MICROBIAL PRODUCTION AND CHARACTERIZATION OF CHITIN DEACETYLASE BY *PENICILLIUM OXALICUM*

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MICROBIAL PRODUCTION AND CHARACTERIZATION OF CHITIN DEACETYLASE BY *PENICILLIUM OXALICUM*** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2007 to June 2011 under the supervision of Dr. R. P. Singh, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

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

Nidhi Pareek

(NIDHI PAREEK)

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

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ABSTRACT

The present investigation was undertaken to isolate a potent microorganism with chitin deacetylase production ability and to develop and economize the process by employing agro-horticultural residual resources.

The fungal strain secreting notably higher levels of extracellular chitin deacetylase was isolated from the residual materials of the sea food processing industry. The isolated strain SA-1 was able to produce notable levels of chitin deacetylase and identified as *Penicillium oxalicum* ITCC 6965 by Indian Agricultural Research Institute, New Delhi. To achieve an improvement in the enzyme production ability, the selected strain was subjected to genetic manipulation using single-stage and mixed mutagenesis. Mutagenesis by microwave irradiation and ethidium bromide had led to the development of strain SAE_M-51 with ability for considerably higher levels of extracellular chitin deacetylase along with lower levels of intracellular enzyme production. Mutant strain had ~2.0 fold improvement in the kinetic variables mainly Q_p, Q_s, $Y_{p/x}$, $Y_{p/s}$, q_p, q_s over the parental strain. Mutagenized strain *P. oxalicum* SAE_M-51 had discrete morphological features. The mycelia of mutant SAE_M-51 were elongated, flattened and smooth as compared to wild type SA-1 which were irregular, rough surfaced and coiled. Similarly, spores from the wild type were crescent shaped while those from mutant strain were oval and irregular.

Analysis of enzyme activity by *P. oxalicum* SAE_M-51 using chitin, colloidal chitin and glucose revealed that chitin comparatively results into lower levels of enzyme activity. Highest CDA levels were attained using glucose as a carbon source under submerged condition. Nutritional and cultural parameters for enzyme production were derived further using response surface methodology under submerged condition. A 6.0 fold increase in enzyme levels was attained under derived conditions. Further in an attempt to see if the process can be made economical and further increase in the production can be obtained, the level of enzyme production was evaluated using solid-state fermentation. Among various solid supports used for

SSF process, mustard oil cake, was found to be the most ideal for fungal colonization and enzyme production. Derivation of fermentation parameters viz. amount of substrate, moisture level and inoculum level using response surface methodology had resulted in 10.9 fold enhancement in enzyme production levels.

To develop an industrially compatible enzyme preparation, CDA from culture supernatants was purified using ultrafiltration, cation and anion exchange chromatography and characterized for its biochemical and molecular properties. Thermodynamic parameters had depicted the notable stability of CDA at higher temperatures (upto 70 ^{0}C). Analysis of kinetic constants had enumerated that enzyme had effectively deacetylated chitin and its oligomers having degree of polymerization more than four. Metal ions i.e. Cu^{+2}, Co^{+2}, Fe^{+2} and Cd^{+2} were observed as inducers of enzyme activity. Developed enzyme preparation was not inhibited with acetate (upto 70 mM concentration), an end product of the enzyme reaction. Enzyme was observed to possess the characteristic secondary structure consisting of both α and β helices at its optimal pH. Significant homology of the enzyme when compared with the existing sequences in the database was observed with CDAs from bacterial, fungal and yeast strains.

Deacetylation potential of the developed enzyme preparation was evaluated for bioconversion of chitin to chitosan in a two stage chemical and enzymatic deacetylation process. Variations in morphology, crystallinity and thermal properties of the deacetylated chitinous substrates were evaluated by scanning electron microscopy, X-ray diffraction, thermogravimetric analysis and differential scanning calorimetry. Degree of deacetylation of the substrates before and following chemical and enzymatic treatments was determined using FTIR and elemental analysis. The pretreatment of the substrate led to the decrease in crystallinity and formation of amorphous chitinous substrates to facilitate enzyme reaction. Among the various substrates analyzed, superfine chitin appeared to be most suitable substrate for enzyme action which had resulted into the chitosan with 79.52 % of the deacetylation. Further, improvement in deacetylation was attained by deriving the reaction variables i.e. substrate amount and enzyme

dose through central composite design. This had led into a further 10 % improvement in deacetylation with the formation of chitosan with 90 % of the deacetylation.

