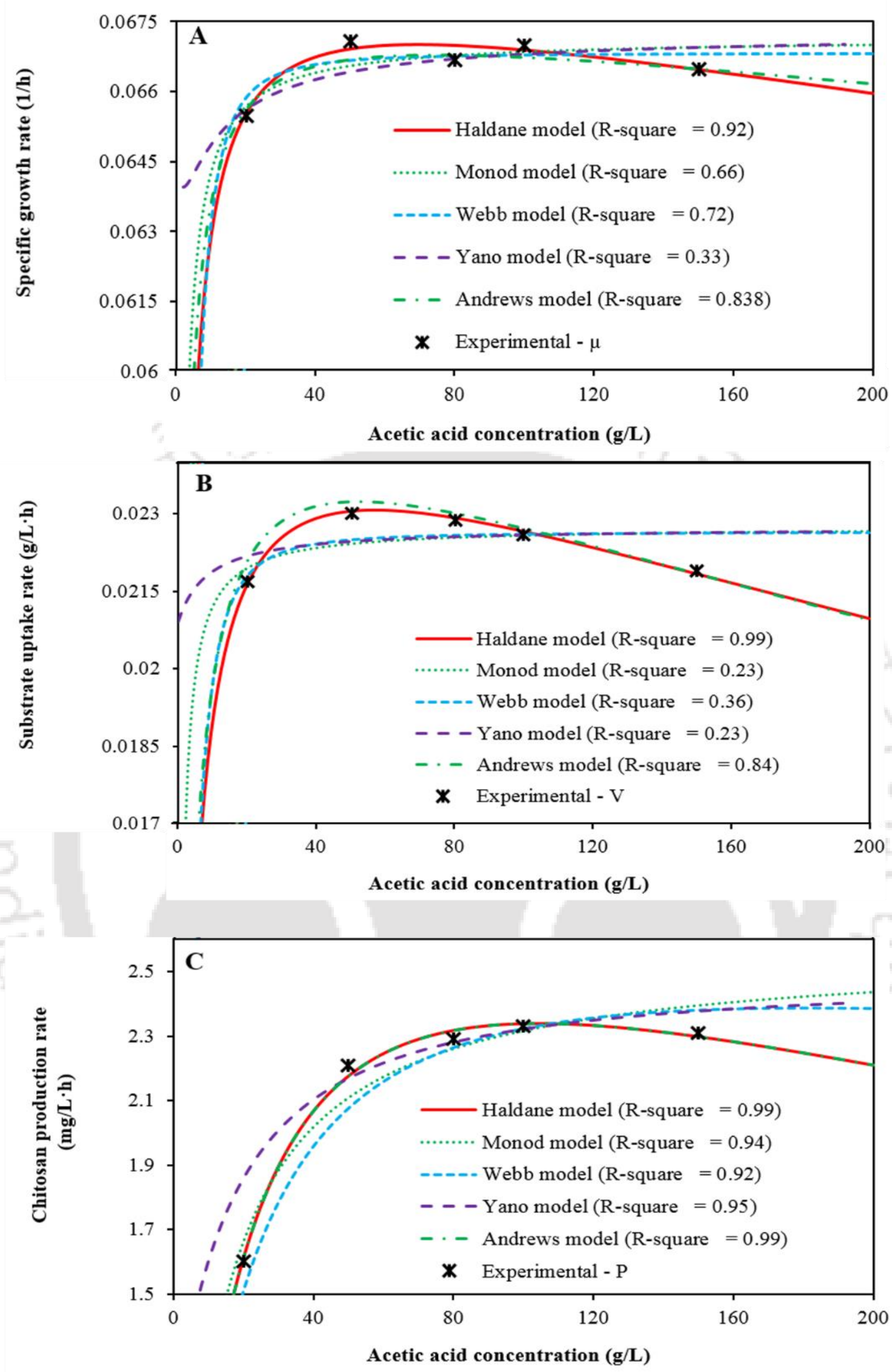


Fig. 4.16 Experimental vs. model predicted values for: (a) specific growth rate, (b) substrate consumption rate and (c) chitosan production rate by *P. citrinum* at different acetic acid concentration

From Table 4.1, it clear that the values of μ_{max}, V_{max} and P_{max} obtained from the biokinetic models are in the range of 0.065-0.069 1/h, 0.023-0.026 g/L·h and 2.57-3.2 mg/L·h, respectively. These values also match with the experimentally observed μ, V and P values. The low values of K_S, i.e. 0.917 mg/L, 3.55 mg/L and 18.89 mg/L, estimated by the Haldane model for specific growth rate, substrate consumption rate and chitosan production rate, respectively, clearly indicate a high affinity of *P. citrinum* biomass towards acetic acid in the medium. A similar value of K_S values in the range 9.57–10.17 mg L^{-1} is observed for a fungal consortium treating the n-hexane and Toluene (Iranmanesh et al., 2015). The degree of inhibition exhibited by any inhibitors present in the media is indicated by the K_I values. A high value of K_I indicates a low inhibitory effect of the substrate even at a high concentration. The very high values of K_I i.e. 5237 mg/L, 910 mg/L and 575 mg/L estimated by the Haldane model for specific growth rate, substrate consumption rate and chitosan production rate, respectively, indicate that acetic acid is less toxic even at high concentration. Naveena et al. (2016) reported a K_I value of 794.9 W min/mL in a study on enhancing the biomass growth rate and the ethanol production rate by an ultrasonic pulsation method, which is to the K_I value (575 mg/L) observed in the present study on chitosan production.

The considerable increase in both chitosan yield and COD removal from paper mill wastewater by *P. citrinum* in the presence of acetic acid can be explained as follows. It is known that paper and pulp wastewater contains cellulose, xylose and hemicellulose residues in low amounts along with polymers, lignin and phenolics. Fig. 4.13 shows that acetic acid at a low concentration contributed to the utilization of xylose, which subsequently enhanced the mycelial growth.

Xylose is diverted to xylulose production and subsequently to the synthesis of biomacromolecules such as polysaccharide and protein via the pentose phosphate pathway

(Jeffries, 2006). Low concentrations of acetic acid has been found to positively influence the activities of xylose reductase and xylitol dehydrogenase enzymes (Table 4.2), leading to formation of xylulose and xylitol in greater amounts. This increase would result in high levels of xylulose 5-phosphate and fructose 6-phosphate (F6P). Due to this enhanced xylose utilization, the majority of the metabolic flux could be directed towards the chitosan synthesis pathway where F6P is an important intermediate for various metabolic pathways (Nwe et al., 2011). A high amount of F6P also leads to enhanced pyruvate synthesis, which in turn enters the TCA cycle, and it is the source point of nitrogen for the chitin and chitosan synthesis pathway. Moreover, the TCA cycle is important for the production of acetyl coenzyme A, which is again a key component of the chitosan biosynthesis pathway. Acetic acid dissociates into hydronium and acetate ions following its entry into the cell cytoplasm, and therefore, is directly involved in promoting the synthesis of acetyl CoA (Morita et al., 2000; Tai et al., 2010). The proposed pathway of acetic acid induced chitosan production by *P. citrinum* is depicted in Fig. 4.17.

Starch based wastewaters have been predominantly exploited for fungal chitosan production in the literature. However, a maximum chitosan yield of only 10-11% has been reported by fungal fermentation of wastewater containing complex carbon sources. The chitosan yield of 13.8 % obtained from this investigation is significantly higher than those reported previously in the literature (Table 4.3).

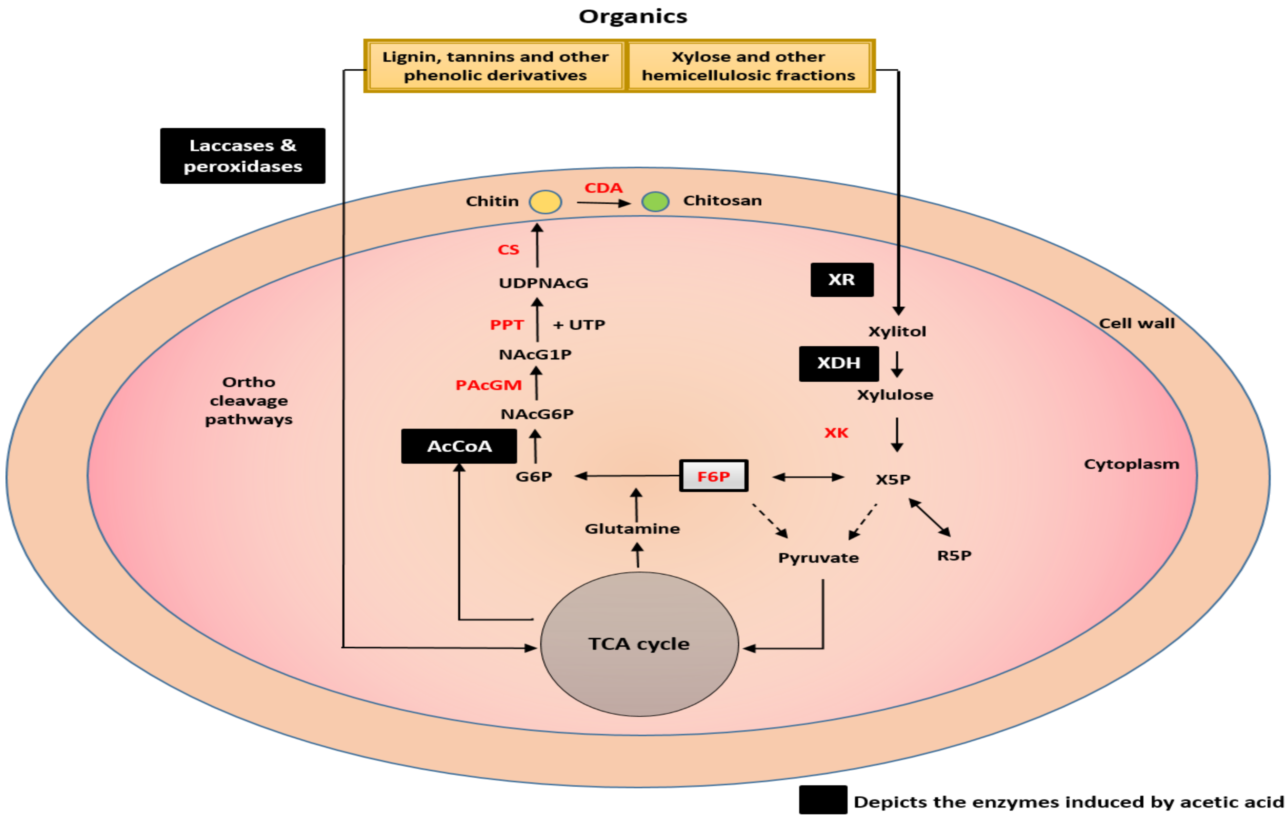


Fig. 4.17 Proposed pathway showing acetic acid induced chitosan production and organic removal from paper mill wastewater by *P. citrinum* (Nwe et al., 2011). XR, Xylose reductase; XDH, Xylitol dehydrogenase; XK, Xylulose Kinase; X5P, Xylulose 5-phosphate; R5P, Ribulose 5-phosphate; F6P, Fructose 6-phosphate; G6P, Glucosamine 6-phosphate; AcCoA, Acetyl coenzyme A; NAcG6P, N-acetylglucosamine-6-phosphate; PAcGM, Phosphoacetylglucosamine mutase; NAcG1P, N-acetylglucosamine 1-phosphate; PPT, Pyrophosphorylase transferase; UDPNAcG, Uridine diphosphate N-acetylglucosamine; CS, Chitin synthetase; CDA, Chitin deacetylase

Table 4.2 XR and XDH activities of *P. citrinum* during fermentation with an initial addition of acetic acid.

Time (h)		**Specific activity (U/mg)**	
		Acetic acid (mg/L)	
		0	**50**
12	XR	0.164	0.366
	XDH	0.177	0.293
24	XR	0.171	0.688
	XDH	0.293	0.528
36	XR	0.216	0.912
	XDH	0.334	0.713
48	XR	0.220	0.534
	XDH	0.275	0.977
60	XR	0.183	0.513
	XDH	0.290	0.744
72	XR	0.179	0.521
	XDH	0.223	0.667

It has been reported that the degradation of tannins is rapid in the presence of other easily metabolizable sugars as in the case of co-metabolism (Bugg et al., 2011; Ganga et al., 1977; Krastanov et al., 2013). Hence, it is likely that the presence of acetate in the system leads to an efficient degradation of the various complex phenolic compounds present in the paper mill wastewater due to enzymes such as laccases and peroxidases. However, the direct effect of acetic acid on the activities of these enzymes leading to a better degradation and utilization of phenolics by the fungi needs to be confirmed by further experiments.

Table 4.3 Fungal chitosan production using liquid waste as substrates.

Organism	Substrate	Chitosan yield	Reference
Penicillium citrinum	Paper mill wastewater	13.8% dcw	This study*
	Rice straw hydrolysate	13.7%	This study*
Aspergillus awamori	Raw distillery thin stillage	7-9% dcw	Ray and Ghangrekar (2016)
C. Elegans	Cassava wastewater+ Corn steep liquor	57.82 mg/g dry mycelia	Berger et al. (2014)
Mucor hiemalis	Wheat hydrolysate	0.46 g/g AIM	Vinche et al. (2013)
Rhizomucor pusillus	Xylose rich wastewater from ethanol plant	45.7 % AIM	Zamani et al. (2007)
Rhizopus oryzae	Hemicellulose hydrolysate of corn straw	0.58 g/L	Tai et al. (2010)

4.3.4 Bioreactor experiments

4.3.4.1 Effect of aeration and agitation

Agitation and aeration are vital parameters that need to be considered for carrying out fungal fermentations, which directly influence the fungal growth and morphology in the reactors. The bioreactors experiments in this study were carried out at different agitation and aeration rates to understand its influence on biomass growth, COD removal, chitosan production and DO levels in the medium.

Oxygen transfer rate and broth circulation in the reactor are directly influenced by volumetric gas flow rate, which in turn affects the fungal biomass growth, chitosan production and COD removal. Chitosan accumulation inside the fungal cell being a growth-associated phenomenon, the main objective of the fermentation is to achieve a high yield of biomass growth, thereby leading to increased chitosan production. One of the major reasons for low biomass growth in a reactor is low dissolved oxygen levels in the fermentation medium. Therefore, to overcome this limitation, the aeration rate was varied from 0.5 to 2 vvm in the reactor at 180 rpm, which was found optimum in batch shake flask experiments). The results are shown in Fig. 4.18. The fungal growth rate in its exponential phase increased with the increase in the aeration rates and similar trends were observed for both COD utilization and chitosan accumulation when the airflow rate was increased. Maximum growth (933 mg/L), chitosan production (121 mg/L) and COD removal (71 %) were achieved at an aeration rate of 2 vvm. The dissolved oxygen levels remained sufficiently low throughout the fermentation when the reactor was operated at a low aeration rate and it was considerably high when operated at 2 vvm aeration rate. The dissolved oxygen concentration started to increase after 36-48 h when the culture reached its late exponential phase, which shows that 2 vvm aeration rate is sufficient for achieving high fungal biomass growth as well as for maximum chitosan production by the fungi.

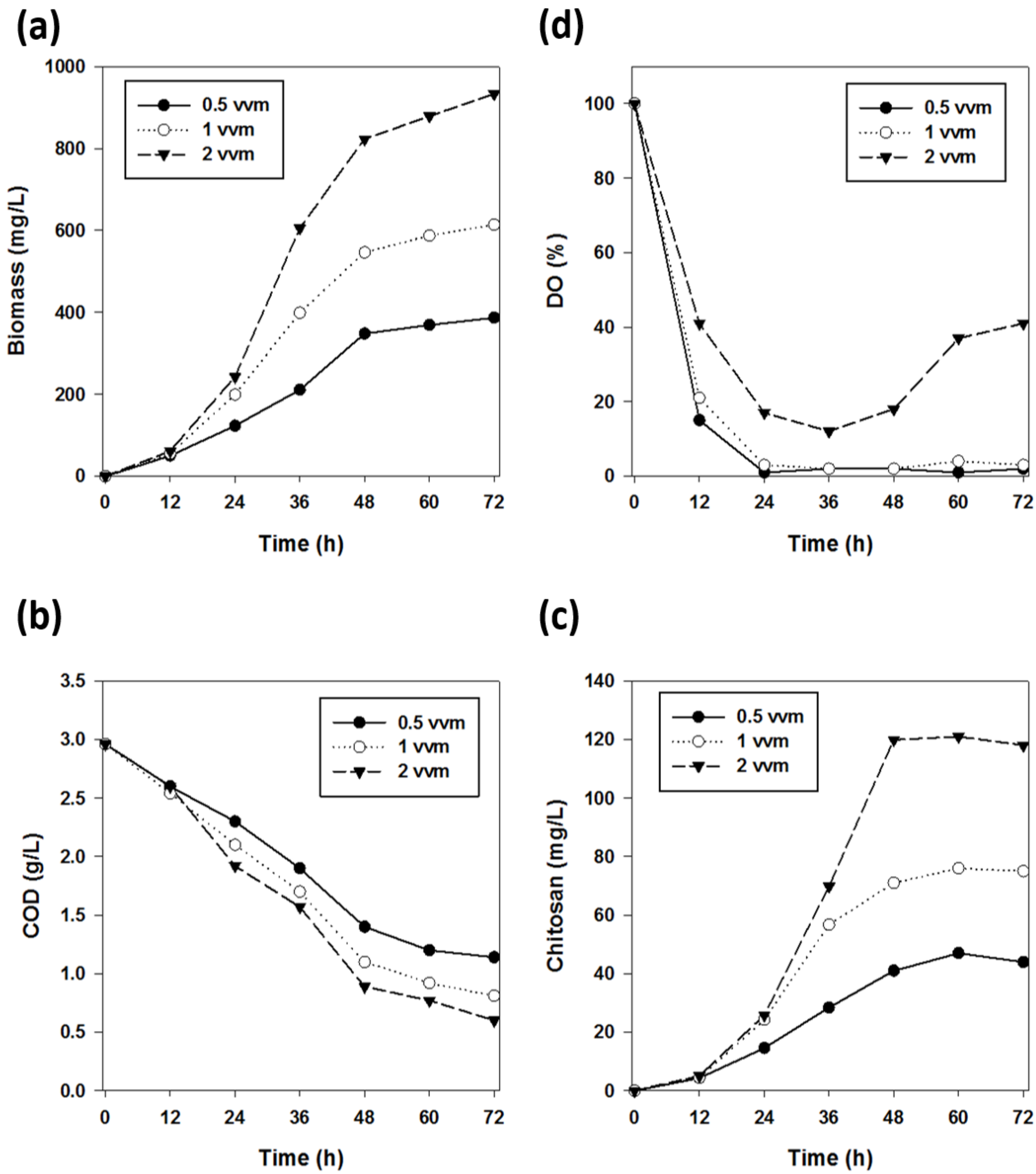


Fig. 4.18 Effect of aeration rate on (a) biomass growth, (b) COD removal (c) chitosan production by *P. citrinum* using paper mill wastewater and (d) DO levels in bioreactor experiments.

Fungi are capable of growing as dispersed mycelia or compact pellets in a stirred tank reactor. Dispersed mycelial growth is known to seriously affect the fermentation process due to its agglomeration and tendency to attach themselves on to the impellers and other internal parts of a reactor. This in turn leads to mass transfer limitations and problems in recovery of the fungal biomass. Therefore, to achieve pelleted growth and further enhance the fungal growth in a STR, three different agitation rates (100, 200 and 300 rpm) were employed to understand its effect on the fungal morphology as well as its growth. Fig. 4.19 shows the effect of the different agitation rates employed on fungal growth, chitosan accumulation, COD removal and DO levels in the fermentation broth. Mycelial aggregation and clump formation was observed when the reactor was operated at 100 rpm agitation rate, which led to settling of the biomass at the bottom of the reactor, and this biomass was difficult to recover from the reactor for further processing. Due to mass transfer limitations and fouling caused by the attachment of fungal mycelia on the impeller, baffles and control probes a low level of dissolved oxygen was observed in the broth. Agitation at 200 rpm resulted in the formation of small compact pellets, better mass transfer and homogeneity in the reactor as compared with agitation at 100 rpm. Maximum chitosan production of 134 mg/L and biomass growth of ~930 mg/L was achieved when the reactor was operated under this condition. Further increase in the agitation rate to 300 rpm, however, led to a drastic decrease in fungal growth, chitosan production as well as COD removal. This could be attributed to the shear stress imparted by the impellers to the fungus, leading to death/inactivity of the fungus.