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"God understands our prayers even when we can't find the words to say them."

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Nidhi Pareek

(Nidhi Pareek)

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ABBREVIATIONS

ANOVA	Analysis of Variance
bp	Base Pair
°C	Degree Celsius
CCD	Central composite design
CD	Circular dichroism spectroscopy
CDA	Chitin deacetylase
cm	Centimeter
CrI	Crystalline index
CV	Coefficient of variance
DA	Degree of acetylation
DDA	Degree of deacetylation
DF	Degree of Freedom
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic Acid
DP	Degree of polymerization
DSC	Differential scanning calorimetry
EMS	Ethyl methane sulfonate
FTIR	Fourior Tranform Infrared Spectroscopy
g	Gram
GlcNAc	N-acetyl glucosamine
HDL	High density lipoprotein
IU	International Unit
kb	Kilobase
kD	Kilodalton

l	Litre
LDL	Low density lipoprotein
MALDI ToF/ToF	Matrix Assisted Laser Desoption/Ionisation Time of Flight
µg	Microgram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MS	Mean sum of square
N	Number of Replicates
NMR	Nuclear magnetic resonance spectroscopy
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PBD	Plackett-Burman design
PDA	Potato Dextrose Agar
RSM	Response surface methodology
rpm	Revolution per Minute
SEM	Scanning Electron Microscope
SS	Sum of Square
TGA	Thermogravimetric analysis
UV	Ultra Violet
v/v	Volume per Volume
w/v	Weight per Volume
w/w	weight per weight
XRD	X-ray Diffraction

CHAPTER I

INTRODUCTION

Chapter 1

INTRODUCTION

Earth has finite resources which are widely used by mankind in one or other ways. A major class of these resources includes biopolymers like starch, cellulose, hemicellulose, lignin, tannin, collagen, pectin, alginate etc. which, due to their biodegradable and renewable nature have a variety of applications in diverse industrial sectors and are explored for their commercial utilization. Chitin is a biopolymer as well, consisting of repeating *N*-acetyl D-glucosamine subunits linked by β- (1,4) glycosidic bond. It is commonly found in the exoskeletons or cuticles of many invertebrates and in the cell wall of fungi. It has been estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates is 3.7×10^4 metric tons (Shaikh and Deshpande, 1993). In spite of its abundance and being biocompatible, no major industrial usage of this vastly available material has so far been developed due to its high degree of crystallinity and insolubility in aqueous and organic solvents.

To augment its commercial feasibility it could be subjected to inter-conversion into derivatives with novel and improved characteristics viz. increased solubility and less crystallinity. Chitosan, the deacetylated, nontoxic, biodegradable, biocompatible derivative of chitin which is a linear biopolyaminosaccharide consisting of β-(1,4) linked D-glucosamine subunits and is soluble in dilute acidic solutions. Chitosan is more tractable material than chitin with a broad and impressive array of applications in diverse industrial sectors, like pharmaceutics (drug delivery), gene delivery, tissue engineering, food and cosmetics industry, water treatment, agriculture, etc.

Chitosan production is a foremost area of interest due to its wide industrial relevance. At present, most of the chitosan is produced by a chemical process which involves thermo-alkaline

deacetylation of chitin. This process is incompatible as the conversion to chitosan, using a strong alkali solution at high temperature results into the products with variable properties and increased cost of production. At the same time, the waste liquid which contain large amounts of base, protein and protein degradation products from the industrial processing leads into environmental pollution. Since the physicochemical characteristics i.e. molecular weight, degree of deacetylation, viscosity etc. of chitosan affects its functional properties, hence a controlled biological process could specifically be developed so as to realize the commercial value of the product. 'Chitin deacetylase' (CDA, EC 3.2.1.41) is the key enzyme employed for bioconversion of chitin to chitosan. It catalyzes deacetylation of *N*-acetyl-D-glucosamine residues under mild reaction conditions and results into production of novel superior-quality chitosan. The enzyme aided production is a vital step towards the chitosan production in the green chemistry realm as the chemical process is engraved with a number of limitations and bottlenecks. Apart from being used in bioconversion reactions, CDA has many biological roles such as formation of spore wall in *Saccharomyces cerevisiae* and vegetative cell wall in *Cryptococcus neoformans*, responsible for pathogenesis of plant pathogenic fungi etc.