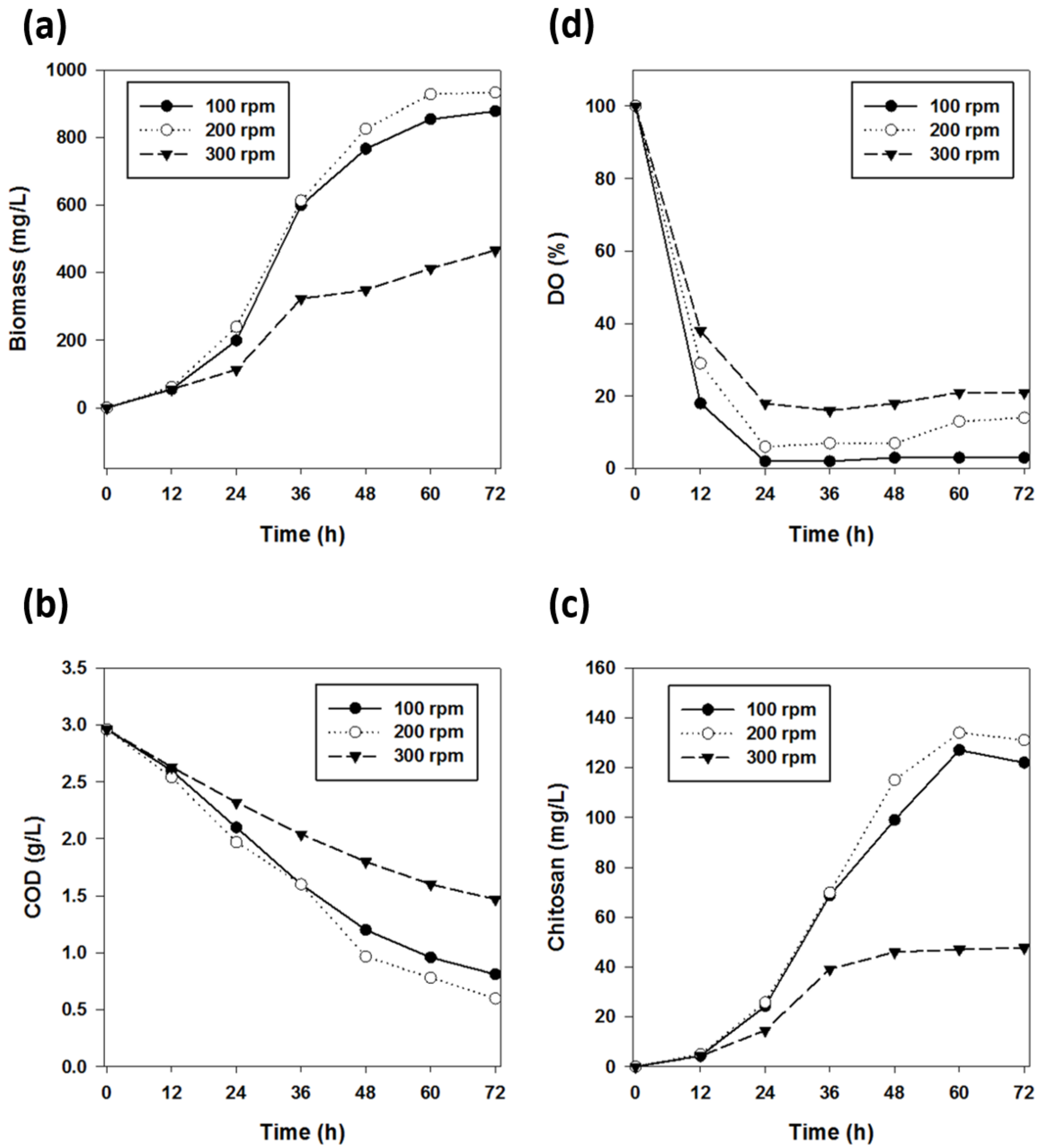


Fig. 4.19 Effect of agitation rate on (a) biomass growth, (b) COD removal and (c) chitosan production by *P. citrinum* using paper mill wastewater as the substrate and (d) DO levels in the bioreactor experiments.

4.3.4.2 Batch, fed-batch and continuous mode of operations

Fig. 4.20 shows the time profile of biomass growth, COD removal and chitosan production by *P. citrinum* in the batch bioreactor experiments. A maximum chitosan production of 141 mg/L was obtained during 48-60 h of the fermentation, whereas the COD removal efficiency was 75% within 72h of fermentation. A total chitosan yield of 13.7% was observed at 48 h batch fermentation. As paper mill wastewater is known to contain a mixture of compounds such as cellulose and hemicellulose residues, lignin and its derivatives, resin acids, fatty acid esters, tannins, etc., analysis of total phenolic content of the wastewater was performed to estimate lignin derivatives, tannins and phenolic acids in the wastewater. Maximum reduction of 70% of phenolics present in the wastewater was achieved after 72 h of fermentation. Thus, about 84% decolourization of the paper mill wastewater was achieved using the *P. citrinum* biomass in the batch bioreactor experiments.

Biokinetic constants, viz. specific growth rate (μ) and biomass yield ($Y_{X/S}$) were calculated from the batch growth profile and, accordingly, the feed flow rate for operating the bioreactor under fed-batch mode was estimated. Continuous feeding was started after 48 h of batch fermentation, i.e. after chitosan production started declining. A feed flow rate of 0.15 L h^{-1} was maintained throughout the feeding stage for 4 h, which was calculated from the following equation (Daverey and Pakshirajan, 2016):

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{X/S} S_0} \qquad (12)$$

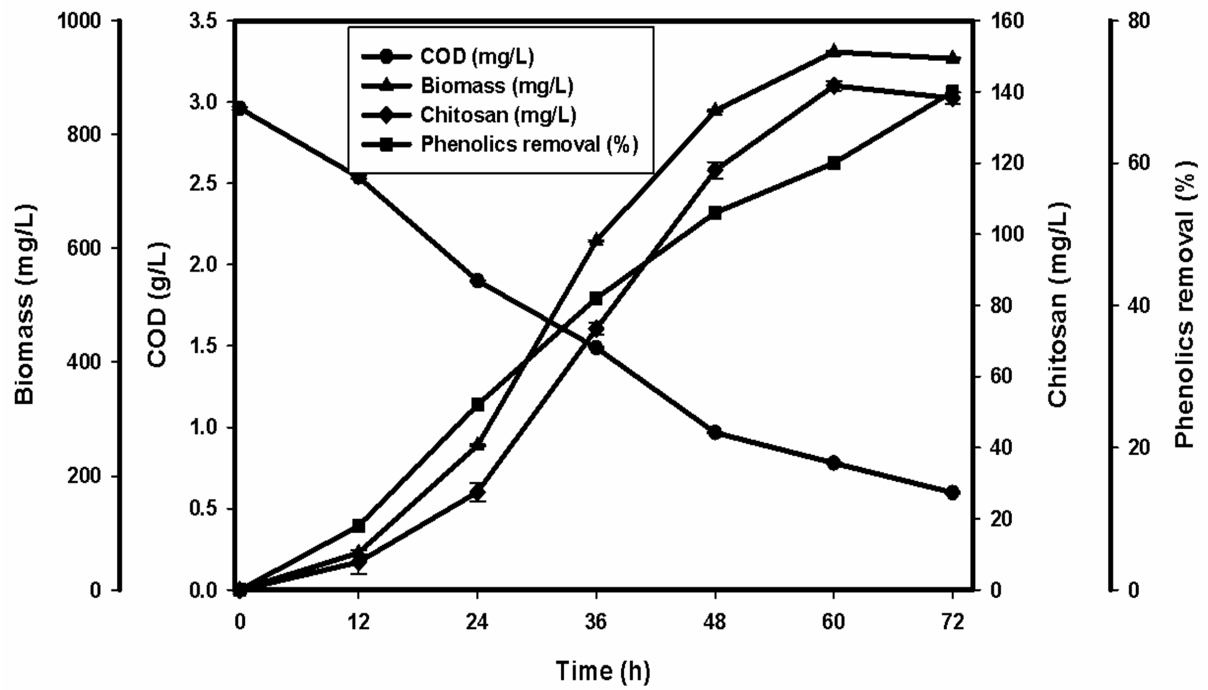


Fig. 4.20 COD removal, biomass and chitosan production by *P. citrinum* in the batch operated bioreactor

The results obtained during the fed-batch experiments with the bioreactor showed that during the initial batch phase (0-48 h) a COD removal of ~70% is achieved followed by a drastic reduction in COD during the feeding stage. A sharp increase in the biomass as well as chitosan was also observed during the feeding stage. The high COD removal efficiency obtained in the case of fed-batch mode of operation is attributed to the fact that fungal biomass quickly reached the stationary growth phase at the end of 48 h in the batch mode of operation due to the low initial substrate concentration. The constant supply of the wastewater during the feeding stage further led to an efficient utilization of COD present in the wastewater (Daverey and Pakshirajan, 2016; Gupta et al., 2018). After the feeding was stopped, the reactor was operated under batch mode to achieve maximum COD removal efficiency and chitosan production. Chitosan production rose to 160 mg/L following the fed-batch operation and a maximum COD removal of 91% was achieved at the end of 72 h of operation (Fig. 4.21). These results further demonstrate that *P. citrinum* can efficiently utilize paper mill wastewater as the substrate for achieving both maximum COD removal efficiency and chitosan production. Maximum reduction of 86% of phenolics present in the wastewater was achieved after 72 h of fermentation. Thus, about 89% decolourization of the paper mill wastewater was achieved using the *P. citrinum* biomass in the fed-batch experiments with the bioreactor.

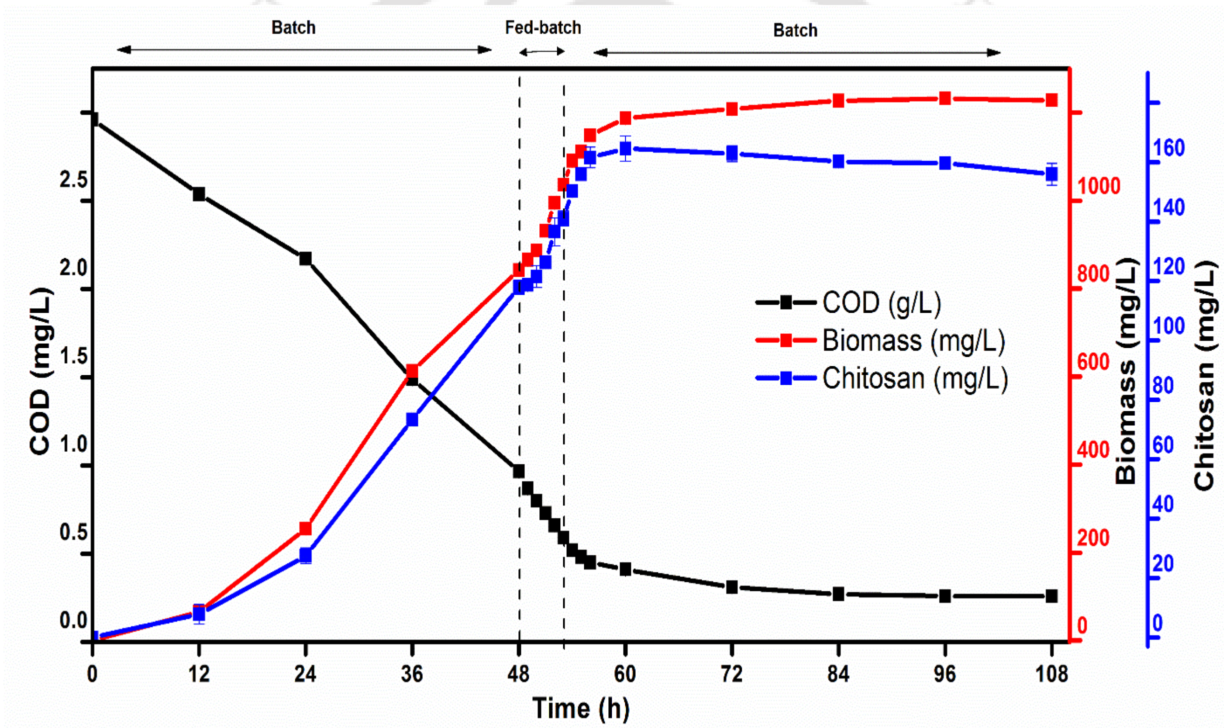


Fig. 4.21 COD removal, biomass and chitosan production by *P. citrinum* in the bioreactor experiments carried out under fed-batch mode.

Continuous mode of fermentation of the paper mill wastewater was carried out with the objective to obtain high fungal biomass and chitosan yield. Under this mode of operation with the bioreactor, highest chitosan production of ~170 mg/L was observed. Although the chitosan production was slightly higher than that obtained under the fed-batch mode of operation, the COD removal efficiency was low (83%) (Fig. 4.22). It was also observed that during the initial phase of operation, fungi grew into small compact pellets, which turned into slightly large hollow fungal pellets and as mycelial agglomerates towards the end of the fermentation.

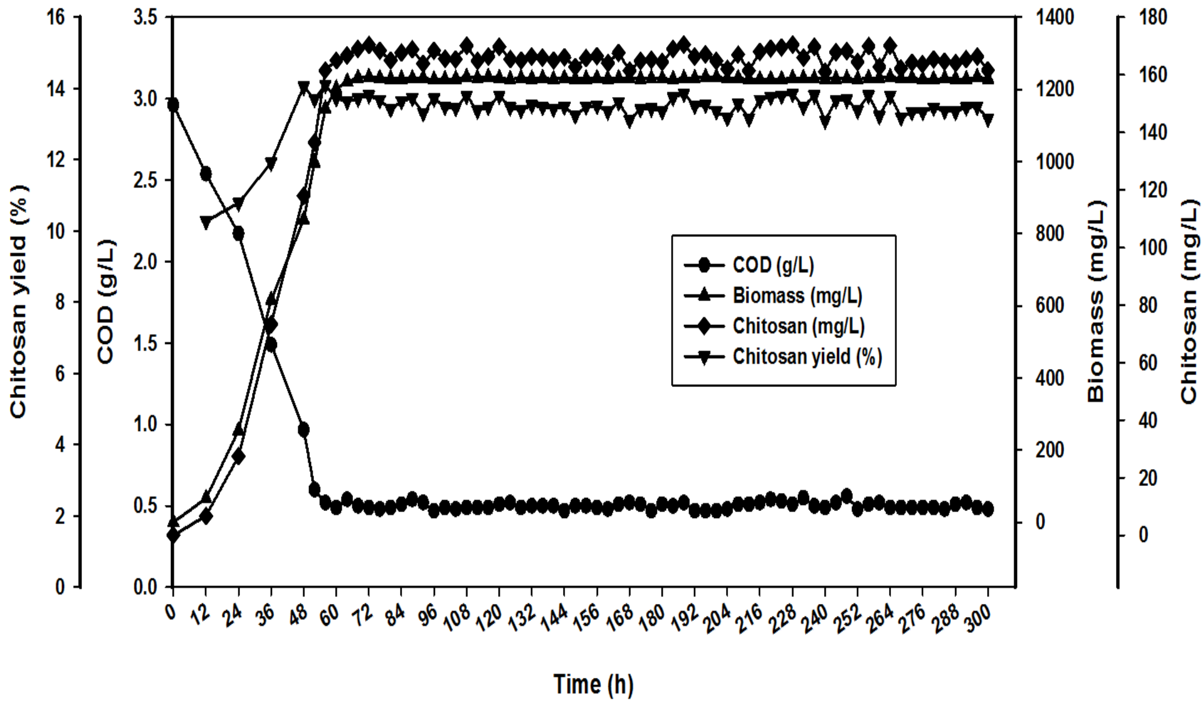


Fig. 4.22 COD removal, biomass and chitosan production by *P. citrinum* in bioreactor experiments carried out under continuous mode of operation.

Under controlled conditions of temperature, pH, agitation and aeration rate, an enhanced COD removal efficiency of 91% and a maximum chitosan production of 160 mg/L were achieved in the bioreactor experiments (Fig. 4.21). Among the different fungi, *Penicillium* and *Aspergillus* species are well known as tannin degraders, which are abundantly found growing on the surface of tannery waste liquids and pits (Rajakumar and Nandy 1977). Most of the fungal species studied for tannery effluent treatment belong to *Penicillium* and *Aspergillus* genera. Studies which have employed fungal based wastewater treatment routes to degrade paper mill wastewaters have shown COD removal efficiencies in the range 60-80% (Freitas et al., 2009; Liu et al., 2011; Rajwar et al., 2017). The high COD removal efficiency obtained in this study using the bioreactor under batch mode of operation proved that *P. citrinum* is efficient in assimilating the various complex substrates present in the wastewater.

A 13.8% yield of chitosan from dry fungal biomass due to acetic acid addition further indicates that the fungal chitosan yield can be enhanced by the addition of such simple and cheaply available inducing agents (Fig. 5). The results obtained from this study suggests that acetic acid is critical for enhancing both COD removal and chitosan production by *Penicillium citrinum*. A 150% increase in chitosan yield achieved in this investigation leading to 13.8% dcw is the highest yield reported in the literature. The optimal conditions for achieving maximum chitosan production (138 g/kg) and COD removal (~91%) under fed-batch mode of operation were 28ºC, pH-4.5, 200 rpm agitation. This strategy could, therefore be applied to overcome the issue of low chitosan production generally associated with fungal fermentation of cheaply available waste resources.

4.4 Fungal chitosan production using rice straw hydrolysate

Fermentation of rice straw hemicellulose hydrolysate was carried out using *P. citrinum* biomass owing to its ability to assimilate xylose and other hemicellulose residues efficiently. Xylose (24.6 g/L) is present as the dominant sugar in the rice straw hydrolysate. Glucose (4.3 g/L) and arabinose (3.9 g/L) were the other sugars that were present in the hydrolysate along with acetic acid and furfurals. Formic acid and furfurals have been reported to be inhibitory to the growth of bacteria, yeasts and fungi in general and therefore removal of these compounds is often necessary for a successful fermentation process (Almeida et al., 2007; Huang et al., 2009). The presence of these compounds depend on the source and type of lignocellulosic residues being utilised. In this investigation, both detoxified and non-detoxified rice straw hydrolysate were employed for fungal chitosan production using *P. citrinum* biomass. Fig. 4.23a shows the time profile of utilization of different sugars present in the detoxified hydrolysate, biomass growth and chitosan production. Optimum growth and chitosan production was achieved at 2.5 vvm aeration rate and 220 rpm agitation. Glucose was the preferred sugar for *P. citrinum* biomass and was utilised almost completely within 24 h of fermentation. This was followed by the utilization of xylose and its concentration dropped significantly after 24 h of the fermentation. The sugar utilization profile corresponded well with the increase in fungal growth and chitosan yields observed in Fig. 4.23b. Maximum biomass concentration of 9.6 g/L and the highest chitosan titre of 911 mg/L were achieved by using the detoxified rice straw hydrolysate. The chitosan yield per dry mycelia increased from 4 % initially to a maximum of 13.4 % at 60 h. The value reduced with time, which is similar to the trend observed in almost all the studies involving fungal chitosan production. This is attributed to the fact that chitin and chitosan crosslink with other polymers of the cell wall following the exponential growth phase and hence, it does not easily yield to extraction from the cell wall fraction. Another important observation was that *P. citrinum* biomass was able to utilise almost 98% of the total sugars present in the hydrolysate within 96 h of fermentation.

Similar trends of sugar utilization and biomass growth were observed in the case of fermentation by *P. citrinum* biomass using non-detoxified rice straw hydrolysate (Fig. 4.24). Glucose was consumed at first, followed by xylose and arabinose. Maximum biomass concentration achieved in this case was 6.3 g/L with a maximum chitosan production of 635 mg/L, which are relatively lower than the values, obtained using detoxified RSH. Chitosan yield from dry mycelia was found to be 13.3 %, which is distinctively similar to the values, obtained using detoxified hydrolysate. However, total sugar consumption reached around 87% after 108 h of fermentation. Fermentation was stopped beyond this point, as the main objective was to achieve maximum chitosan production though complete utilization of the sugars present could be possible if the fermentation time was prolonged.

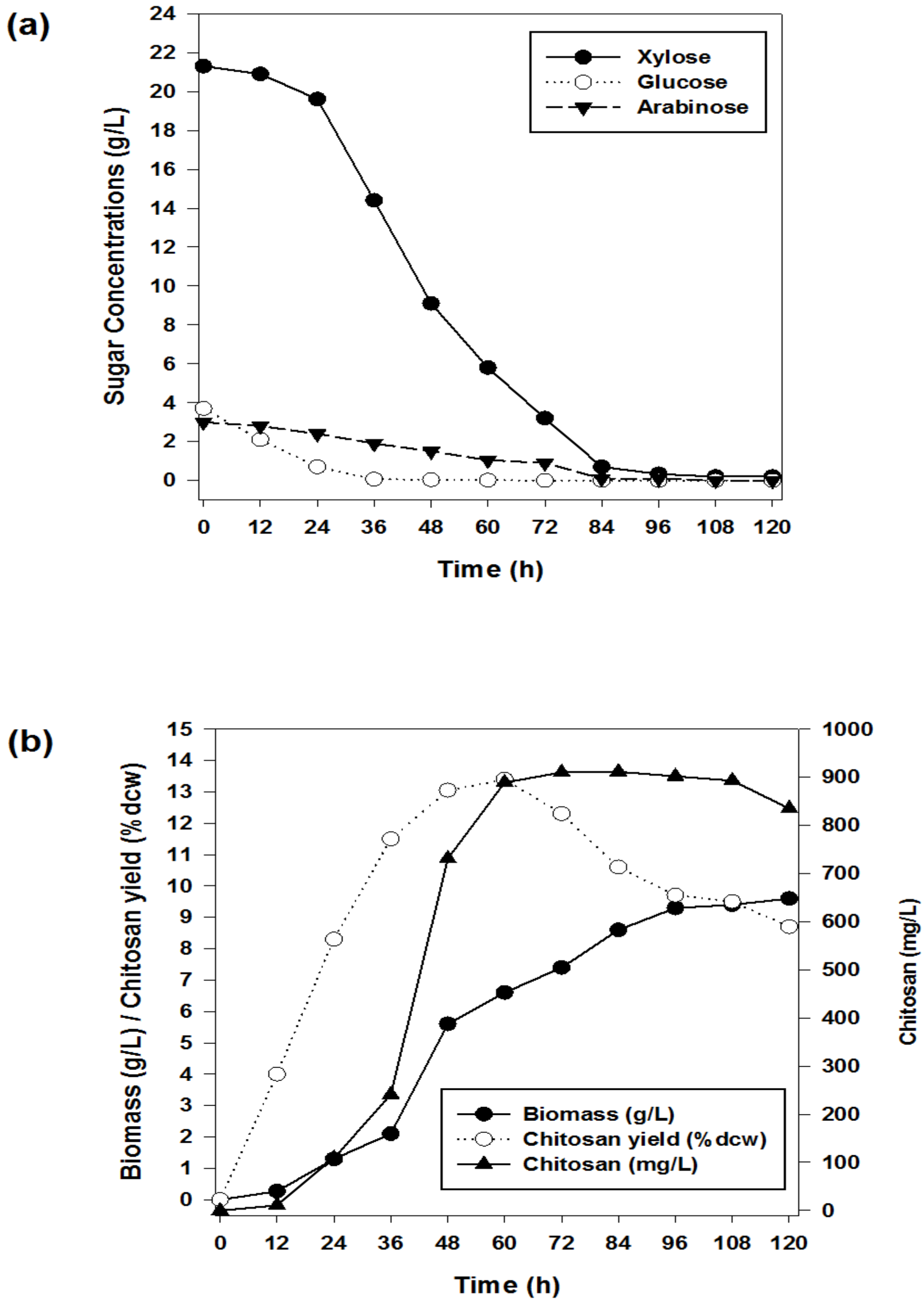


Fig. 4.23 Time profile of (a) sugar consumption and (b) biomass growth and chitosan production by *P. citrinum* using detoxified rice straw hydrolysate as the substrate in the batch bioreactor experiments.

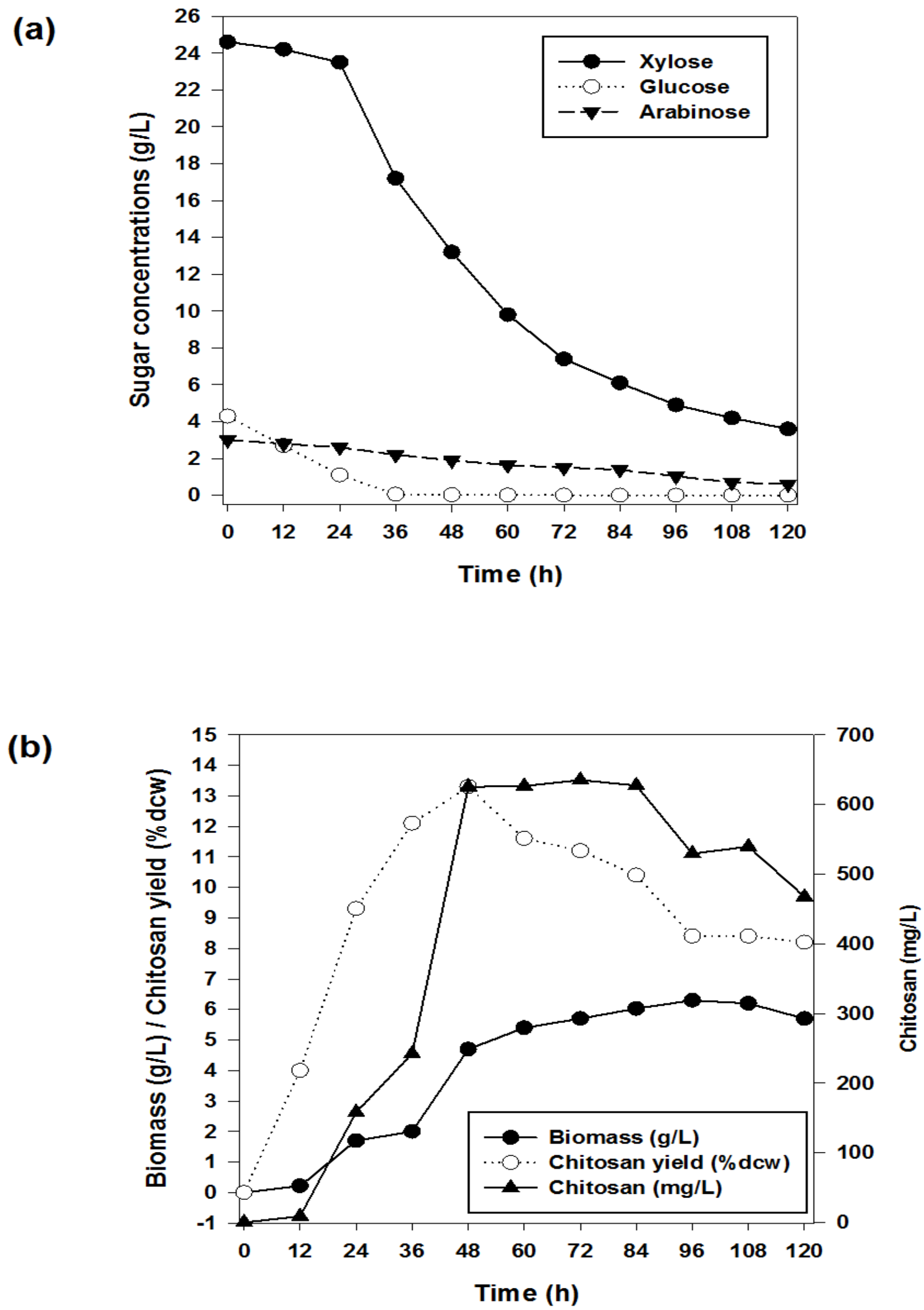


Fig. 4.24 Time profile of (a) sugar consumption and (b) biomass growth and chitosan production by *P. citrinum* using non-detoxified rice straw hydrolysate as the substrate in the batch bioreactor experiments.

Fermentation carried out under fed-batch mode of operation in the bioreactor was unsuccessful due to the agglomeration of fungal biomass, which resulted in drastic reduction in DO levels. This in turn resulted in reduced biomass growth and most of it could not be recovered efficiently for chitosan extraction. High sugar concentration in the RSH lead to relatively high fungal biomass during the batch mode of operation, which led to agglomeration when feed containing the concentrated RSH was introduced into the reactor. Therefore, batch reactor mode of operation was considered best for fungal chitosan production using detoxified RSH. High chitosan yields of above 13% using detoxified or non-detoxified hydrolysates is attributed to acetic acid present in the hydrolysates, which was earlier shown to induce chitosan production by the fungus. Hence, rice straw hydrolysate was used as a substrate as it avoids the addition of any inducers for chitosan production by *P. citrinum*.

4.5 Fungal chitosan production using agricultural and industrial wastes

Solid-state fermentation is ideal for filamentous fungal growth as these microorganisms are naturally adapted to grow on moist solid substrates. It also overcomes the problem with substrate inhibition for achieving high productivity, which could be encountered in submerged fermentation. Agro-industrial residues such as rice straw, paper mill sludge and citrus peels were chosen for this study as they are considered as ideal substrates to keep the process cost low. These substrates were initially screened for chitosan production and effect of pre-treatment and various process parameters were investigated, followed by fermentation in lab scale tray fermenters.

4.5.1 Screening of substrates

For initial screening of rice straw, citrus peels and paper mill waste sludge as alternate carbon source for chitosan production by *P. citrinum*, experiments were carried out using Erlenmeyer shake flasks for 9-11 days by pre-adjusting the pH to 4.5. Due to the high ash content in paper mill sludge, repeated washing with deionised water was carried out to bring the ash content down to 7% prior to inoculation. Among the three substrates examined for chitosan production, rice straw and paper mill sludge were very efficiently utilized by *P. citrinum* biomass. These substrates resulted in a chitosan yield of ~3 g/kg substrate (Fig. 4.25). However, with a low yield of just around 1.5 g/kg substrate, citrus peels of the sweet lemon was not found to be a suitable substrate for chitosan production by solid state fermentation using *P. citrinum.* Therefore, rice straw and paper mill sludge were chosen for further experiments to enhance chitosan production.

4.5.1.1 Effect of mineral salt media

Mineral salt medium serves as the source of nitrogen, salts and trace elements that are vital for efficient growth of fungi and other microorganisms. Chitin/chitosan is a part of the fungal cell wall and therefore, is considered a growth-associated product. Nutrient rich conditions thus result in high biomass and chitosan yields (Maghsoodi et al., 2008; Mondala et al., 2015). Chitosan yield from rice straw increased with the supplementation of MSM till 20% (v/v) (Fig. 4.26). Further increase in MSM volume, however, did not improve the chitosan yield. In case of paper mill sludge, maximum chitosan yield was achieved at 10% v/v MSM with no further enhancement observed with an increase in the MSM concentration. Thus, maximum chitosan yields from rice straw and paper mill sludge were 3.9 and 3.7 g/kg substrate, respectively. This could be due to the relatively lower nitrogen content in rice straw when compared with paper mill wastewater (Table 3.3).

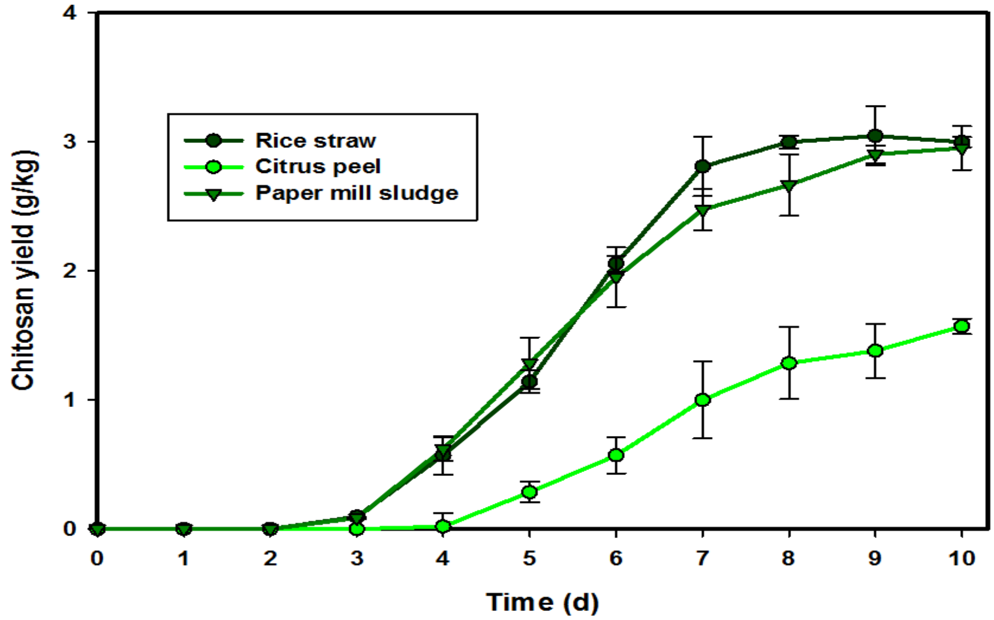


Fig. 4.25 Screening of solid substrates for chitosan production by *P. citrinum*.

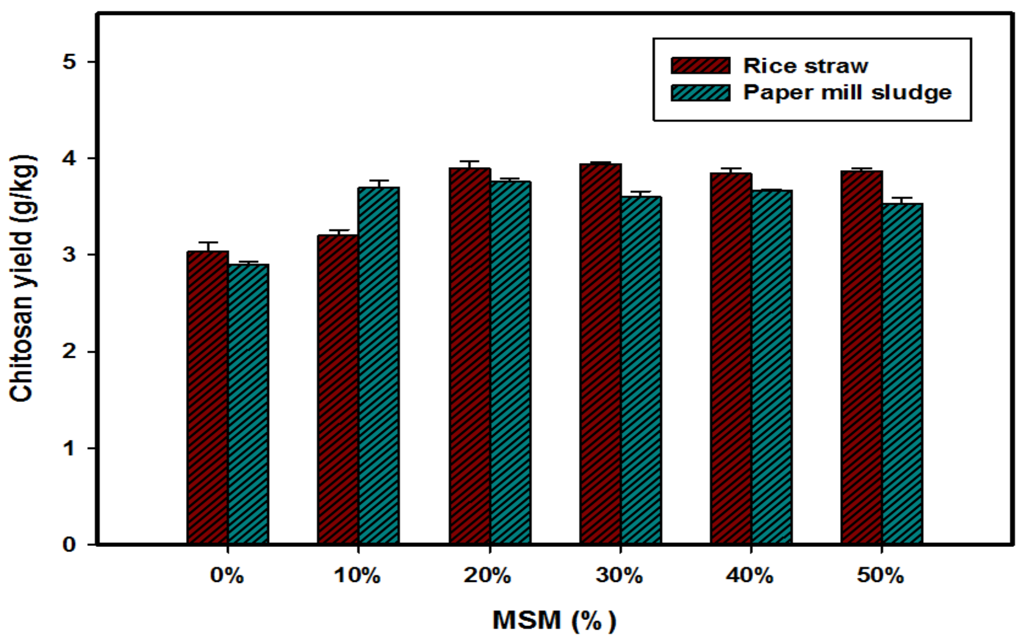


Fig. 4.26 Effect of MSM supplementation on fungal growth and chitosan production using rice straw and paper mill sludge as substrates.

4.5.2 Fungal chitosan production using rice straw

Rice straw showed promising results of chitosan production using *P. citrinum* biomass after preliminary screening, and, therefore, further investigations were carried out to enhance the chitosan yields by subjecting the rice straw to different pre-treatment methods. The effect of different physical parameters such as particle size and moisture content on fungal chitosan production further examined. The effect of acetic acid as an inducer of chitosan production was also evaluated. SSF using a tray fermenter under controlled conditions of relative humidity was finally carried out to achieve maximum chitosan yield.

4.5.2.1 Effect of pre-treatment

Rice straw was subjected to alkaline and acid pre-treatment to investigate the effect on fungal growth and chitosan production. Alkaline treatment clearly showed a better result with a chitosan yield of ~7 g/kg substrate following 7 days of growth (Fig. 4.27). Alkali pre-treatment of lignocellulosic wastes is known to remove lignin and a part of hemicellulose, and it efficiently increases the accessibility of cellulose present to degrading enzymes. The separated and fully exposed micro-fibrils further increased the external surface area and the porosity of rice straw, thus facilitating enzymatic hydrolysis. Dilute acid pre-treatment, on the other hand, predominantly affects hemicellulose with little impact on lignin degradation. The micro structure and surface morphology (transverse sections) of untreated and pre-treated rice straw were examined using FESEM (Fig. 4.28). The untreated rice straw showed a compact and rigid structure with the microfibrils in an ordered pattern. Compared to the untreated rice straw, considerable changes of pre-treated rice straw were observed with the surface showing rough aggregated irregular structures. Disruption of microfibril structure in alkali treated rice straw was found to be the most profound with microfibrils completely exposed when compared with that of the untreated rice straw. Compared with acid treatment, alkali treatment is an effective method to break ester bonds between lignin, hemicellulose and cellulose, and it avoids fragmentation of hemicellulose polymers (Gáspár et al., 2007). Alkali treatment in conjugation with microwave helps in dissolution of lignin, which results in formation of pores on rice straw, which then explode due to the microwave treatment. This simultaneous action of alkali and microwave pre-treatment results in enhanced removal of lignin from the straw.

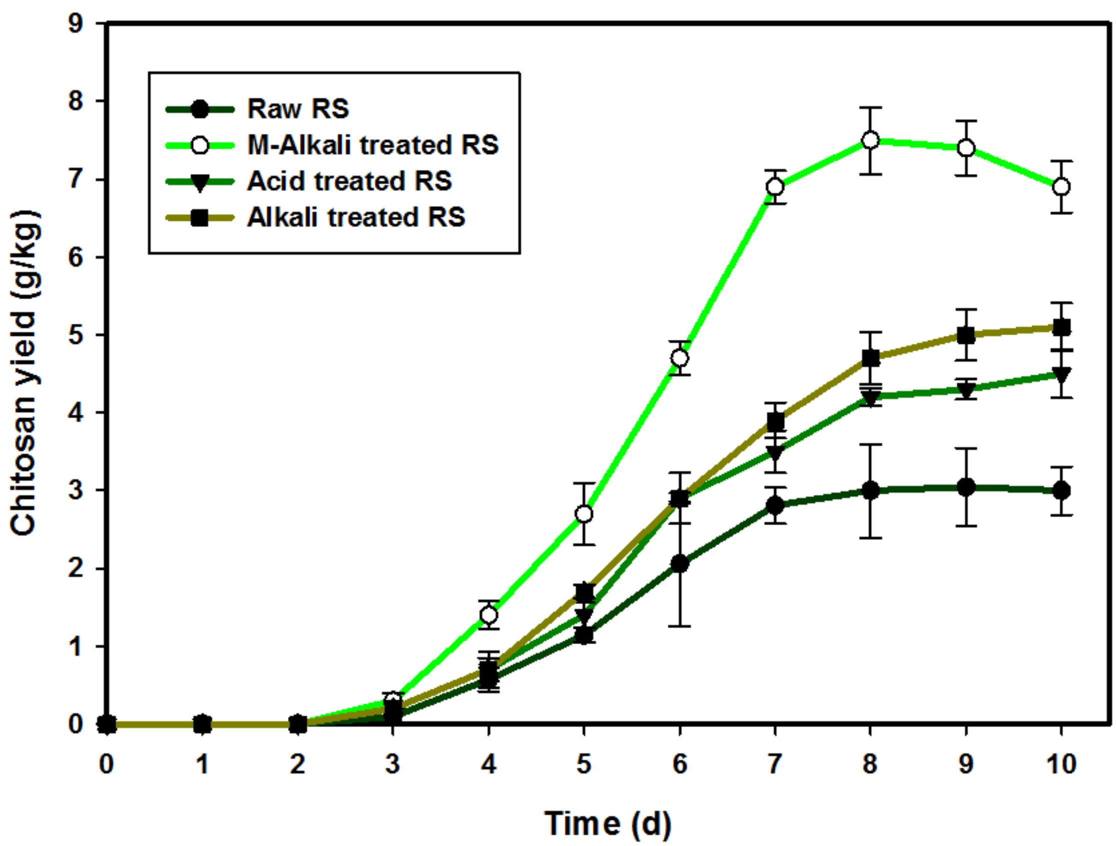


Fig. 4.27 Effect of different pre-treatment methods on fungal growth and chitosan production by *P. citrinum* using rice straw as the substrate.

Table 4.4 shows that similar trends in the increase and decrease of the content of cellulose and lignin, respectively in the rice straw is observed for alkali and microwave associated alkali treatments. Cellulose content in the rice straw increased due to alkali treatment with a corresponding decrease in the lignin percentage. Hemicellulose content is found to be high with alkali pre-treated rice straw; however, a decrease in both hemicellulose and lignin content was observed for microwave-alkali treated rice straw. This loss in hemicellulose is likely because lignin is tightly bound to hemicellulose structures and, therefore, disruption of the lignin-hemicellulose structure lead to the partial removal of hemicellulose from the rice straw. Microwave alkali pre-treatment method have been shown to reduce hemicellulose and cellulose components of rice straw into reducing sugars which are easily assimilated by fungi for its growth and metabolite production (Cheng et al., 2011; Zhu et al., 2005).

Table 4.4 Composition of rice straw following different pre-treatment methods.

Pre-treatment	**Composition (%)**		
	Cellulose	**Hemicellulose**	**Lignin**
Untreated	34.1	27.3	11.7
Acid	26.3	21.1	16.7
Alkali	43.9	32.6	10.4
Microwave-alkali	56.2	24	6.7

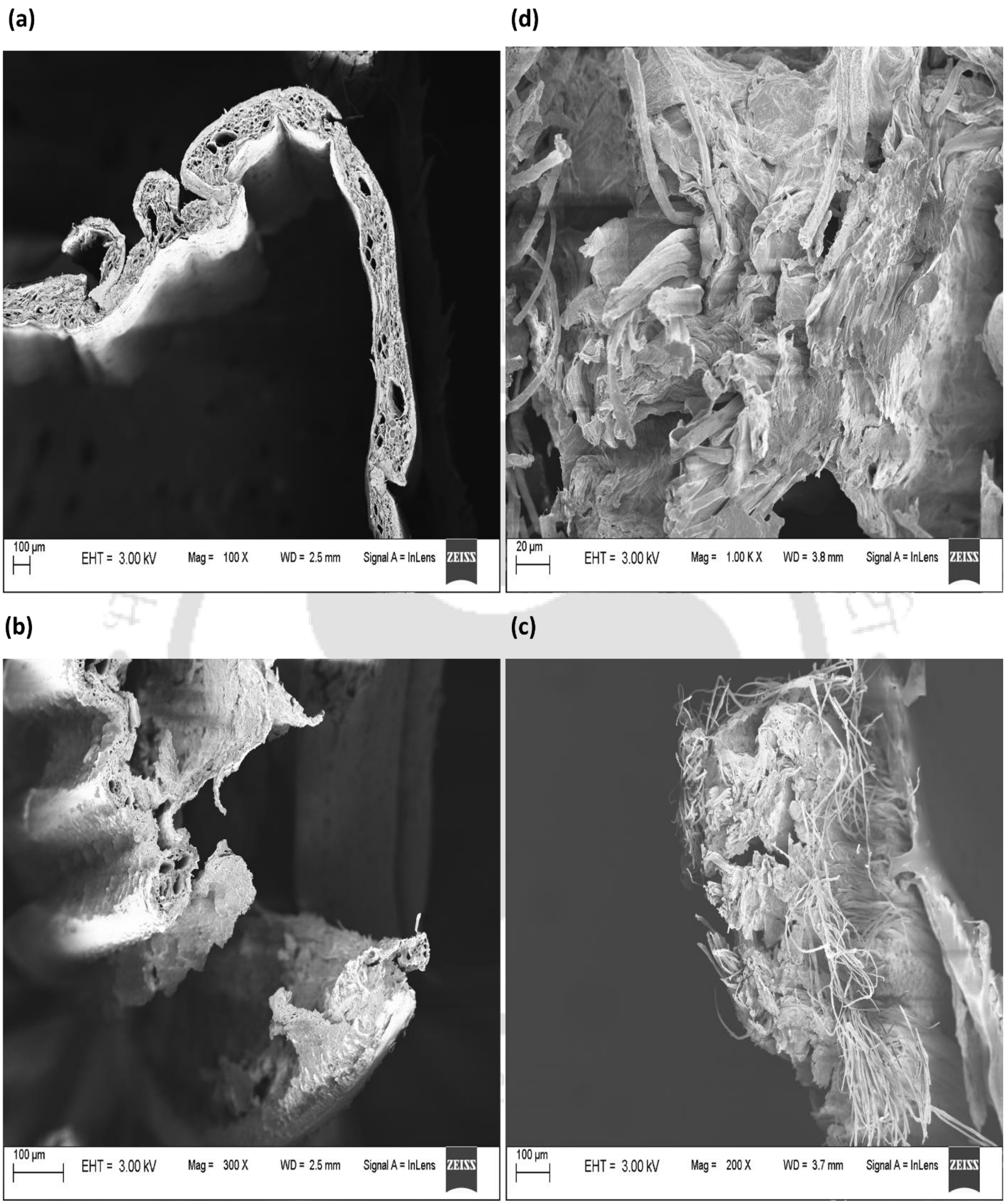


Fig. 4.28 FESEM images showing (a) raw, (b) acid-treated and (c) alkali-treated (d) microwave alkali rice straw

4.5.2.2 Effect of particle size and moisture content on chitosan production

The effect of various process parameters on chitosan production by *P. citrinum* grown on pre-treated rice straw was investigated by varying the levels of moisture content and particle size one at a time. Fig. 4.29 shows that an increase in the moisture content (solid to liquid ratio) enhanced fungal growth and chitosan production. A moisture content of 70% was found to be optimum for maximum chitosan production at all particle sizes. At ~4-5 mm size (PS2) of pre-treated rice straw, maximum biomass growth and chitosan yield were obtained for different solid to liquid loading ratios.