In nature there is a repertoire of organisms viz. bacteria, yeast and fungi producing CDA but fungi are considered superior, because of higher titre, multifunctional and extracellular nature of enzyme. CDA assisted enzymatic conversion to chitosan, needs intensive screening of novel CDA hyper-producers as most of the strains observed so far are intracellular producers with lower activity and yield, which limits their performance for bioconversion reactions. There are limited reports related to the extracellular production of enzyme, moreover, lower and insignificant levels of enzymatic production have been observed.

Enzyme production, characterization and application is an enduring, fundamental and vital area of current research. The potential and biotechnological significance of CDA is mainly due to its role in the chitin deacetylation. So, it would be pertinent to look for novel strains that have ability to produce higher levels of extracellular CDA. Furthermore, the CDA thus obtained should preferably be stable under standard conditions of pH, temperature, etc. which therefore might enable the enzyme to be an economically attractive proposition for its utilization for commercial purposes. Study of enzyme kinetics and thermodynamic parameters further would provide information to decode the factors regulating the thermal deactivation of the enzyme and its mode of action.

Enzyme production by microorganisms is greatly influenced by multiple factors, such as medium constituents and cultivation conditions. Developing a productive and robust CDA production process would be a prerequisite not only to achieve a higher product yield but also to render the process more economical. Optimization studies can be conducted by using either conventional or statistical approaches. In conventional method, a single factor affecting the production levels is analyzed at a time while keeping the other variables fixed for the process. This method may lead into unreliable results and inaccurate conclusions as it does not guarantee the determination of optimal conditions and is unable to detect the frequent interactions occurring between the factors. To overcome these limitations, response surface methodology (RSM) could be employed for optimization studies. Optimization of the crucial variables by statistical experimental designs can eliminate the limitations of 'one variable at a time' approach. RSM, a collection of mathematical and statistical techniques for building empirical models, is gaining momentum for optimizing conditions for the production of commercially viable products. Usually, this process employs a polynomial equation in a pre-determined region of variables, which is later analyzed to locate the optimum values of the independent variables for the best response. It can give

information about the interaction between variables, provide necessary information for process optimization and give multiple responses at the same time. It also aids in understanding interactions among the variables at varying concentrations and in calculating the optimal value of each variable for a given response.

Superior productivity, improved product recovery, reduced risk of contamination, low capital investment, simple technique, reduced energy requirement, closeness to the natural way of life for many microorganisms, low waste water output are some advantages of solid-state fermentation (SSF) over submerged fermentation. SSF may be defined as growth of microbes in absence or near absence of free flowing water on the surface of a natural substrate or inert solid support. The solid substrate not only supplies the nutrients to the microbial cultures, but also serves as an anchorage for the cells. A large number of agro-residues (sugar cane bagasse, wheat bran, wheat straw, rice bran, oil cakes and many more) which are generated in huge quantities during agricultural practices are considered as the suitable and promising substrates for SSF. Utilization of these agro-residual materials in bioprocesses can be part of environmental pollution control on one hand and their consumption for production of cost effective products of commercial significance on the other hand, thus changing its status from waste to a potential provider.

In order to develop a robust and high yielding microbial system for over production of CDA and to analyze the enzyme preparation for its application in generating the high quality chitosan, the work proposed has the following as the major objectives,

a. Exploration and isolation of microbial strains from different natural sources for chitin deacetylase production.

b. Screening of the isolated strains for selecting a strain with higher enzyme yield; genetic manipulation of the selected fungal strain to achieve a further enhancement in the enzyme production level.

c. To analyze the major fermentation systems mainly submerged and solid-state systems for chitin deacetylase production; evaluation of the critical parameters for maximizing the levels of production.

d. Purification of the enzyme and its biochemical and molecular characterization.

e. Evaluation of purified enzyme for its efficacy for deacetylation for the production of chitosan.