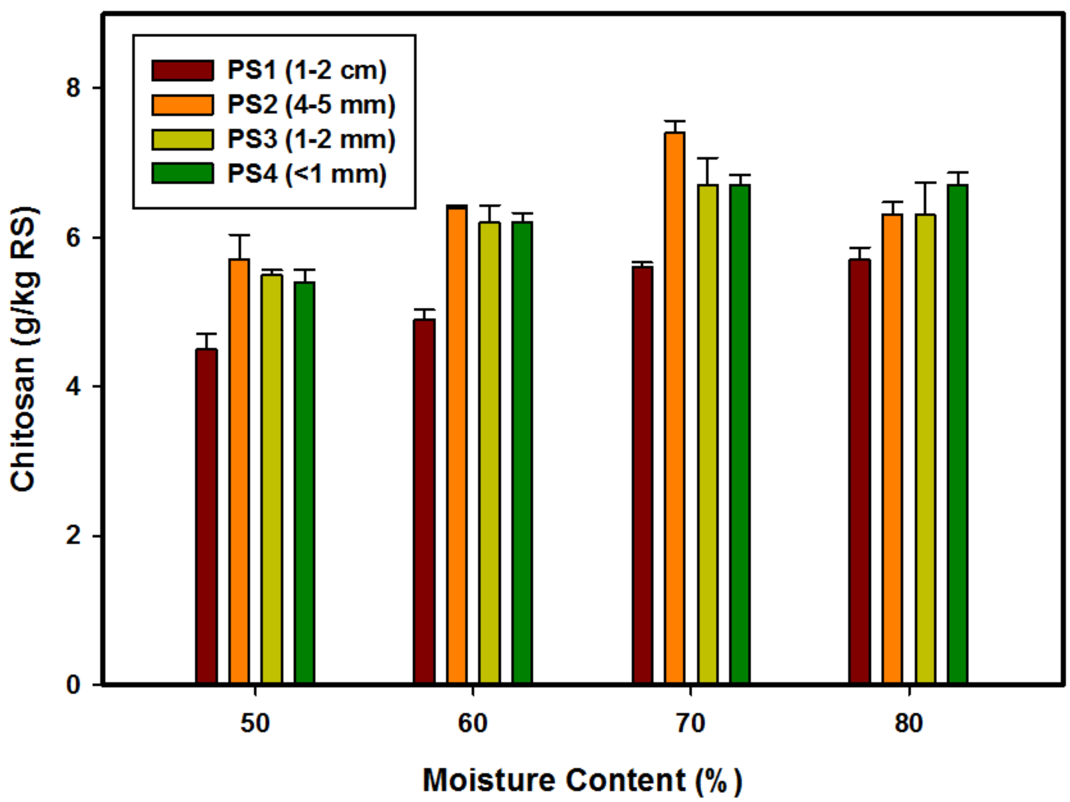


Fig. 4.29 Effect of different particle size and moisture content on fungal growth and chitosan production by *P. citrinum* using pre-treated rice straw.

Particle size is critical for enhanced mass transfer of oxygen and other nutrients to the void space that affects fungal growth on solid media. Generally, small-sized substrate particles provide a large surface area for microbial action but too small particles may result in substrate agglomeration, which may interfere with microbial respiration thus resulting in poor growth. Hence, in this study a low chitosan yield was obtained in the case of PS3 and PS4 particle sizes. Large-sized particles on the other hand have limited surface area for microbes to grow on with very less accessibility of fungal enzymes to act upon the substrates as could be seen in the case of PS1 for chitosan production in the study (El-Mansi et al., 2018).

4.5.2.3 Effect of acetic acid on chitosan production using pre-treated rice straw

Fig. 4.30 clearly shows the enhancement in chitosan production due to acetic acid addition. Chitosan yield from rice straw increased to a maximum of ~8 g/kg substrate in the presence of acetic acid at 80 mg/L concentration. Alkali pre-treated rice straw is an excellent source of cellulose and hemicellulose and, acetic acid addition proved efficient in enhancing the activities of various enzymes by *P. citrinum*, which resulted in maximum utilisation of the substrate by the fungi for fungal growth and chitosan production in this study. Moreover, acetic acid itself may serve as a primary carbon source for an efficient fungal growth and release of hydrolyzing enzymes to act upon other complex substrates present. A further increase in the acetic acid concentration, however, did not improve the chitosan yield which is consistent with the results obtained previously on the effect of acetic acid on chitosan production using paper mill wastewater under submerged conditions (Namboodiri and Pakshirajan, 2019).

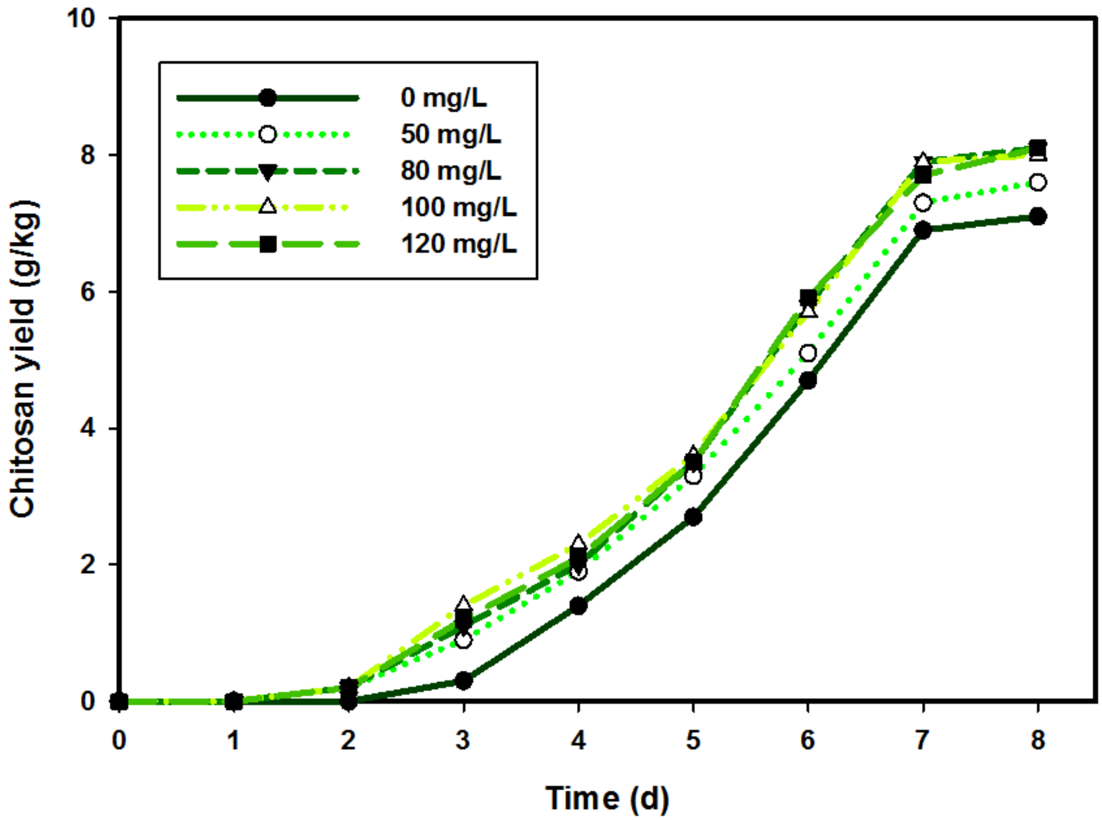


Fig. 4.30 Effect of different acetic acid concentration on fungal growth and chitosan production by *P. citrinum* biomass using pre-treated rice straw as the substrate

4.5.2.4 Solid state fermenter studies

A lab scale tray fermenter with three trays was employed to carry out the solid-state fermentation under controlled condition of relative humidity. Effect of inoculum size was first investigated by maintaining a RH of 70%, and the results are shown in Fig. 4.31. The effect of maintaining different relative humidity conditions on chitosan production revealed an optimum value of 65% using the tray fermenter (Fig. 4.32). Relative humidity plays a vital role in solid-state fermentations which are generally carried out at relatively low levels of water. However, an optimum level of water is essential for fungi to carry out its biochemical functions including enzymatic hydrolysis of substrates for easy uptake. Among the various fermenters designed for SSF, tray fermenters such as the one used in this study have been reported to be the most viable design for chitosan production owing to easy recovery of the fungal biomass (Nwe et al., 2011). Moreover, tray fermenters are easy to operate due to their simple design and its scale up cost is also low (Durand, 2003).

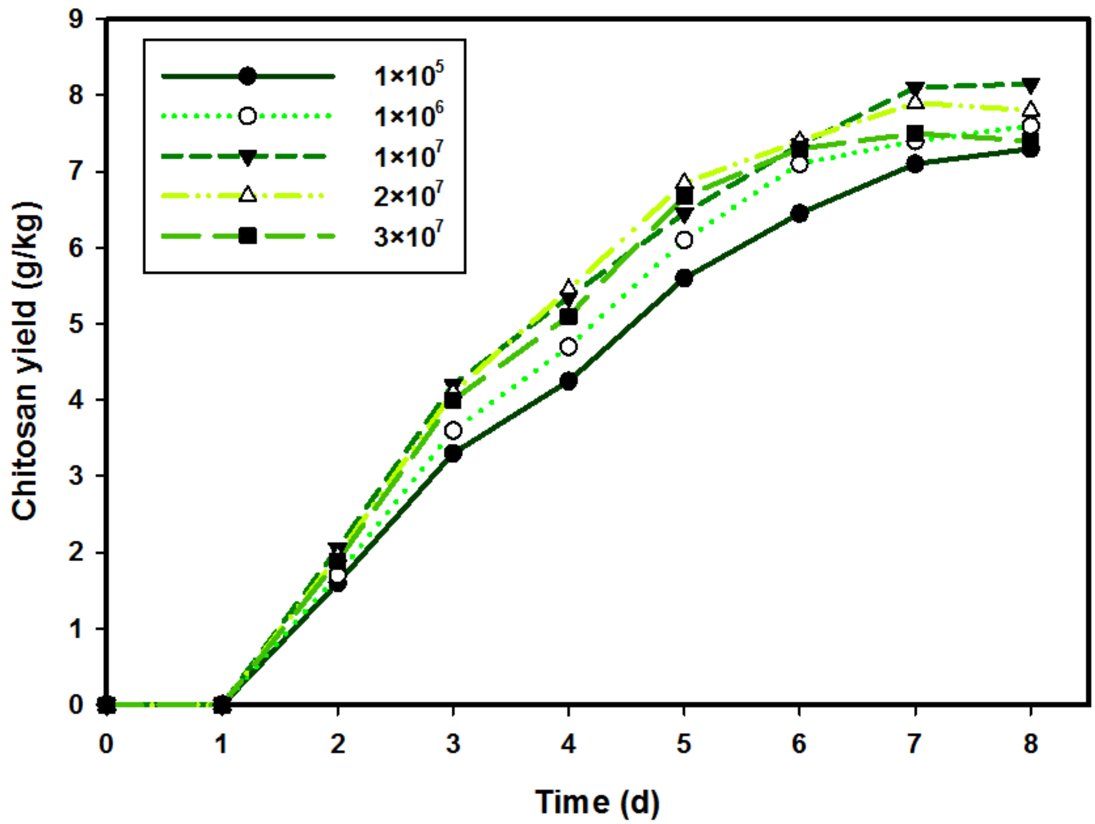


Fig. 4.31 Effect of different inoculum size on fungal chitosan production by *P. citrinum* biomass on pre-treated rice straw under 70% relative humidity conditions.

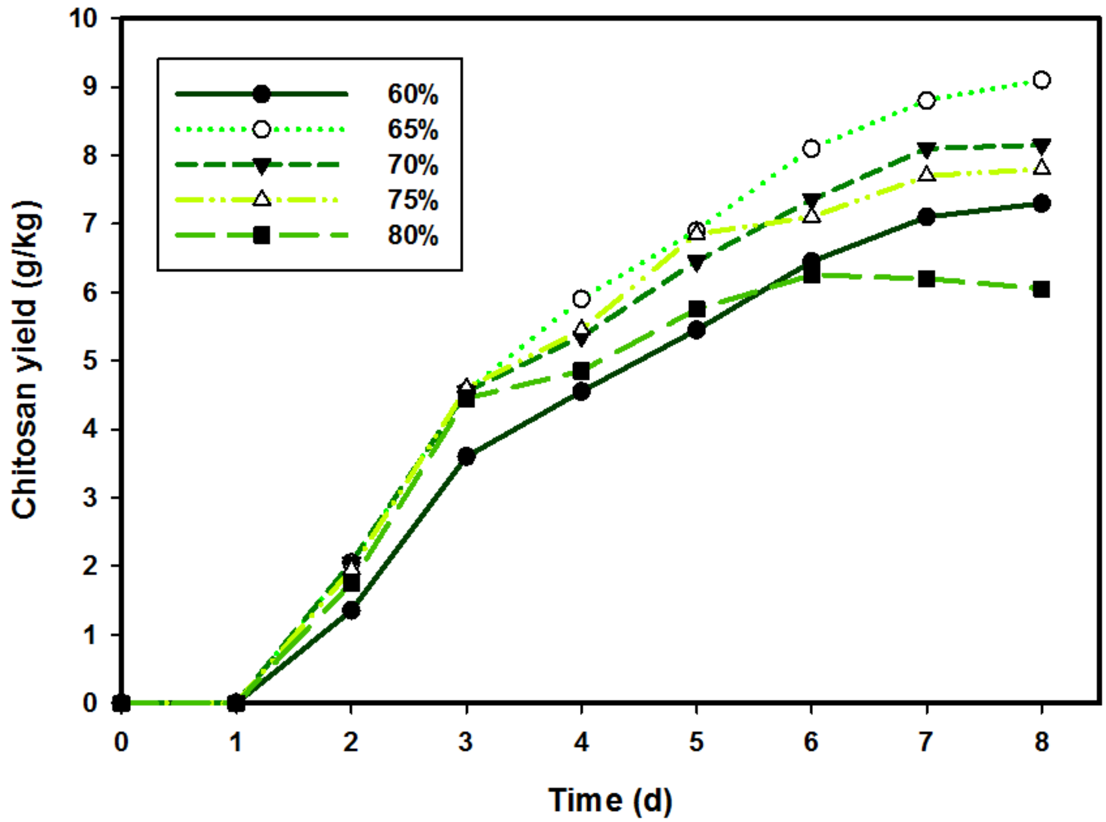


Fig. 4.32 Fungal chitosan production by *P. citrinum* biomass on pre-treated rice straw under different relative humidity conditions.

Among the limited studies available on solid-state fermentation (Table 4.5) for fungal chitosan production, a highest chitosan yield of 5.63 g/kg from rice straw is previously reported by Vida et al. (2008). Thus, maximum chitosan yield of 9.3 g/kg of pre-treated rice straw reported in this study indicates the ability of *P. citrinum* to efficiently utilise hemicellulosic and lignocellulosic residues present in the rice straw by solid-state fermentation. Hence, this study suggests that rice straw is a viable feedstock for fungal chitosan production, which could reduce the cost of production and simultaneously mitigate environmental risk due to the burning of rice straw.

Table 4.5 Fungal chitosan production by solid-state fermentation.

Organism	Substrate	Chitosan yield	Reference
Lentinus elodes	Wheat straw	6.18 g/kg biomass	(Crestini et al., 1996)
Rhizopus oryzae	Soybean and mung bean	4.3 g/kg soybean 1.6 g/kg mung bean residue	(Suntornsuk et al., 2002)
Rhizopus oryzae	Rice straw	5.63 g/kg fermented media	(Khalaf, 2004)
Aspergillus niger	Canola residue	12.7 g/kg canola residue	Vida et al. (2008)
Mucor rouxii	Soybean meal	34.4 g/kg substrate	(Mondala et al., 2015)
Penicillium citrinum	Rice straw	9.3 g/kg rice straw	This study*
	Paper mill sludge (PMS)	8.5 g/kg PMS	This study*
	PMS with paper mill wastewater	10.6 g/kg substrate	This study*

4.5.3 Fungal chitosan production using paper mill sludge

In addition to rice straw, paper mill sludge showed promising results for chitosan production using *P. citrinum*. Therefore, investigations aimed at enhancing the chitosan yield from paper mill sludge were carried out by subjecting it to different pre-treatment methods and understanding the effect of different process parameters on fungal chitosan production.

4.5.3.1 Effect of pre-treatment

Paper mill sludge was primarily subjected to alkaline and acid pre-treatment to investigate the effect on fungal growth and chitosan production. Although alkali pre-treatment showed better results for chitosan production with PMS as observed previously with rice straw, the increase in chitosan production was not significant in this case, and the reason could be due to a high percentage of lignin in PMS as compared with that in rice straw. The sludge is also highly compact due to the binding properties of lignin with glucan and xylan present in it. In order to overcome this problem, an efficient pre-treatment technique was explored to disrupt the structural integrity of PMS, thereby enabling the fungal enzymes to act upon the substrate. Microwave assisted alkali treatment have been found to enhance enzymatic hydrolysis of lignocellulosic wastes by removing the recalcitrant lignin (Cheng et al., 2011). Thus, chitosan production increased drastically from 3 g/kg to ~ 6 g/kg substrate when paper mill sludge was subjected to microwave-alkali pre-treatment at 500 W for 15 min (Fig. 4.33). Alkali treatment helps in the dissolution of the lignin, which results in the formation of pores and grooves on PMS. However, the simultaneous microwave treatment leads to explosion of these pores due to the presence of water. This simultaneous action of alkali and microwave pre-treatment results in enhanced removal of lignin from the PMS. However, some loss in the hemicellulose content present in the sludge is observed (Table 4.6) which could be because lignin is tightly bound to hemicellulose structures and disruption of the lignin-hemicellulose structure leads to their partial removal. Similar observation was made in the case of microwave-alkali pre-treatment of rice straw for chitosan production by *P. citrinum*.

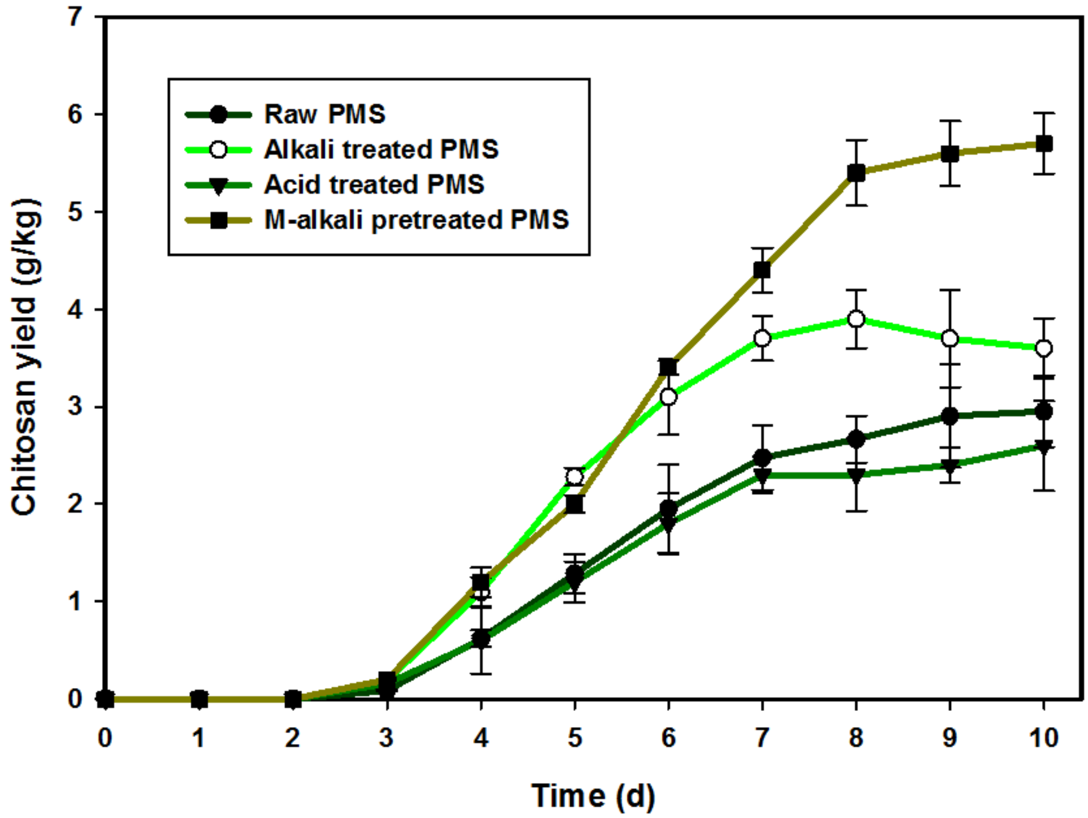


Fig. 4.33 Effect of different pre-treatment methods on fungal growth and chitosan production by *P. citrinum* using PMS as the substrate.

Table 4.6 Composition of PMS after employing different pre-treatment methods.

Pre-treatment	**Composition (%)**		
	Cellulose	**Hemicellulose**	**Lignin**
Untreated	17.9	4.3	26.3
Acid	14.3	3.9	31.1
Alkali	24.5	9.5	21
Microwave-alkali	36	4.1	12.3

4.5.3.2 Effect of particle size and moisture content on chitosan production

Fig. 4.34 shows that increasing the moisture content (solid to liquid ratio) from 50% to 60% resulted in increased fungal growth and chitosan production and, a moisture content of 60% was found to be optimum for maximum chitosan production at all particle sizes. A particle size of ~4-5 mm (PS2) yielded maximum growth and chitosan at all values of the solid to liquid ratios, which is similar to the results obtained with rice straw.

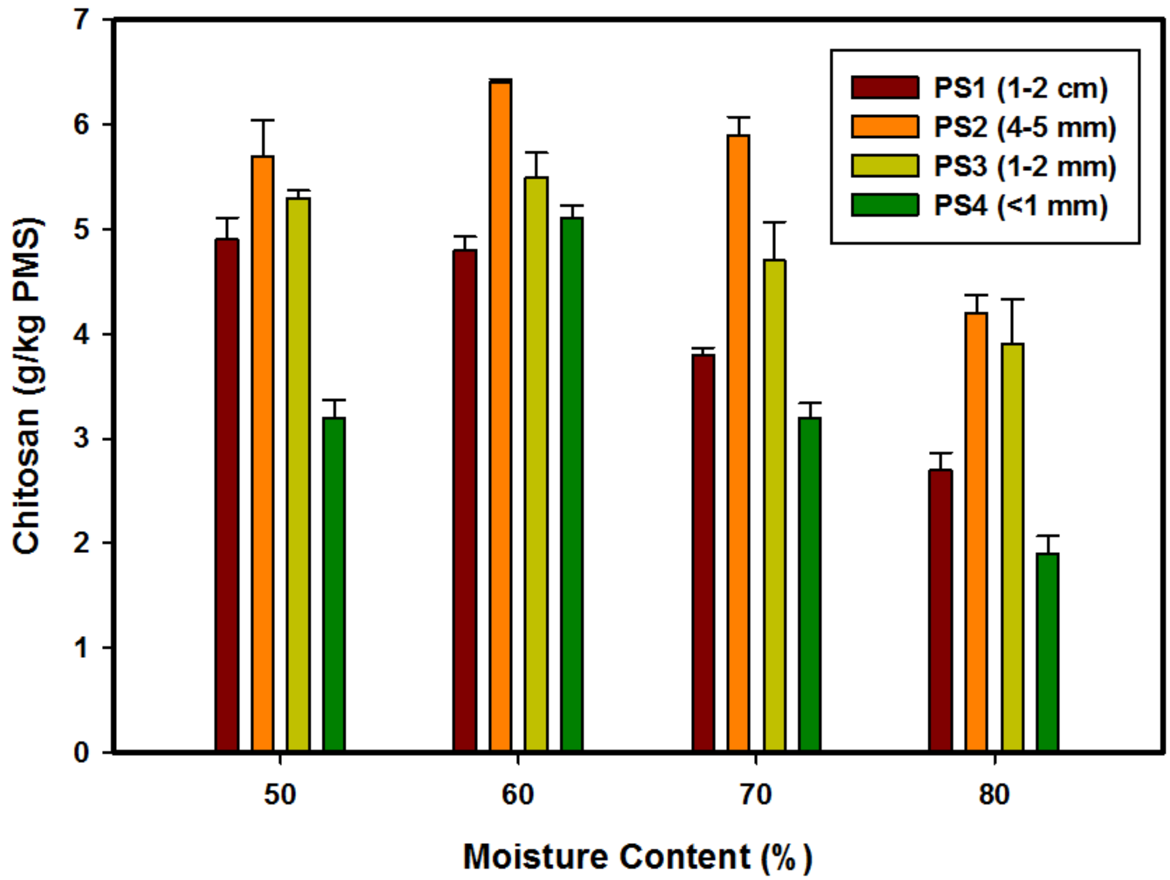


Fig. 4.34 Effect of different particle size and moisture content of PMS on fungal growth and chitosan production by *P. citrinum*.

Drastic reduction in chitosan yield was observed at 70% and 80% moisture content with PS4 particle size. This is consistent with the fact that a high amount of water causes agglomeration of small-sized particles, thereby leading to reduced mass transfer of nutrients and heat dissipation. Thus, maximum chitosan yield of 6.5 g/kg PMS was achieved at 60% moisture content with a particle size of 4-5 mm, and the same conditions were followed in further experiments.

4.5.3.3 Effect of acetic acid addition on chitosan production

Fig. 4.35 shows the effect of acetic acid concentration on chitosan production by *P. citrinum* using paper mill sludge as the substrate. Only a slight increase in the chitosan yield from 6.5 g/kg to 6.8 g/kg substrate is observed with the addition of acetic acid. However, lag phase in fungal growth was reduced due to the addition of acetic acid. This could be reasoned based on the low levels of hemicellulose present in PMS, as enhancement of fungal growth as well as chitosan production has been observed with substrates rich in hemicelluloses.

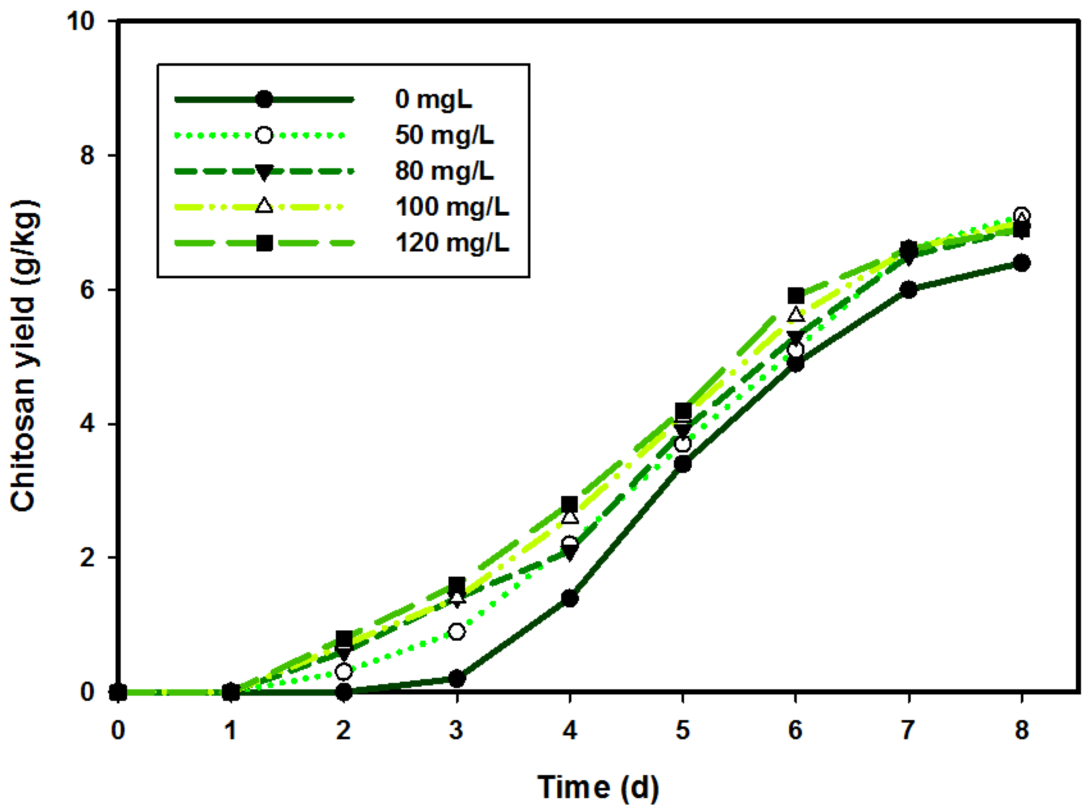


Fig. 4.35 Effect of different acetic acid concentration on fungal growth and chitosan production by *P. citrinum* using pre-treated PMS as substrate.

4.5.3.4 Solid state fermenter studies

Optimum inoculum size was first established in a tray fermenter to carry out the solid-state fermentation of paper mill sludge under controlled condition of relative humidity of 65% as depicted in Fig. 4.36. The fermenter was then operated at different relative humidity setting by passing humidified air through the reactor to achieve high chitosan yield and a maximum chitosan yield of 8.5 g/kg was achieved at a relative humidity of 60% (Fig. 4.37).

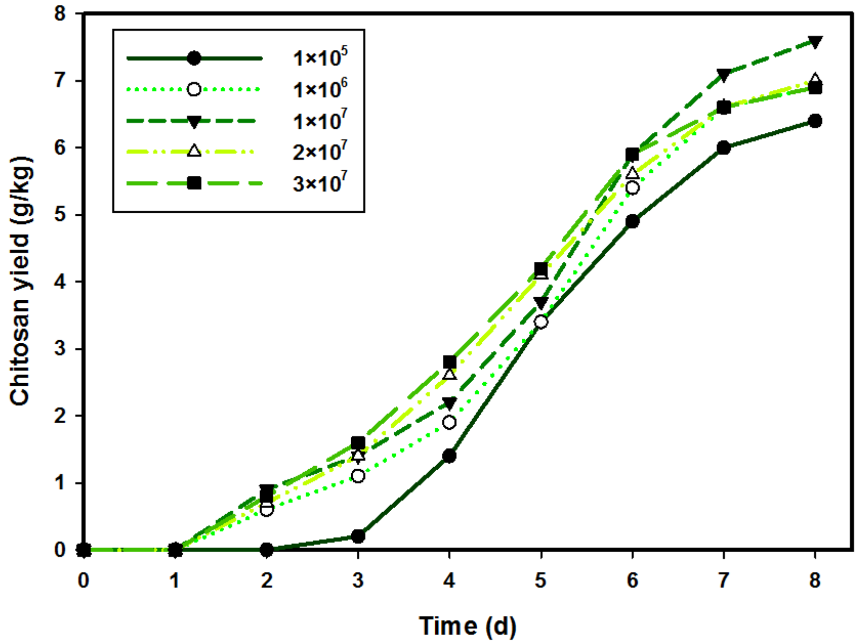


Fig. 4.36 Effect of different inoculum size on fungal chitosan production by *P. citrinum* using pre-treated PMS in the tray fermenter under 65% relative humidity conditions.

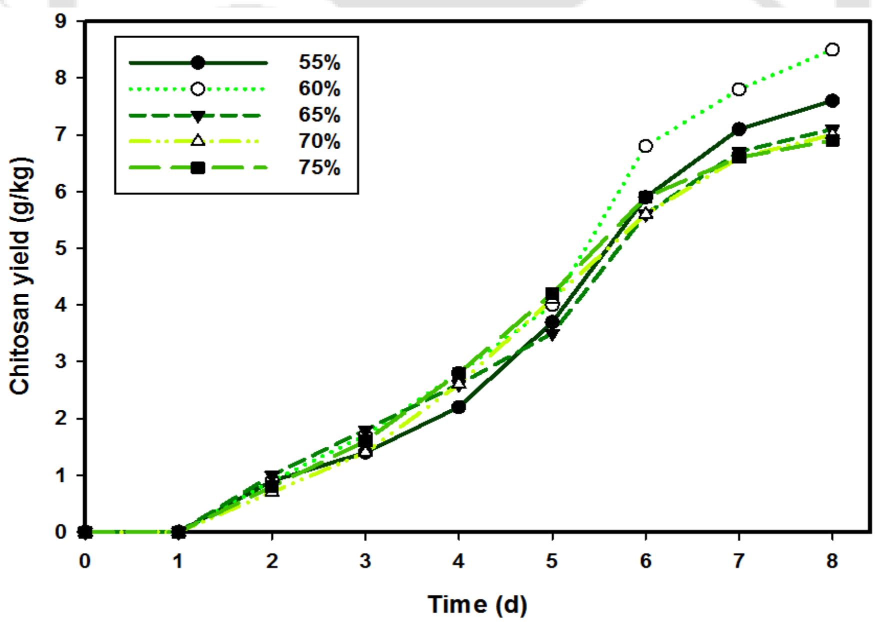


Fig. 4.37 Fungal chitosan production by *P. citrinum* biomass on PMS under different relative humidity conditions.

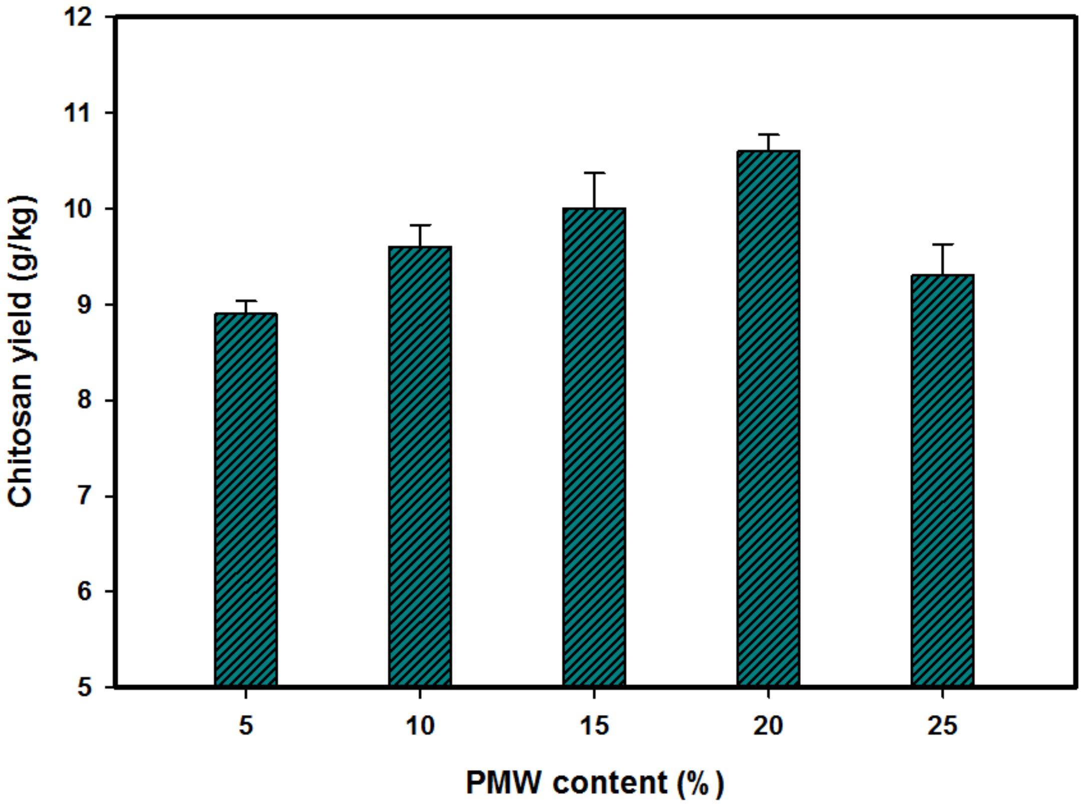


Fig. 4.38 Fungal chitosan production by *P. citrinum* on pre-treated PMS supplemented by paper mill wastewater.

Based on the previous results obtained using paper mill wastewater as the substrate under submerged condition, an integrated approach to simultaneously utilize both paper mill sludge as well as wastewater for chitosan production was investigated. In this approach, solid-state fermentation of paper mill sludge was carried out with paper mill wastewater/MSM for the initial moisture content instead of only mineral salt media. Paper mill wastewater at different volumes (5%, 10%, 15%, 20% and 25%) were added to the MSM and supplemented to the paper mill sludge for carrying out the fermentation and a maximum chitosan yield of 10.6 g/kg substrate was achieved by following this approach (Fig. 4.38). The decrease in chitosan yield above 20% v/v of the wastewater in MSM is attributed to low levels of nitrogen available in the mixture solution, which is vital for the fungal growth as well as chitin/chitosan synthesis pathway. Compared with the literature reports on fungal chitosan production, the value obtained using pre-treated PMS supplemented with the wastewater is very high (Table 4.5). Hence, the process could possibly be integrated in paper mill industry where large amounts of wastewater and sludge is generated for achieving sustainable fungal chitosan production. Simple cost estimation of the biological process developed in this study its comparison with chemical process for chitosan production was done using the following assumptions:

1. In both chemical (commercial) and biological processes for chitosan production, the amount of input raw material is considered the same. Based on Gómez Ríos (2016) 260 kg dried shrimp shell waste (1 ton wet weight) per cycle was considered as the input for chemical production and for the biological process 260 kg of paper mill sludge per cycle was considered as the input raw material.

2. 1.4 L diesel per ton shrimp shells is required for its transport using a tractor with an open trailer (Muñoz et al., 2018)

3. The machinery and equipment cost was calculated for the chemical process as per Gómez Ríos (2016). Assuming the other costs (piping, storage and other investments) are the same for both the processes, the machinery and equipment cost for the fungal fermentation process is almost 50% lower than that required to set up a chemical process industry.

The economic analysis of both the processes show that the cost of fungal based chitosan production process using paper mill sludge is ~35% lower than that incurred in the chemical process. This study shows that the fungal chitosan production route is more feasible than the chemical route for chitosan production.

Table 4.7 Simple cost estimation of the biological process developed in this study its comparison with chemical process for chitosan production

Item	Chemical Process	Biological Process
	Cost (USD)	Cost (USD)
Material	9.13	7.29
Transportation	0.0215	0
Energy and Utilities	2.1	1.09
Machinery and Equipment	3.883	1.94
Land	1.927	0
Vehicles and Furniture	2.280	2.280
Total	19.34	12.60

4.6 Chitosan characterization

In the FTIR spectra of chitosan samples shown in Fig. 4.39, a band corresponding to the vibrations due to amide II band (N-H stretching) is observed in the range 1555 cm^{-1} to 1557 cm^{-1} which confirms that the chitosan containing samples are deacetylated. The peak at 3440 cm^{-1} represents OH group stretching and at 2900-2921cm^{-1} represents C-H stretching. The band near 1620 cm^{-1} is due to the stretching of C-N bond vibration of the superimposed C=O group. The peak at 1307 cm^{-1} to 1311 cm^{-1} is attributed to the formation of CO-NH group. The degree of deacetylation (DD) for chitosan obtained from *P. citrinum* was 77%. Chitosan obtained by the addition of acetic acid to paper mill wastewater showed a slightly high DD of 81%, whereas standard (commercial) chitosan showed a DD of only 71%.

The viscosity average molecular weight (M_v) of commercial standard chitosan and chitosan obtained in this study were estimated to be 44 × 10^3 Da and 3 × 10^5 Da, respectively, by the Mark-Houwink-Sakurada equation. Molecular weight of chitosan is dependent on NaOH concentration used during deacetylation. The use of a high concentration of NaOH (70%) leads to disruption of the chitosan backbone and, therefore, results in its depolymerization.

The chitosan obtained from *P. citrinum* showed properties similar to that of a commercially available chitosan. Degree of deacetylation (DD) and molecular weight of the chitosan are very important factors, which regulate its use in various industries. High DD and low to medium molecular weight of chitosan produced by *P. citrinum* further demonstrates its potential for applications in agricultural, environmental and medical fields.

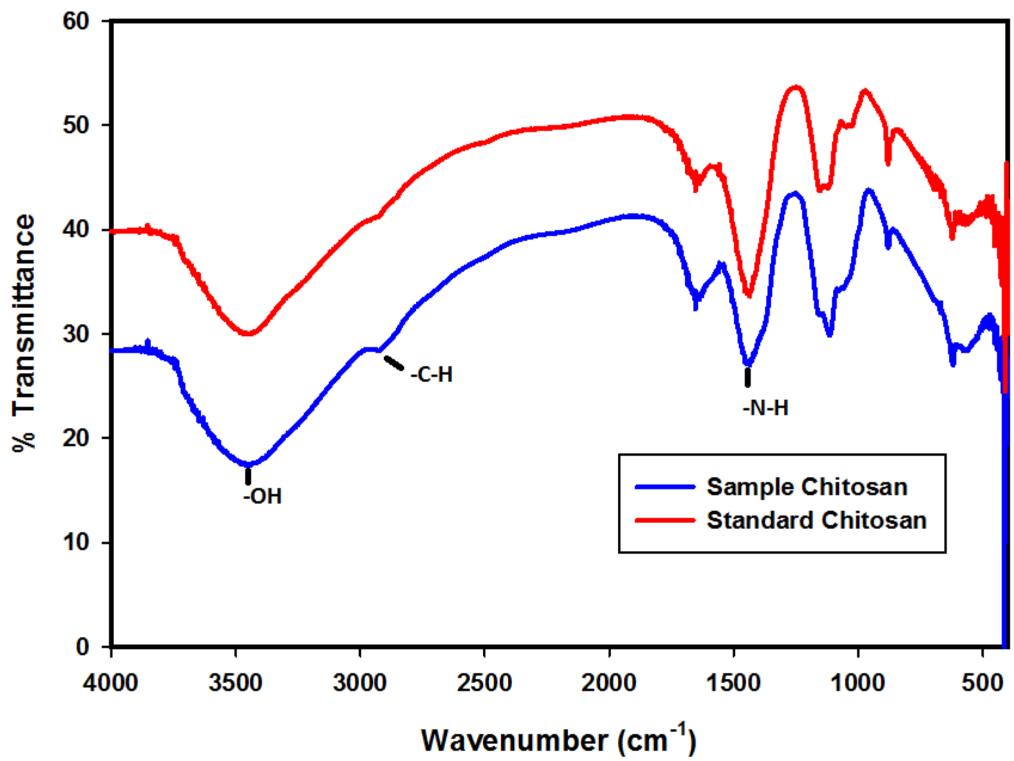


Fig. 4.39 FTIR spectra of chitosan samples

4.7 Application of chitosan based nanosorbents for heavy metal removal

Hydroxyl and amino groups on chitosan are active sites for the sorption of various dyes and heavy metal ions, which make it an excellent choice of biosorbent. Owing to its sensitivity towards pH, several studies have cross-linked chitosan with other compounds such as polyurethane, activated clay, poly vinyl alcohol etc., to improve its sorption capacities (Chang and Juang, 2004; Lee et al., 2009; Zhu et al., 2010). In this study, magnetic nanoparticles coated with chitosan and carboxymethyl chitosan were synthesised for improving the biosorption efficiency and easy separation of the biosorbent for reuse.

4.7.1 Characterization of CNP and CMCNP

Physical properties

Fig. 4.40 shows FETEM micrographs of CNP and CMCNP, which reveal that the nanoparticles were spherical in shape with a size in the range 20-30 nm. Selected area diffraction (SAD) analysis showed that the nanoparticles are polycrystalline in nature, which is attributed to the coating of Fe_3O_4 nanoparticles by a polymer film of chitosan and carboxymethyl chitosan, respectively. Agglomeration of the nanoparticles was observed in FETEM images, probably due to the magnetic dipoles in effect, and the van der Wall forces (Rajput et al., 2016). The size range of these agglomerates are in less than a few microns.