Details of the investigations on the above defined aspects have been enumerated in the following chapters of the present investigation.

CHAPTER II

LITERATURE REVIEW

Chapter - 2

LITERATURE REVIEW

One of the major objectives of present age of biotechnology is headed towards production and application of a range of bio-based value-added products. Biopolymers i.e. cellulose, xylan, lignin, chitin, tannin, pectin, etc. are considered as the major resource materials for a number of industrial sectors due to their renewable and biodegradable nature. Among all the polymers, in spite of its abundance in nature, chitin is the most under-utilized one due to its high degree of crystallinity and insolubility in most of the solvents (Ruiz-Herrera, 1978). Chitosan, the N-deacetylated derivative of chitin has enormous commercial potential due to its characteristically superior properties like biodegradability, biocompatibility, solubility, non-toxicity etc (Kurita, 2006). Chitosan is present in nominal amounts in animal biomass, in the shells or cuticles of many crustaceans and also in the fungal cell wall, it is therefore mainly derived from chitin by chemical or bio-catalytic alkaline deacetylation process. Chemical route utilizes larger amounts of concentrated alkali that in turn eventually leads into environmental deterioration (Chang et al., 1997). Bioconversion is a comparatively superior alternative to the chemical one due to the various advantages it has over the chemical process. CDA catalyzes the deacetylation of monomeric acetyl glucosamine residues of chitin by multiple attack mechanism and produces high quality chitosan (Tsigos et al., 2000). CDA-assisted chitosan production had initially begun as a thought and now considered as worthwhile promising technology of the future. Intense research and developmental activities are underway so as to have a technology that would eventually be independent from using bulk amounts of chemicals for deacetylation of chitin would be a cherished dream come true.

2.1 CHITIN

Chitin is one of the most abundant biopolymers in nature after cellulose and also considered as the most widespread amino-polysaccharide, consisting largely of alternating *N*-acetylglucosamine residues, linked by β-(1,4) glycosidic bonds. Hydrolysis of chitin by chitinase leads into release of *N*-acetylglucosamine and chito-oligosaccharides. It is widely distributed in nature as the principal component of exoskeletons or cuticles of many invertebrates, primarily of crustaceans like crabs, shrimps, prawns, lobsters (Muzzarelli, 1977; Herring, 1979) and also a major constituent of the fungal cell wall (Blumenthal and Roseman, 1957). Epidermal cuticle of *Paralipophrys trigloide* contains chitin as structural material (Wagner et al., 1993). Annual synthesis of this polysaccharide in fresh water and marine ecosystems is estimated to be 600 and 1600 million tons, respectively (Synowiecki and Al-Khateeb, 2003). Derivatives of chitin oligomers have also been implicated as morphogenic factors in the communications between leguminous plants and rhizobium and even in vertebrates, where they may be important during early stages of embryogenesis (Merzendorfer and Zimoch, 2003). Chitin is such a characteristic component of fungal cell walls that it has been used for assessing the fungal content of natural materials such as decomposing organic matter, infected plants or even the infected tissues of animals (Newell, 1992).

2.1.1 *Structure*

Chitin contains 6–7 % nitrogen (Mathur and Narang, 1990) and unlike cellulose, it can be a source of nitrogen as well as carbon (C:N, 8:1) (Struszczyk, 2006). Chitin polymer tends to form microfibrils of ~3 nm in diameter that are stabilized by hydrogen bonds formed between the amine and carbonyl groups. Chitin micro fibrils of peritropic matrices may exceed 0.5 μm in length and frequently associate in bundles containing parallel groups of 10 or more single microfibrils (Atkins, 1985). X- ray diffraction analysis suggested that chitin is a polymeric substance that occurs in three different crystalline modifications, termed α, β and γ (Mazeau et al., 2002; Sikorski et al., 2009). These forms are mainly differ in the degree of hydration, size of the unit cell and the number of

chitin chains per unit cell. In the α form, all chains exhibit an anti-parallel orientation; in the β form the chains are arranged in a parallel manner and in the γ form sets of two parallel strands alternate with single anti-parallel strand (Merzendorfer and Zimoch, 2003; Khoushab and Yamabhai, 2010). The α-form is mainly obtained from crab and shrimp shells, is widely distributed; β-form is obtained from molluscs such as squid, and the fungal chitin is belongs to the γ-form (Atkins, 1985). The anti-parallel arrangement of chitin molecules in α form allows tight packaging into chitin microfibrils, and the whole structure is stabilized by a number of hydrogen bonds formed between the molecules. This arrangement may contribute significantly to the physicochemical properties of cuticle such as strength and stability (Merzendorfer and Zimoch, 2003). In contrast, the packaging tightness and number of inter-chain hydrogen bonds of the β and γ chains are reduced, resulting in an increase in the number of hydrogen bonds with water. The high degree of hydration and reduced packaging tightness resulted in more flexible and soft chitinous structures, as are found in peritropic matrices. Conversion from the β-form to the α-form is possible, but not the reverse (Lavall et al., 2007; Mazeau et al., 2002; Schiffman et al., 2009). γ chitin can also be converted into α-chitin by treatment with lithium thiocyanate (Rudall and Kenchington, 1973).

2.1.2 *Production*

Chitin was first isolated in 1811 by the French chemist and pharmacist Henri Braconnot, as the alkali-resistant fraction from fungi (Braconnot, 1811). Since chitin is present along with proteins, minerals, lipids, and pigments, these components have to be quantitatively removed to achieve the high purity necessary for biological applications and also for its use as a raw material to make high quality chitosan. The extraction procedures invariably involve demineralization and deproteinization along with decolorization for extraction of chitin. Demineralization is frequently carried out by treatment with dilute acids or chelating agents, while deproteinization involves treatment with dilute alkali or proteolytic enzymes (Muzzarelli, 1977; Shimahara et al., 1989). Hydrochloric acid seems to be the preferred reagent for demineralization and is applied at a

concentration between 0.2 - 2 M for 1 - 48 hrs at temperatures varying from 0 - 100 ^{0}C (Percot et al., 2003a). In most instances, protein separation precedes demineralization (Hackman, 1954). However, the deproteinization process may precede the demineralization step without any adverse effects on the product (No et al., 1989a, b; Sandford, 1989). Subsequent to these processes, the product may be decolorized with acetone and/or hydrogen peroxide, or NaOCl (Austin, 1988; No et al., 1989a, b).

Although many research groups had suggested different extraction procedures for the removal of proteins and minerals, detrimental effects on the molecular weight and fraction of acetylated (F_A) units cannot be avoided with any of these extraction processes (Percot et al., 2003b). Therefore, a great deal of interest still prevails for the optimization of the extraction procedure to minimize the degradation of chitin, while at the same time, reducing the impurities down to a level satisfactory for specific applications. Some investigators have attempted to deproteinize crustacean waste intended for chitin production by enzymatic digestion (Takeda and Abe, 1962; Takeda and Katsuura, 1964; Broussignac, 1968) and reported that enzymatic methods produce better chitin with consistent and well defined properties as compared to chemical methods. However, the residual protein in the chitin produced, often remains relatively high and the reaction time is longer compared to chemical deproteination. These drawbacks make the enzymatic method unlikely to be applied on an industrial scale unless progress is made in making the process more efficient (Percot, Viton & Domard, 2003b).

2.1.3 *Chitin biosynthesis pathway*

Carbohydrates are essentially needed for various physiological roles viz. as an energy source for metabolism and in biosynthesis. Polysaccharide synthesis in fungi is similar to that of in other organisms. Chitin biosynthesis has been studied in a large variety of organisms viz. fungi, crustaceans and insects and was observed to follow the same route using uridine diphosphate *N*-acetylglucosamine as the glycosyl donor (Bartnicki-Garcia and Nickerson, 1962a, b; McGarrahan

and Maley, 1965; Camargo et al., 1967) with the enzyme chitin synthase (CS, EC 2.4.1.16) catalyzing the synthesis process. Initially, fructose-6-phosphate is converted to *N*-acetylglucosamine-6-phosphate through consecutive action of two enzymes viz. aminotransferase and *N*-acetyltransferase by successive additions of an amine group and an acetyl group. *N*-acetylglucosamine-6-phosphate further reacts with uridine triphosphate to form uridine diphosphate - GlcNAc which is added stepwise to the elongating chitin chain through the action of enzyme chitin synthase.