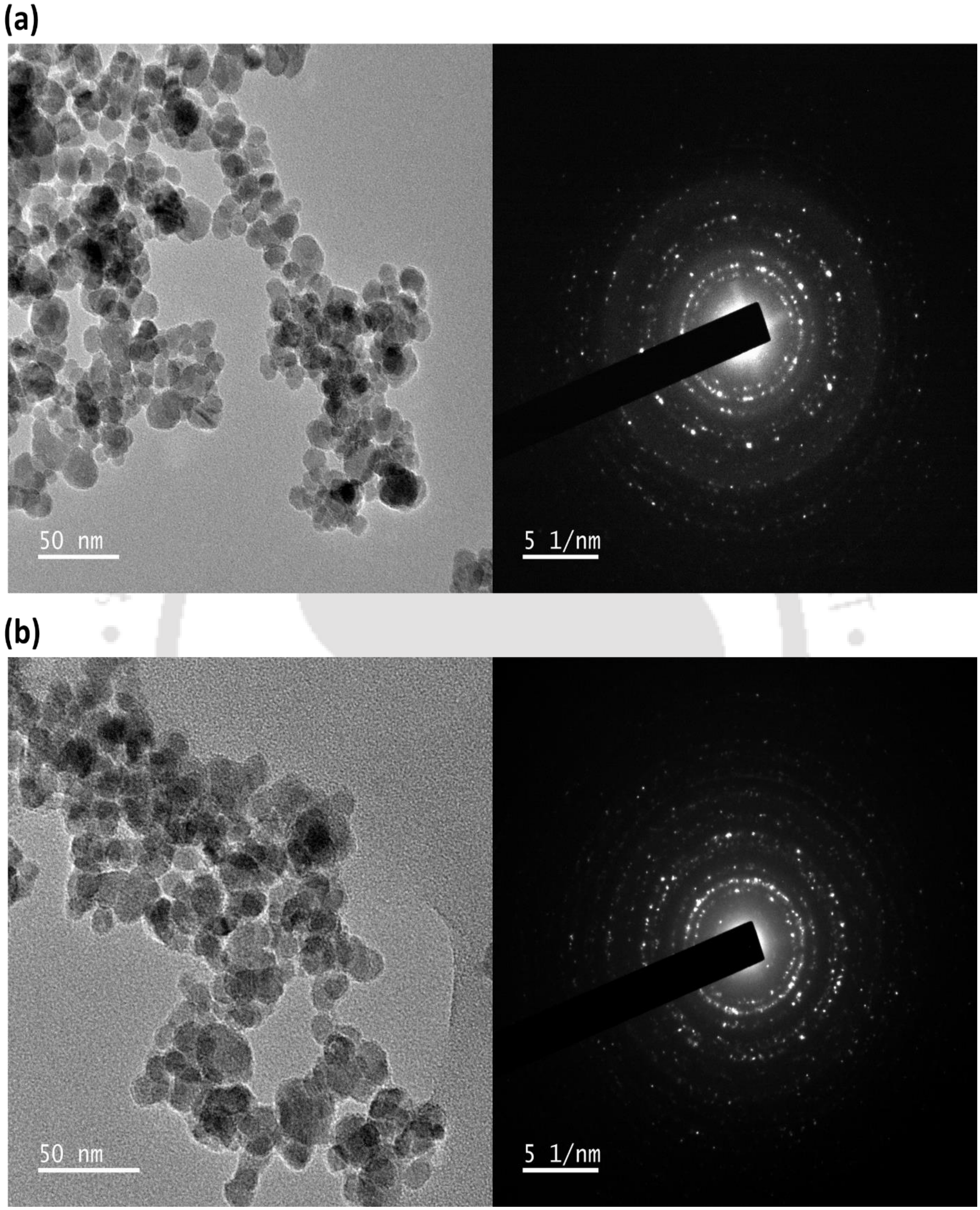


Fig. 4.40 FETEM image and SAD pattern of (a) CNP and (b) CMCNP.

Chemical properties

XRD patterns were observed to characterize the structures of CNP and CMCNP as depicted in Fig. 4.41. The peaks with miller indices at 30.1 (220), 43.08 (400), 53.45 (422), 56.98 (511), 62.57 (440), 70.99 (533) and 74.02 (444) confirmed the pattern obtained with magnetite (Fe_3O_4) nanoparticles (Feng et al., 2012; Yuwei and Jianlong, 2011). This result confirms the successful synthesis of the chitosan based Fe_3O_4 nanoparticles.

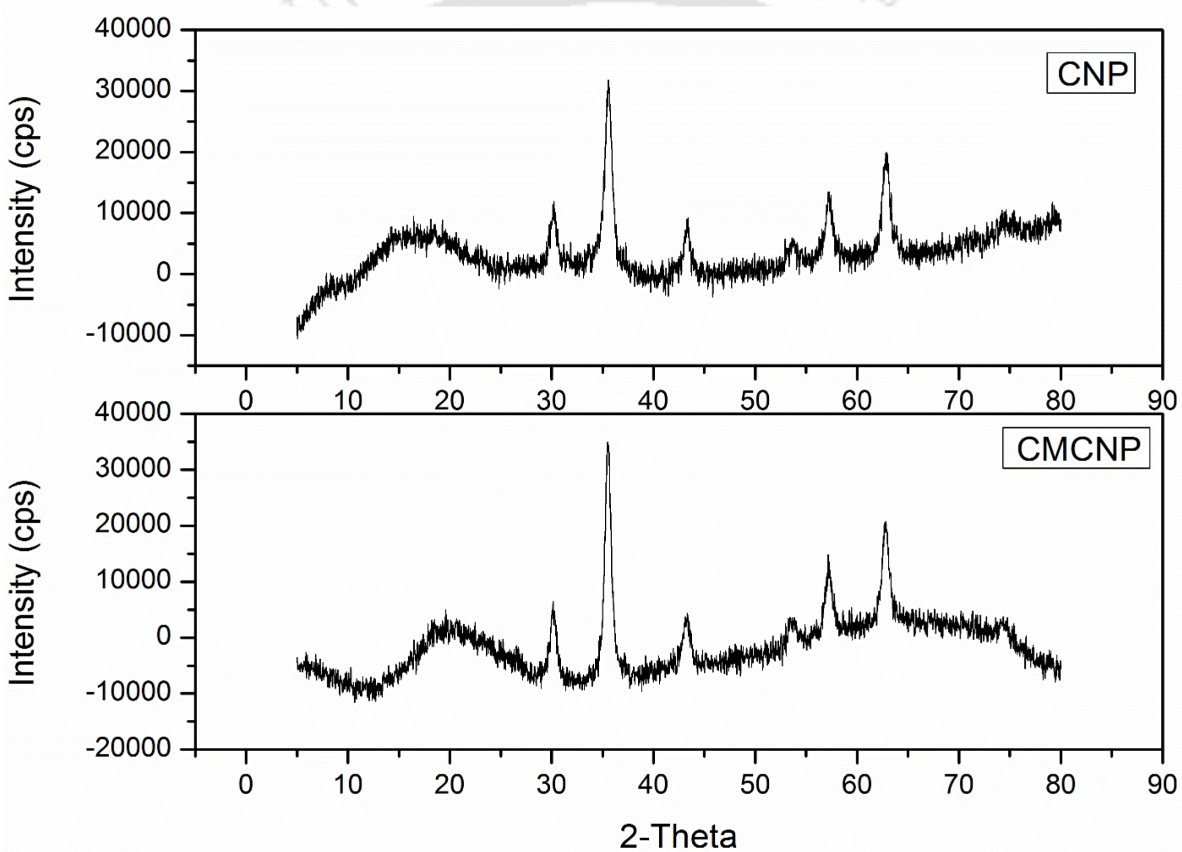


Fig. 4.41 Powder XRD spectra of CNP and CMCNP

To confirm the successful coating of chitosan and carboxymethyl chitosan onto the magnetic iron nanoparticle surface, functional group characterization of CNPs and CMCNPs were carried out by Fourier transform infrared spectroscopy (Fig. 4.42). Characteristic peaks of chitosan were observed at 3440 cm^{-1} that represents the OH group stretching, and the peaks in the range 2920-2940cm^{-1} represent C-H stretching due to methyl and methylene groups. The peak at 1630 cm^{-1} in both the spectra of CNP and CMCNP is due to the stretching of C-N bond vibration of the superimposed C=O group and the main difference in the IR spectra of chitosan and carboxymethyl chitosan due to its symmetrical and asymmetrical stretching. The peak at 545 cm^{-1} in the spectra of both CNP and CMCNP is consistent with Fe-O vibrations present in the spectra of Fe_3O_4 particles. All these results of FTIR spectra confirms successful coating of chitosan and carboxymethyl chitosan on Fe_3O_4 nanoparticles (Feng et al., 2012).

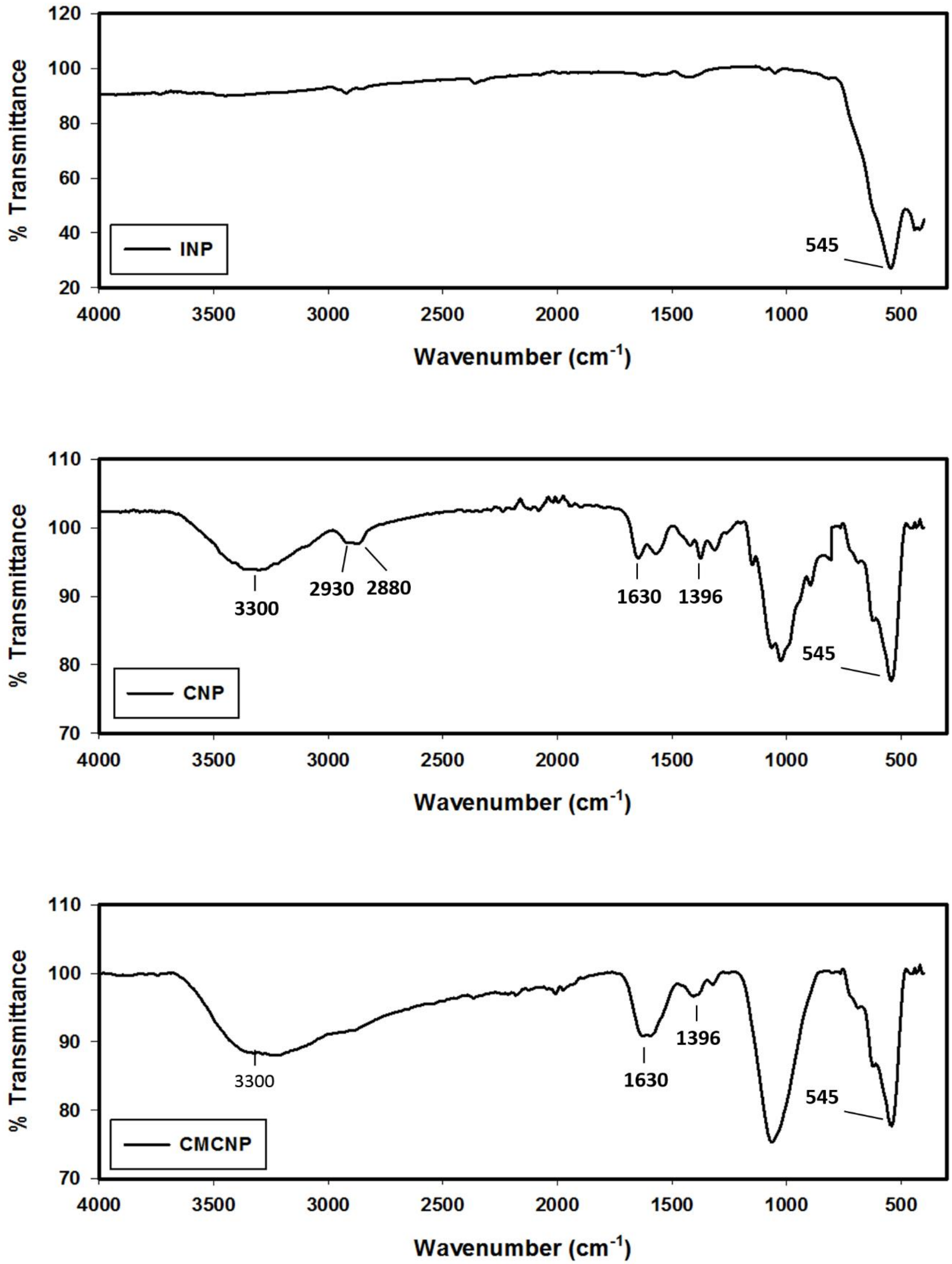


Fig. 4.42 FTIR spectra of (a) Fe_3O_4 nanoparticles, (b) CNP and (c) CMCNP.

Magnetic properties

One of the objectives of preparing chitosan coated Fe_3O_4 nanoparticles is to achieve efficient separation of the biosorbent following heavy metal sorption. Magnetization curve analyses of the nanoparticles established the separation of these nanoparticles under the influence of applied magnetic field. Fig 4.43 shows magnetic hysteresis loop for both CNP and CMCNP, which revealed saturation magnetization values of 63 and 40 emu/g, respectively. These results suggest that the nanoparticles could be easily separated from solution by the use of strong magnet (Charpentier et al., 2016).

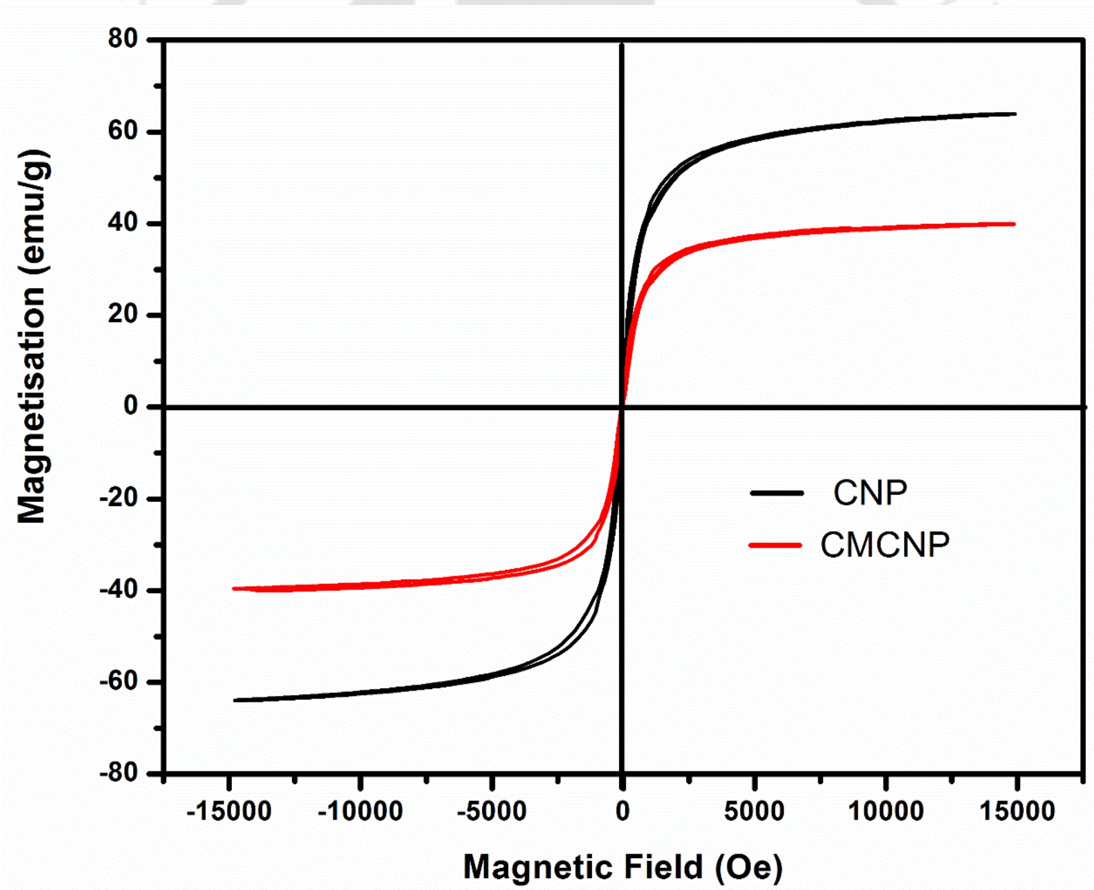


Fig. 4.43 Magnetic hysteresis loops of CS and CMC magnetic nanoparticles

4.7.2 Heavy metal removal experiments

In order to evaluate the potential of CNP and CMCNP to remove heavy metals from aqueous solution, experiments were carried out under batch mode. The effect of important parameters, viz. solution pH (2-6), initial concentration of Cr (VI) and Pb (II) (50- 300 mg/L) and sorbent dose (0.1-2 mg/ml) were first examined and their levels optimized.

4.7.2.1 Effect of pH

Initial pH of the solution is one of the most influential parameters for heavy metal biosorption as it determines protonation or deprotonation of biosorbent active sites, speciation of heavy metal ions and interactions between biosorbent with the heavy metal ions. In this study, maximum removal of Cr (VI) by the biosorbents was achieved for an initial pH 2.0 (Fig. 4.44). At an acidic pH Cr(VI) predominantly occurs in the forms of hydrogen chromate ($HCrO_4{}^{2-}$), chromate ($CrO_4{}^{1-}$) or dichromate ($Cr_2O_7{}^{2-}$), whereas CNP and CMCNP are positively charged in acidic conditions (Kumari et al., 2015; Wu et al., 2008). Hence, the anionic Cr (VI) forms show affinity towards the protonated biosorbent surface, causing electrostatic force of attraction and favouring Cr (VI) biosorption. An increase in the pH from 2 to 10, resulted in a low biosorption capacity due to the reduction of Cr (VI) anions as well as protons on the biosorbent surface for an effective binding of the metal ions. Based on the results of removal efficiency of Cr (VI), pH value of 2.0 was considered as the optimum for further experiments with the nano biosorbents.

In the case of Pb (II) sorption, it is cationic at low pH. The Pb (II) removal efficiency thus increased with an increase in solution pH due to reduction in competitive binding by H^+ ions and the reduction in positively charged species on the nanoparticles. However, Pb (II) precipitated at pH above 6; therefore the solution pH for Pb (II) biosorption was kept at 5 for further experiments (Kumari et al., 2015; Rajput et al., 2016).

4.7.2.2 Effect of biosorbent dosage

Effect of biosorbent dosage on the removal of Pb (II) and Cr(VI) from aqueous solution was carried out by varying the dose in the range 0.2-2 g/L with an initial concentration of 50 mg/L and the results are shown in Fig. 4.45. Removal efficiency of Pb (II) increased with increase in biosorbent dosage to a different extent based on the type of biosorbent and maximum Pb (II) and Cr (VI) biosorption efficiencies were observed at 1.2 and 1.6 g/L, respectively, using CMCNP. The results are due to the availability of a high number of binding sites as the biosorbent dose is increased. However, above a certain dose, the metal removal efficiency reaches a maximum saturation value due to binding of all metal ions to active sites on the biosorbent.

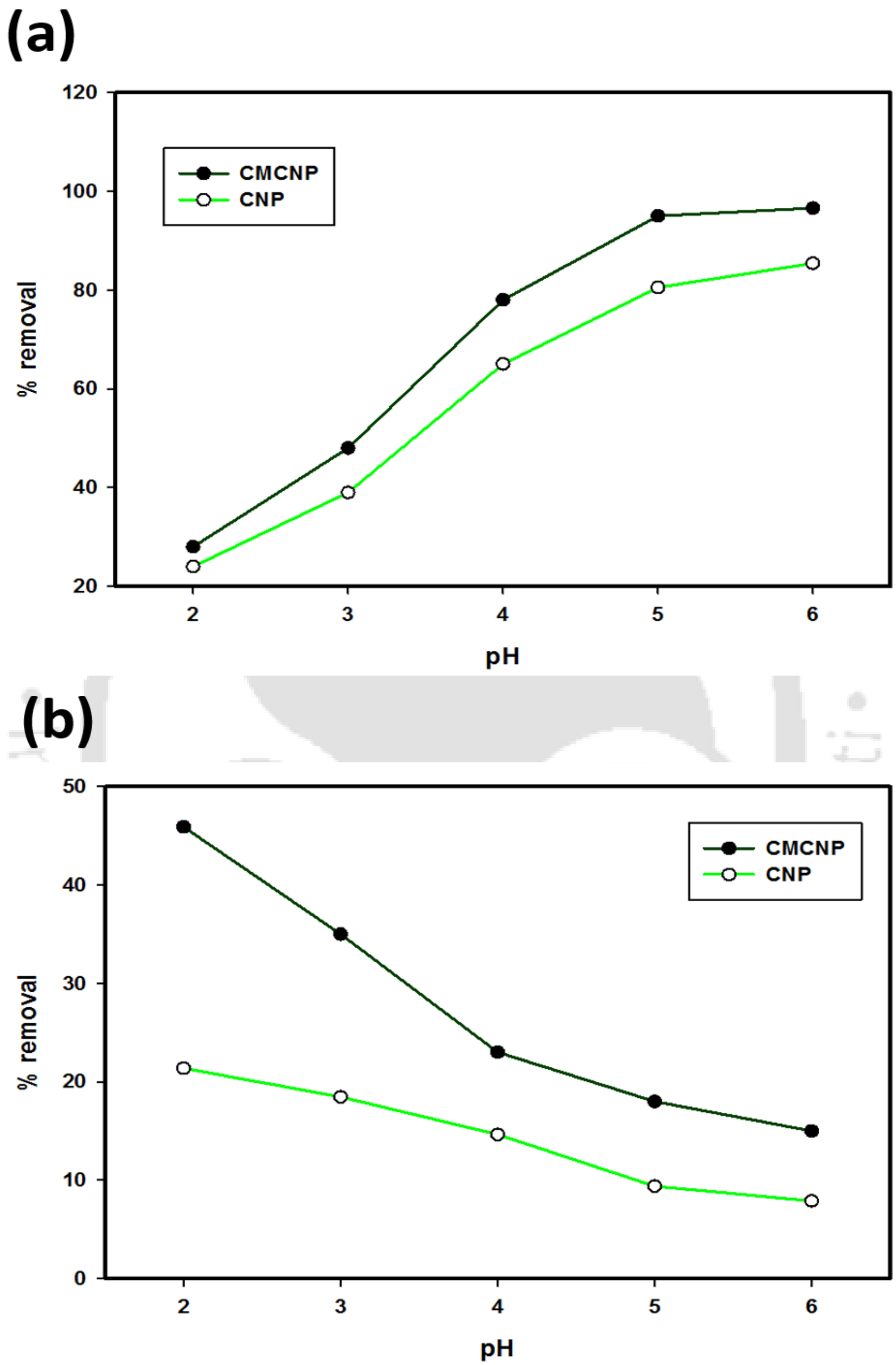


Fig. 4.44 Effect of pH on (a) Pb (II) and Cr (VI) biosorption on magnetite nanoparticles.