Three chitin synthases (CS) i.e. CS I, II and III have been found in *Saccharomyces cerevisiae* that are different from each other in terms of function and catalytic activity. CS III is considered as the most active enzyme, responsible for 90 % of the chitin synthesis (Bulawa, 1993). Fungal chitin synthases are grouped into two families and five classes (Roncero, 2002). There are two chitin synthases in insects (Merzendorfer, 2006; Kato et al., 2006). Nematodes also possess two chitin synthase genes, which are differentially expressed (Veronico, 2001). Therefore, it appears that different forms of this enzyme demarcate distinct ability to catalyze the reaction. CSs use UDP-*N*-acetylglucosamine as substrate to produce chitin fibrils (Cohen, 2001), the same demonstrated in *Mucor rouxii* (McMurrough et al., 1971). The genomes of some chloroviruses contain a chitin synthase gene (*chs*). The *chs* gene of chlorovirus CVK2 when introduced into *Chlorella* cells, an algae that does not possess a chitin synthase gene, enables later to produce chitin (Kawasaki, 2002).

2.1.4 ***Chitin degradation***

A consortium of enzymes is involved in complete hydrolysis of chitin. Chitinase (EC 3.2.1.14), the major enzyme of the pathway, catalyzes the degradation of water-insoluble chitin into water-soluble chitin oligosaccharides by hydrolytic reaction (Patil et al., 2000). Chitodextrinases (EC 3.2.1.14) and *N*-acetylglucosaminidases (EC 3.2.1.52) act on the oligosaccharides produced by the action of chitinases and release monomeric *N*-acetylglucosamine subunits (Tsujibo et al., 1998; Howard et al., 2003; Suginta et al., 2010). CDA (EC 3.2.1.41) catalyzes the deacetylation reaction

and produces chitosan (Henrissat, 1991; Tsigos et al., 2000) which can be further metabolized by the action of chitosanase (EC 3.2.1.132) into monomeric glucosamine subunits (Howard et al., 2003). All of the above mentioned enzymes constitute the chitinolytic enzyme system.

2.1.5 *Derivatives of chitin*

In spite of its production in astronomical quantities, till now, fewer industrial applications of chitin are known. A high degree of crystallinity and insolubility in most of the standard polar and non-polar solvents hinders to its industrial applicability. Because of the abundance of chitin on earth's surface and possibility of generating various soluble chitin derivatives, has led researchers to explore their applications for various sectors. Among the different chitinous compounds, chito-oligosaccharides (Choi et al., 2001; Jung et al., 2007a), chitosan (Shahidi et al., 1999; Tsigos et al., 2000) and glucosamine are the major products with diverse set of applications.

2.1.6 *Global demand*

In the present era of bio-based products, the demand for chitin derivatives viz. chitosan, chitooligosaccharides, glucosamine, etc. is steadily increasing. As estimated 75 % of the chitin produced is used to manufacture products for the nutraceutical market. Currently the major driving force in the market is the increasing sales of glucosamine as a dietary supplement (Sandford, 2002). Approximately 65 % of the chitin produced is converted into glucosamine, ~25 % is converted into chitosans, ~9 % is used to produce oligosaccharides and approximately 1 % is utilized for the production of *N*-acetylglucosamine (Mustaparta, 2006) (Table 1).

Table. 1 Estimated global production and consumption of chitin-derived products

Product	Annual production (tons)	Chitin consumption (tons)	Market price (USD/kg)
Glucosamine	4500	9000	7-35
Chitosans	3000	4000	10-100[a]
Oligosaccharides	500	1000	50-100[b]
***N*-acetylglucosamine**	100	200	20-140[c]

[a]Ultra pure/GMP (Good Manufacturing Practice - Quality System) products can have prices of up to more than 50.000 USD/kg; [b]Ultra pure and well characterized products can have prices up to more than 10.000 USD/g; [c]Chemically manufactured: 20 USD/kg and enzymatically manufactured: 100-140 USD/kg.

Variation in the estimated market prices depends not only on the quality of the product but also on to the demand of product in the market. The major raw materials for the production of chitin today are cuticles of various crustaceans, mainly crab and shrimp (Kim & Rajapakse, 2005) and the market price is approximately 5-8 USD/kg for average quality chitin (Mustaparta, 2006).

2.2 CHITOSAN

The major industrially tractable polycationic biopolymer, occurs naturally or is obtained by the *N*-deacetylation of chitin. It refers to partially or fully *N*-deacetylated derivatives of chitin which are soluble in dilute acidic solutions. Chitosans are a family of linear, binary polysaccharides consisting of β-(1,4) linked *N*-acetylglucosamine and glucosamine subunits in varying proportions (Tsigos et al., 2000). It was first discovered by Rouget in 1859, by boiling chitin in a concentrated potassium hydroxide solution. There has been a substantial momemtum in chitosan research during

the past decade due to its biocompability, biodegradability, non-toxicity and other unique properties such as ability to form films, chelation, adsorption properties and antimicrobial activity (Kumar, 2000). Chitosan is considered as a much more tractable material than chitin with a range of applications in multiple fields mainly in pharmaceutics (drug delivery), protein and gene delivery, tissue engineering, food, water treatment, cosmetics and agriculture etc. (Tsigos et al., 2000). In 2001, the company Primex Ingredients ASA (Norway) promoted its shrimp-derived chitosan as "Self-affirmed GRAS (Generally Recognised As Safe)" under FDA regulations (US Food and Drug Administration). Many had expectations that the affirmation of chitosan as GRAS would open up considerable market for high-quality chitosans for human consumption. However, the self-affirmation was withdrawn in 2002 (FDA, 2006). Still, it is expected that the use of chitosan in both biomedical and food sectors will go up with increasing availability of high quality chitosans (Sandford, 2002).

2.2.1 *Chitosan: why novel and versatile?*

Chitosan is fundamentally derived from chitin either by chemical or biological means. The major problem which restricts the use of chitin is its crystallinity and insolubility in most of the biological solutions. Chitin has two hydroxyl groups while chitosan has one amino and two hydroxyl groups in the repeating hexosaminide residues generated by the removal of acetyl groups from chitin (Fig. 1). It is considered as a novel and versatile material because its physical and chemical properties are very much different from the parent material, chitin, and due to which it is more useful and tractable. Some of the novel and versatile features of chitosan are as below:

a. Due to the availability of free amino groups in chitosan, it carries a positive charge and thus in turn reacts with many negatively charged surfaces/polymers and also undergoes chelation with metal ions.

b. Chitosan is a weak base and is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH < 6.5), which can convert the glucosamine units into a soluble form i.e. $R–NH_3^+$.

c. It gets precipitated in alkaline solution or with polyanions and forms gel at lower pH.

d. It is available as dry flakes, solution and fine powder.

e. It is biodegradable, non-toxic, and biocompatible.

f. Possess mucoadhesive properties due to electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces.

The above mentioned features make chitosan as one of the most widely explored biopolymer and thus it has applications in diverse sectors ranging from biomedical field to environmental engineering.