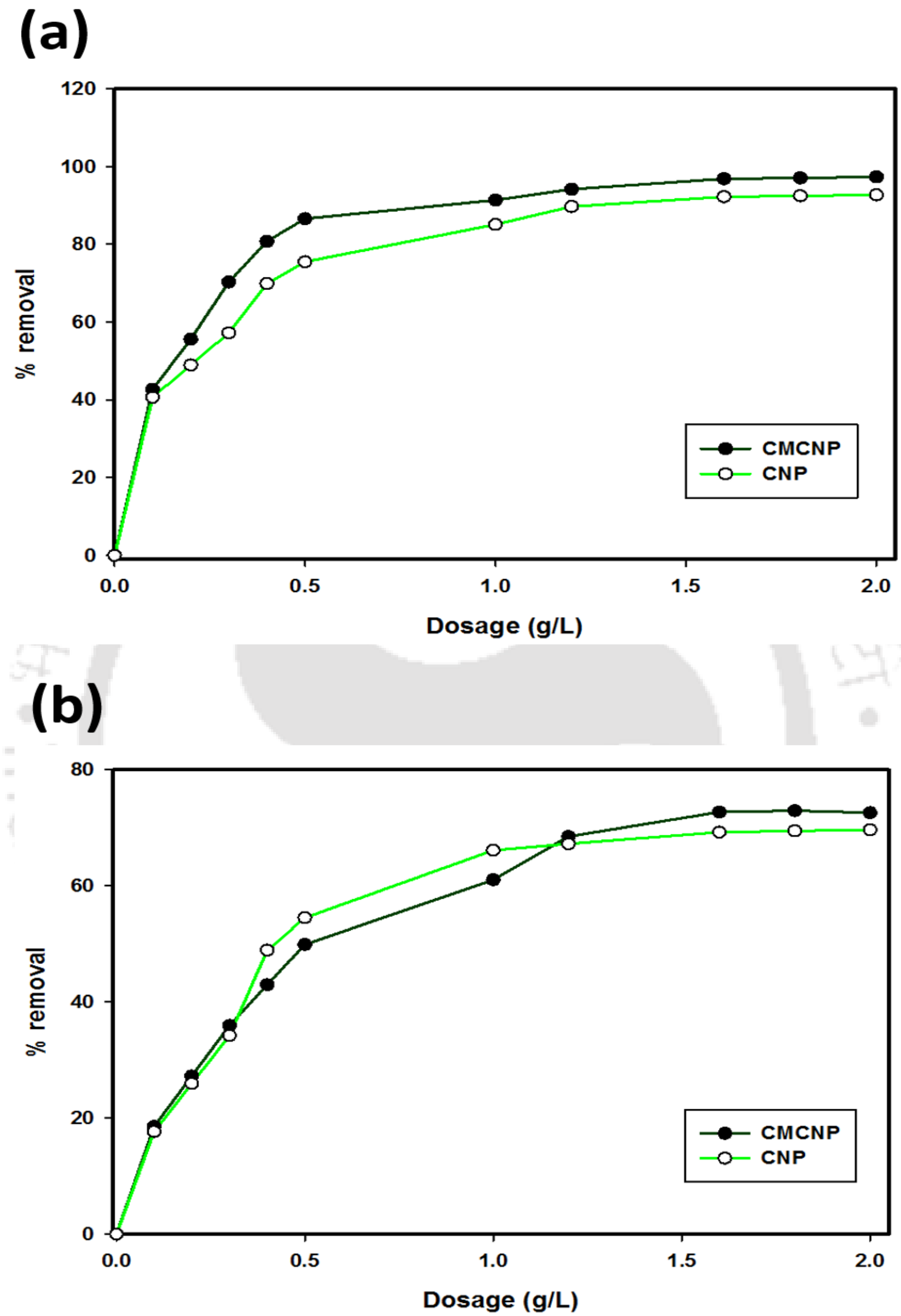


Fig. 4.45 Effect of biosorbent dose on (a) Pb (II) and Cr (VI) biosorption on magnetite nanoparticles.

4.7.2.3 Effect of initial concentration and contact time

Effect of different initial Pb (II) and Cr (VI) concentration on the adsorptive capacity of the biosorbents was evaluated in the range 50 - 300 mg/L, at an optimal biosorbent dosage and initial solution pH of 5 and 2 for Pb (II) and Cr (VI) for an incubation period of 240 minutes (Fig. 4.46). Results indicate that the amount of Pb (II)/Cr (VI) adsorbed increased with an increase in initial concentration and attained saturation beyond a certain value. Thus, the maximum biosorption capacities of 159.8 and 34.2 mg/g for Pb (II) and Cr (VI) were obtained at 250 mg/L initial metal concentration. The results of performance of CNP and CMCNP on metal sorption at different contact times shown in Fig 4.47 indicate spontaneous uptake of the ions by the biosorbent and equilibrium reached within 30 minutes contact time.

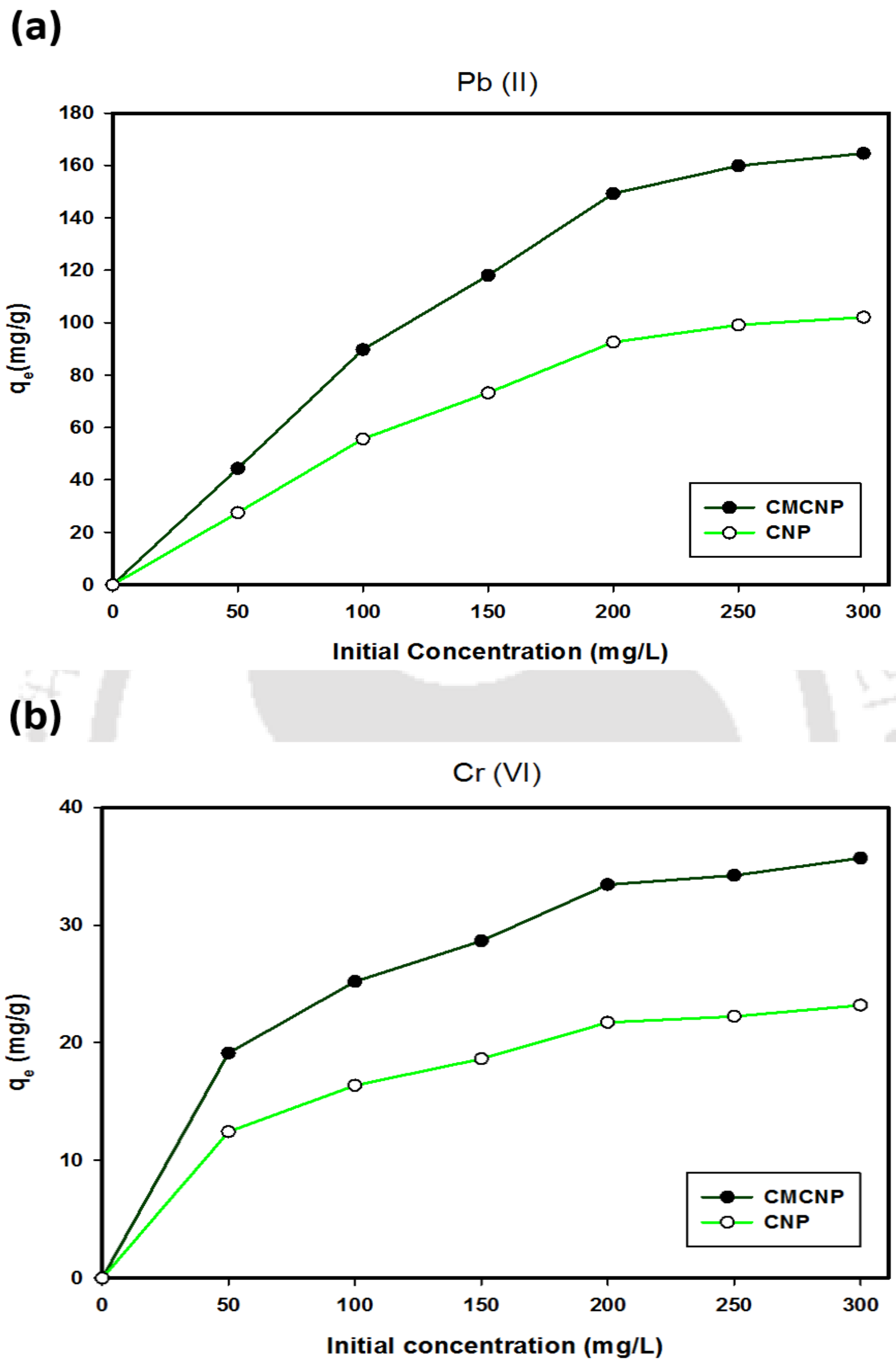


Fig. 4.46 Effect of initial concentration on (a) Pb (II) and (b) Cr (VI) biosorption by CNP and CMCNP

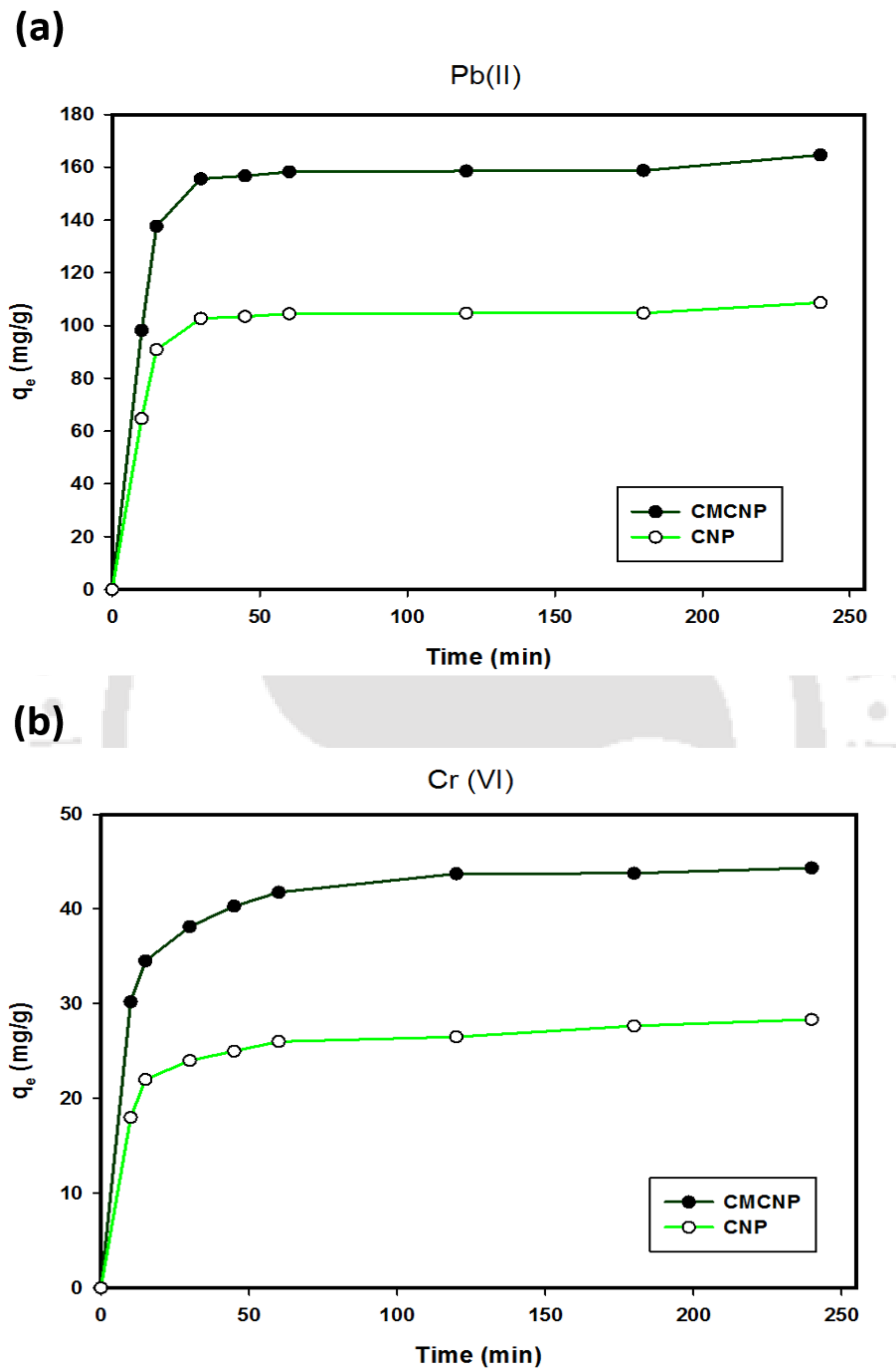


Fig. 4.47 Effect of contact time on (a) Pb (II) and (b) Cr (VI) biosorption by CNP and CMCNP

The data was fitted to various isotherms models, viz. Langmuir, Freundlich, Dubinin-Radushkevich, Frumkin and Redlich-Peterson, in order to gain understanding of the sorption process (Fig. 4.48 and 4.49). Table 4.7 presents the different isotherm parameters estimated in this study along with coefficient of determination (R^2) values for each model. The Langmuir isotherm model gave the best fit for Cr (VI) and Pb (II) biosorption onto CNP at 303 K temperature. Thus, a monolayer formation between metal ion species viz., Cr (VI)/Pb (II) and the biosorbent can be proposed as the sorption mechanism. The theoretical maximum sorption capacity (Q_{LM}) of CNP, estimated using the Langmuir model, for Cr (VI) and Pb (II) was 30 mg/g and 100 mg/g, respectively, at 303 K.

In the case of CMCNP as the biosorbent, Freundlich model gave the best fit with R^2 values of 0.991 and 0.998 for for Cr (VI) and Pb (II). Carboxymethyl chitosan has two binding sites for metal ion biosorption, carboxylate ($-COO^-$) and amine ($-NH_2$) functional groups. Due to the presence of these different functional groups, the nanoparticle surface properties are heterogeneous, and this is best described by the Freundlich model. However, the exact mechanism of metal sorption, i.e. monolayer formation or multilayer sorption onto such biosorbents cannot be simply deduced based on the R^2 values obtained and it requires further experiments to confirm.

Table 4.8 Estimated biosorption isotherm parameters and coefficient of determination (R^2) values obtained using different models for bio-sorption of Cr (VI) and Pb (II) by CNP and CMCNP

Isotherm Models		CNP		CMCNP	
		Cr(VI)	Pb(II)	Cr(VI)	Pb(II)
Langmuir	Q_{LM} (mg/g)	30.36	100.3	42.43	178.25
	L (L/mg)	0.022	0.322	0.025	0.06
	R^2	0.997	0.995	0.958	0.984
Freundlich	K_F	4.52	45.55	6.62	25.93
	n	3.081	5.965	3.23	2.33
	R^2	0.986	0.977	0.991	0.998
Dubinin-Radushkevich	Q_{DR} (mg/g)	24.12	94.25	31.50	142.18
	K_{DR} (mol^2/J^2)	119.5	0.95	81.02	7.12
	E_{DR} (kJ/mol)	0.06	0.07	0.08	0.27
	R^2	0.965	0.978	0.839	0.957
Frumkin	K_{FK} (mg/g)	2169	190.4	13.64	0.04
	α_{FK} (mol^2/J^2)	1.5	4.57	6.65	8.23
	R^2	0.947	0.9002	0.980	0.951
Redlich- Peterson	A_{RP} (mol^2/J^2)	3.72	40.05	3.08	12.07
	K_{RP} (mg/g)	0.639	0.489	0.29	0.06
	g	0.72	0.96	0.77	1.00
	R^2	0.994	0.993	0.992	0.990

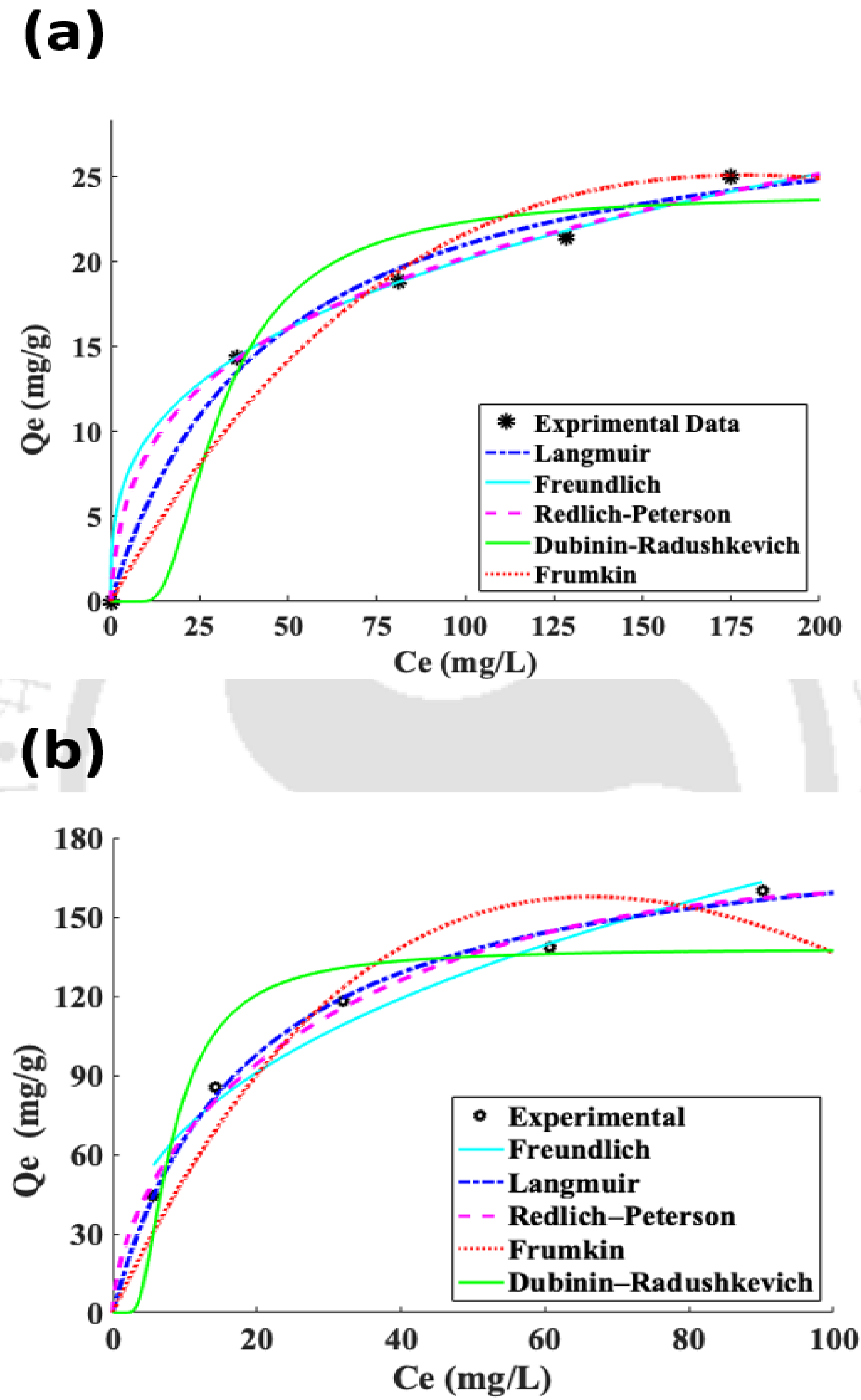


Fig. 4.48 Experimental and predicted (a) Cr (VI) and (b) Pb (II) sorption capacity at different initial metal concentrations due to different isotherm models using CNP.

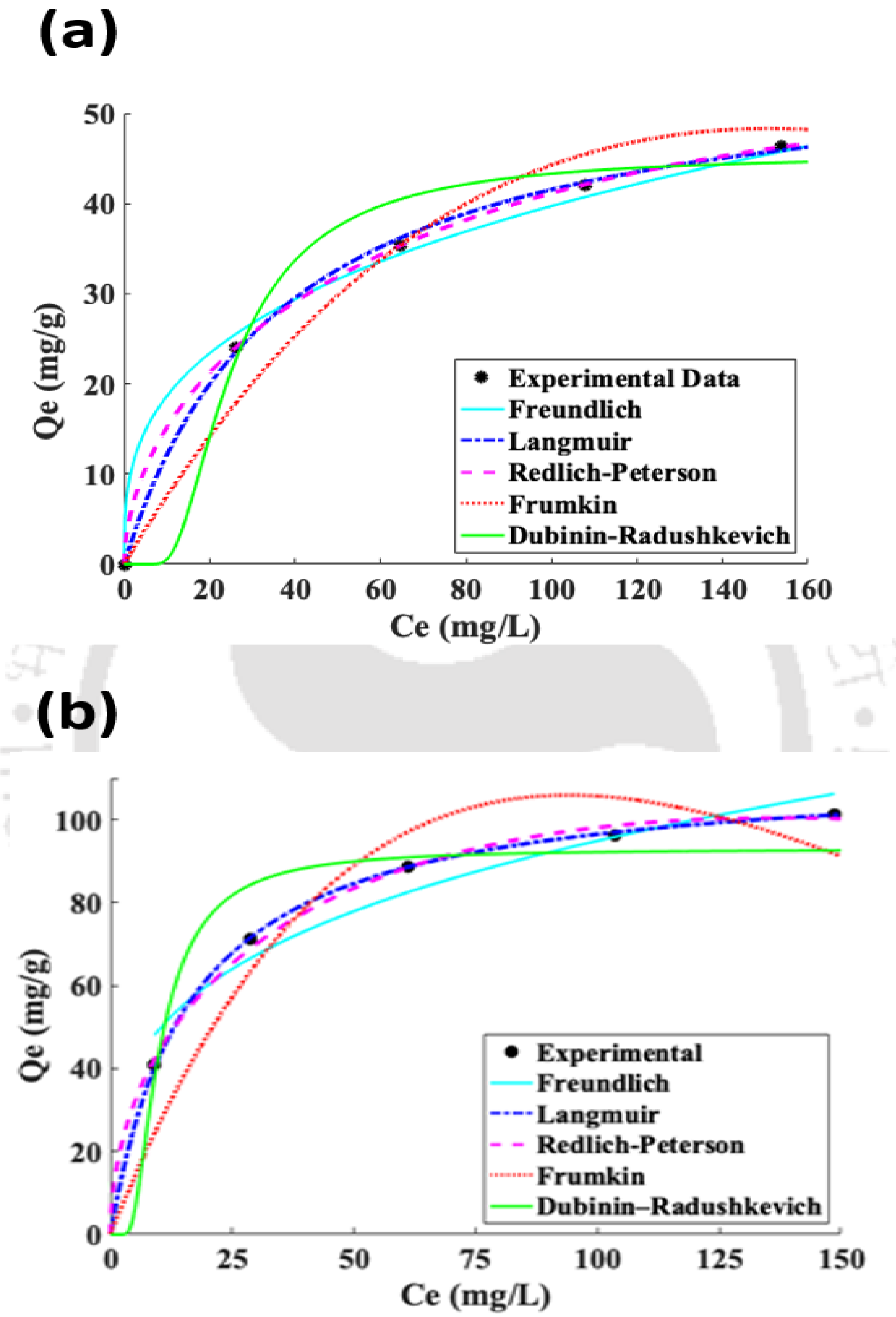


Fig. 4.49 Experimental and predicted (a) Cr (VI) and (b) Pb (II) sorption capacity at different initial metal concentrations due to different isotherm models using CMCNP.

In order to evaluate rate limiting step involved in Cr(VI) and Pb(II) sorption by the nano-biosorbents, the experimental data obtained at different contact time was fitted to various kinetic models, viz. pseudo-first order, pseudo-second order and intra-particle diffusion models. Parameters estimated from these kinetic models and their coefficient of determination values (R^2) values are presented in Table 4.8. R^2 values due to the pseudo first order model are relatively low, and thus the relevance of pseudo-first order with the bio-sorption of Cr (VI) and Pb (II) species by CMCNP is minimal. Coefficient of determination (R^2) values for the pseudo-second order kinetics are comparatively high (R^2>0.99) for both the metals and with the two biosorbents. These results confirm that the pseudo second order model, which is based on chemisorption, governs the rate-limiting step for bio-sorption of Cr (VI) and Pb (II) species by CMC nanoparticles. The results obtained in this study are comparable with other reports where magnetic chitosan based nanoparticles have been applied for Cr (VI) and Pb (II) sorption (Table 4.9).

Table 4.9 Estimated kinetic parameters of Cr (VI) and Pb (II) biosorption in the study.

C_i (mg/L)	Pseudo-first order			Pseudo-second order			Intra-particle diffusion		
	k_1 (1/min)	q_e (mg/g)	R^2	k_2 (g/mg min)	q_e (mg/g)	R^2	k_{id} (g/mg min)	C (mg/g)	R^2
Cr(VI)					**CMCNP**				
50	0.04	20.39	0.5494	0.0022	21.26	0.9945	0.95	6.63	0.7917
100	0.03	37.53	0.6303	0.0018	27.11	0.998	1.2	8.78	0.8082
150	0.03	43.43	0.6849	0.0013	31.86	0.9988	1.48	8.45	0.8852
200	0.03	47.59	0.7077	0.0015	36	0.9986	1.49	13.08	0.8523
250	0.01	1.18	0.2195	0.0068	35.04	0.9975	0.73	25.31	0.264
Pb(II)									
50	0.01	7.91	0.7763	0.0044	45.12	0.9999	0.94	31.84	0.7699
100	0.01	13.7	0.6903	0.0024	90.44	0.9994	2.06	62.26	0.5347
150	0.01	15.37	0.8277	0.0022	120.53	0.9997	1.51	97.98	0.8544
200	0.02	19.97	0.7274	0.0013	152.51	0.9991	3.88	100.16	0.4972
250	0.01	24	0.6998	0.0016	160.6	0.9992	3.17	117.7	0.4545
Cr(VI)					**CNP**				
50	0.042	14.06	0.9647	0.0031	15.97	0.9728	0.86	3.18	0.8169
100	0.039	17.98	0.9803	0.0025	20.41	0.9924	1.11	4.20	0.8226
150	0.034	20.59	0.9752	0.0018	23.65	0.9937	1.32	4.04	0.8821
200	0.032	23.72	0.9484	0.0023	26.54	0.9826	1.43	6.26	0.8275
250	0.095	26.18	0.9073	0.0014	28..04	0.9833	1.56	8.12	0.8734
Pb(II)									
50	0.02	19.15	0.7243	0.001	47.50	0.9992	1.06	29.40	0.9677
100	0.02	28.55	0.9519	0.001	73.02	0.9989	2.44	36.35	0.8916
150	0.01	13.65	0.7022	0.002	95.90	0.9993	2.04	67.94	0.474
200	0.02	39.59	0.974	0.001	99.27	0.9986	2.76	57.02	0.9098
250	0.01	9.53	0.5608	0.003	103.93	0.9994	2.36	72.53	0.5029

Table 4.10 Adsorption capacity of magnetic chitosan based adsorbents for Cr (VI) and Pb (II)

Adsorbent	**Adsorption capacity (mg/g)**		**References**
	Pb (II)	Cr (VI)	
Chitosan/cellulose magnetic microsphere	46		(Luo et al., 2015)
Magnetic chitosan nanoparticle		55.8	(Thinh et al., 2013)
Magnetic cyclodextrin–chitosan/graphene		67.66	(Li et al., 2013)
Chitosan/magnetite composite beads	63.3		(Tran et al., 2010)
Chitosan magnetite nanobiosorbent	100	30	This study
CMC magnetite nanobiosorbent	178.25	42.43	This study

FESEM-EDX analyses (Fig. 4.50) of heavy metal laden nanoparticles reveal distinct peaks due to chromium and lead thereby providing direct evidence of Cr (VI) and Pb (II) sorption onto the nano-biosorbents.

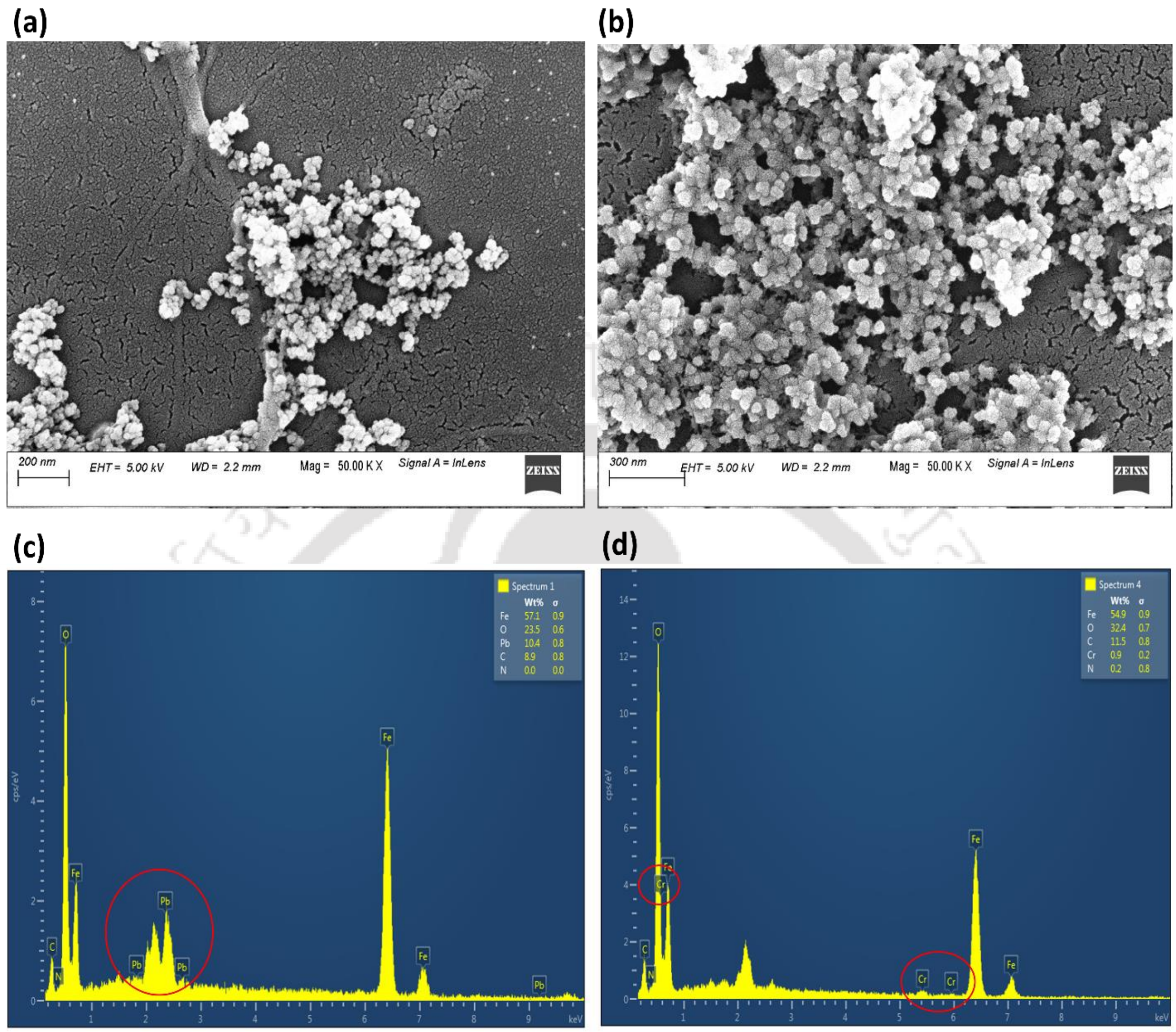


Fig. 4.50 FESEM images of (a) CNP and (b) CMCNP along with EDX analysis results of (c) Pb (II) and (d) Cr (VI) sorption onto the nanobiosorbents.

4.7.2.4 Reusability of CNP and CMCNP

The ability of the biosorbents to be recycled and reused is an important characteristic which determines its practicality for the application of heavy metal removal. It also ensures in the reduction of overall cost of the process. Sorption-desorption of Cr (VI) and Pb (II) ions from CNP and CMCNP were evaluated for up to 4 cycles and the results are shown in Fig 4.51. Slight reduction in the removal efficiency with the biosorbent is observed at the end of each cycle and it reduced to a very low value of 50% at the end of the 4th cycle. This may be due to the degeneration of chitosan and carboxymethyl chitosan, and continuous wear of the surface functional groups on the biosorbent after every cycle. Leaching due to Fe in the aqueous solution was below the detection limit (0.5 µg/L) for the first two cycles, however, the amount of Fe^{3+} in the solution reached up to 0.56 ppm at the end of the 4th cycle. Based on these results, it could be said that the nano biosorbents could be safely reused for up to 3-4 without significant loss in metal removal efficiency.

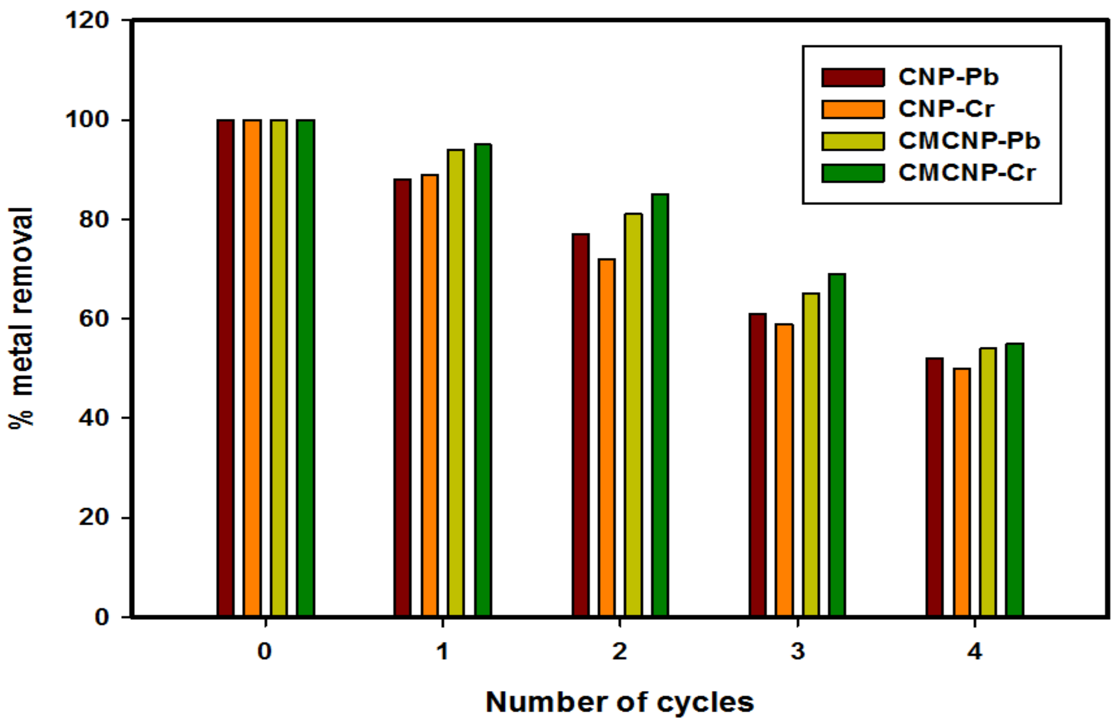


Fig. 4.51 Reusability of CNP and CMCNP for biosorption of Cr (VI) and Pb (II).

CHAPTER FIVE

SUMMARY & CONCLUSIONS

Marine sources such as crustaceans have been extensively explored for the production of chitosan. However, limited availability as well as variations in the properties of the derived chitosan is a serious drawback for utilizing marine sources for chitosan production. Furthermore, chitosan obtained from marine sources require a series of downstream operation steps to attain the standard commercial quality for application at an industrial scale. Compared with the marine sources, fungi seem to be more attractive for the production of chitosan owing to its fast growth rate and capability to grow on cheap substrates. In addition, inexpensive feedstock for the fungal chitosan extraction may help keep the process economically viable. Majority of the investigations utilised waste sources rich in sugar, starch or nutrient content. However, very little attention has been paid to non-starch or lignocellulosic wastes for fungal chitosan production, which are available cheaply and abundantly from agricultural and industrial sectors.

This study therefore focused towards utilizing cheap and abundantly available non-starch based wastewater, viz. domestic, dairy, paper mill wastewaters and agricultural residues i.e. rice straw, and lemon peels so as to keep the production cost low.

The fungus *Penicillium citrinum,* was first isolated from an infected bamboo shoot and cultured on potato dextrose agar plates. The fungal isolate was later identified based on the sequence variation present in 18s rRNA which revealed that the isolate was phylogenetically related to members of the genus *Penicillium citrinum.* Among the different wastewaters screened for chitosan production, paper mill wastewater served as an excellent substrate for *P. citrinum* when compared with domestic and dairy wastewaters. Process parameters such as temperature, agitation and pH were optimized to achieve maximum fungal growth and chitosan production. Paper mill wastewater supplemented with mineral salt media (MSM) in the ratio 9:1 yielded maximum COD removal and chitosan production by *P. citrinum.* Moreover, these results were better as compared with those obtained from *Cunninghamella elegans*, a fungus that has already been reported in the literature for chitosan production.

Addition of acetic acid at 50 mg/L further enhanced the chitosan production and COD removal along with ~70% removal efficiency of phenolics. The experimental data on chitosan production rate in presence of acetic acid was further fitted to different biokinetic models viz., Haldane, Monod, Webb and Yano models. Among these models analysed to explain the effect of acetic acid addition, Haldane model was found to accurately fit the data with a very high coefficient of determination (R^2) value. The positive effect due to acetic acid addition on the utilization of xylose present in the paper mill wastewater was further confirmed by analysis of XR and XDH enzyme activities. The activities of both the enzymes were found to be very high due to acetic acid addition, which resulted in high levels of xylulose 5-phosphate and fructose 6- phosphate (F6P). Due to this enhanced xylose utilization, majority of the metabolic flux could be directed towards the chitosan synthesis pathway wherein F6P is an important intermediate for many other metabolic pathways. A high amount of F6P also leads to enhanced pyruvate synthesis, which in turn enters the TCA cycle that serves as the source point of nitrogen for the chitin and chitosan synthesis pathway.

Submerged fermentation using a bioreactor operated under fed-batch mode at controlled conditions of temperature, pH, agitation and aeration rates resulted in maximum COD removal from paper mill wastewater along with high chitosan yields. Even under continuous mode of operation with the bioreactor, chitosan production was high, but the COD removal efficiency was low when compared with the value obtained under fed-batch mode.

Fermentation of rice straw hemicellulose hydrolysate (RSH) carried out using *P. citrinum* biomass resulted in a maximum chitosan yield after detoxification. Batch reactor mode of operation was considered to be best for fungal chitosan production using detoxified RSH. High chitosan yields of above 13% using detoxified or non-detoxified hydrolysates is attributed to acetic acid present in the hydrolysates.

Solid-state fermentation of agro-industrial wastes as alternate carbon source for chitosan production by *P. citrinum* showed that rice straw (RS) and paper mill waste sludge (PMS) were efficiently utilized by the fungus. Among the different pre-treatment methods investigated, microwave alkaline pre-treatment further enhanced utilization of both PMS and RS. Chitosan production by the fungal isolate using rice straw as the substrate was investigated by varying the moisture content (50-80%) and particle size and an increase in the moisture content enhanced the fungal growth and chitosan production. Solid-state fermentation of both rice straw and paper mill carried out using a lab scale tray fermenter under controlled condition of relative humidity resulted in substantial increase in the chitosan yields by the addition of paper mill wastewater to MSM. Hence, the process could possibly be integrated in paper mill industry where large amounts of wastewater and sludge is generated for achieving sustainable fungal chitosan production.

Synthesis, characterization heavy metal removal using chitosan based nano-biosorbents were finally carried out to establish the application of the fungal chitosan produced in the study. Magnetic chitosan and carboxymethyl chitosan (CMC) iron (Fe_3O_4) nanoparticles were prepared by the co-precipitation method and employed for Cr (VI) and Pb (II) removal from aqueous solutions. The nanoparticles obtained were in the range of 20-40 nm and poly crystalline in nature. The heavy metal sorption mechanism followed pseudo second order kinetics, which confirmed that the rate-limiting step for bio-sorption of Cr (VI) and Pb (II) species by CMC nanoparticles is governed by chemisorption. Among the different isotherm models tested in this study, the Langmuir isotherm model gave the best fit for Cr(VI) and Pb(II) adsorption onto CNP which suggests a monolayer formation between metal ion species viz., Cr(VI)/Pb(II) and the adsorbent, whereas, in the case of CMCNP, Freundlich model gave the best fit. Sorption-desorption results revealed that the chitosan based nano-biosorbents could be reused for 3-4 times with minimal loss in heavy metal removal efficiency. This study established an excellent potential of the cheaply produced chitosan based biosorbent for heavy metal removal from wastewaters.

Scope for future work

The present work focused on achieving fungal chitosan production using inexpensive waste substrates like paper mill wastewater, rice straw and paper mill sludge and its potential application. Some suggestions for future work based on this thesis are:

- ✓ Genetic engineering or metabolic engineering of the *P. citrinum* strain to further improve the chitosan yields.
- ✓ Use of rotating drum fermenter for solid-state fermentation for improving fungal chitosan production
- ✓ Techno-economic feasibility of fungal chitosan production from agro-industry wastes.

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LIST OF PUBLICATIONS

Book Chapter

1. P. Mullai, M.K. Yogeswari, S. Vishali, M. M. Tejas Namboodiri, B.D. Gebrewold, E.R. Rene, K. Pakshirajan (2017). Aerobic treatment of effluents from textile industry. In Current Developments in Biotechnology and Bioengineering (pp. 3-34). Elsevier.
2. M. M. Tejas Namboodiri & Kannan Pakshirajan. Valorisation of waste biomass for chitin and chitosan production. Waste Biorefinery: Potential and Perspectives - Integrating biorefineries for waste valorisation -Volume II. (Submitted)

Publications in International Journals

1. M. M. Tejas Namboodiri and Kannan Pakshirajan, K. (2019). Sustainable and green approach of chitosan production from *Penicillium citrinum* biomass using industrial wastewater as a cheap substrate. Journal of environmental management, 240, 431-440.

Presentation in international/national conferences

1. M. M. Tejas Namboodiri and Kannan Pakshirajan. Fungal chitosan production by solid state fermentation of rice straw using *Penicillium citrinum* biomass. RABEB-2019. March 15-16, 2019, IIT BHU, Varanasi.
2. M. M. Tejas Namboodiri and Kannan Pakshirajan. Solid state fermentation of rice straw for chitosan production by a novel *P. citrinum* isolate Bioprocessing India 2017, December 9-11, IIT Guwahati, Assam.
3. M. M. Tejas Namboodiri and Kannan Pakshirajan. Chitosan production from *Penicillium citrinum* biomass for value addition and resource recovery from industrial wastewater. Conference on Challenges in Environmental Sciences and Engineering 2017, November 11-15, Kunming, China.
4. M. M. Tejas Namboodiri and Kannan Pakshirajan. Chitosan production by *Aspergillus niger* using cheaply available domestic wastewater**.** Recycle 2016, 1-2nd April, IIT Guwahati Assam.
5. M. M. Tejas Namboodiri and Kannan Pakshirajan. Fungal chitosan production using cheaply and abundantly available waste substrates**.** RAER 2016, 4-5th June IIT Guwahati Assam.
6. M. M. Tejas Namboodiri and Kannan Pakshirajan. Chitosan production from domestic wastewater using *Aspergillus niger*. Chemcon 2015, 27-30 December, IIT Guwahati Assam.

Manuscripts under preparation

1. M. M. Tejas Namboodiri, Arul Manikandan, Kannan Pakshirajan and G. Pugazhenthi. Acetic acid induced chitosan production by *Penicillium citrinum* wastewater and rice straw hydrolysate in a stirred tank bioreactor operated under different modes.

2. M. M. Tejas Namboodiri and Kannan Pakshirajan. Fungal chitosan production by *Penicillium citrinum* using rice straw and paper mill sludge in a tray fermenter

3. M. M. Tejas Namboodiri, Raj Mohan Naidu Medisetti, Kannan Pakshirajan and Selvaraju Narayanasamy. Heavy metal removal using fungal chitosan derived nano-biosorbents.