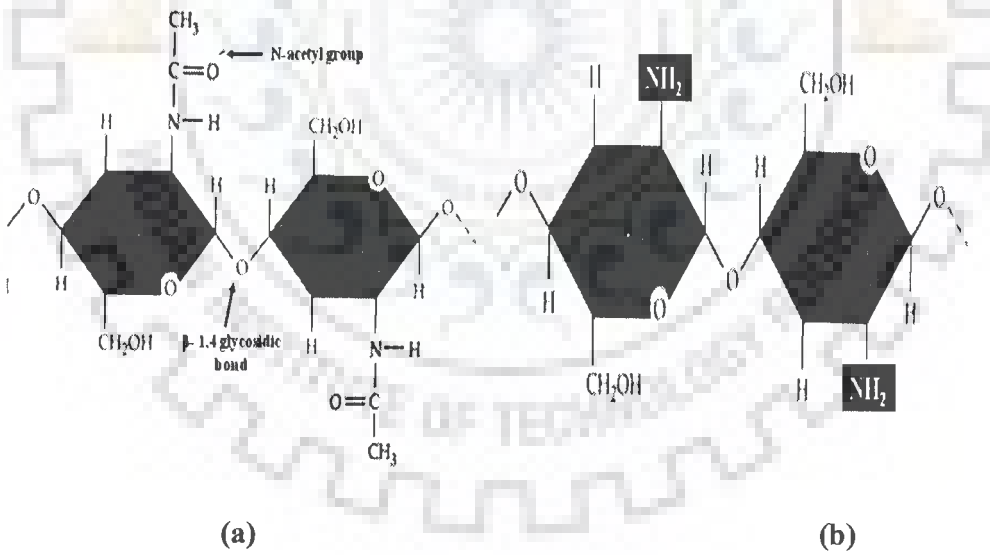


Fig. 1 Polymeric structure of chitin (a) and chitosan (b)

2.2.2 *Production*

Currently major portion of the chitosan is produced by the thermo-alkaline deacetylation of chitin with highly concentrated sodium hydroxide solution (40 - 50 %) at high temperature (100 - 150 ^{0}C), exclusive of air, for about an hour. Up to 70 % of deacetylation may be achieved with this

treatment (Johnson and Peniston, 1982). Extended treatment with hot concentrated sodium hydroxide solution could result in an almost completely *N*-deacetylated (90 %) product (Wu and Bough, 1978). Some researchers have used potassium hydroxide in place of sodium hydroxide at 180 ^{0}C, to achieve up to 95 % deacetylation preferably in nitrogen atmosphere (Barker et al., 1958; Horton and Lineback, 1965). Extent of deacetylation is governed by the concentration of alkali used, temperature, duration of reaction, particle size and density. Since treatment with alkali leads to depolymerization of the material, so it is preferred to be carried out in an inert atmosphere to alleviate the depolymerization problem and thereby recover chitosan of higher viscosity and molecular weight (Muzzarelli, 1977). Repeated washing of the intermediate products with water also attains effective deacetylation during the alkali treatment. The washing results into reduction of the alkali concentration between the chitin particles, thereby increasing the accessibility of the chitin during the subsequent deacetylation treatment. The procedure didn't significantly degrade the polymer, and led into the recovery of a product of about 5 x 10^5 daltons and up to 100 % deacetylation (Mima et al., 1982). Some researchers had suggested that the multiple treatments are more effective than a single treatment to prepare fully deacetylated chitosan (Domard and Rinaudo, 1983).

Despite of being used at commercial scale, the process shares various disadvantages of that of a multi-step chemical procedure mainly: use of large volumes of concentrated alkali with simultaneous production of large quantities of liquid waste containing protein and other non-protein nitrogenous compounds, process is conducted at higher temperature and therefore entails high energy cost, environmentally unsafe, tedious to control and resulting into heterogeneous range of products (Shimahara et al., 1989; Chang et al., 1997).

2.2.3 *Biological conversion to chitosan*

Chitosan production has received worldwide attention in both industrial and scientific communities, mainly because of its wide spectrum of applications in pharmaceutical, food,

agricultural sectors (Zhao et al., 2010a). An alternative or complementary procedure to the conventional chemical process is the enzymatic deacetylation of chitin that can potentially be employed, which is a controlled, non-degradative and a defined process leading into the production of novel and superior quality chitosan. Bioconversion to chitosan can be achieved enzymatically by using CDA (EC 3.5.1.41), which catalyzes deacetylation of *N*-acetyl-D-glucosamine residues under mild reaction conditions and results into superior-quality chitosan (Araki and Ito, 1975; Tsigos et al., 2000). Enzyme aided production could prove to be promising, eco-friendly and results into high quality desired product, which may accordingly be utilized for broad range of applications (Kauss and Bausch, 1988).

2.3 CHITIN DEACETYLASE

Chitin deacetylase (CDA, EC 3.5.1.41) catalyzes the removal of acetyl groups from the nascent chitin chain, resulting into the production of a polymer composed of both glucosamine and *N*-acetylglucosamine monomers (Cai et al., 2006). It was initially identified and partially purified from the mycelial extracts of *Mucor rouxii* by Araki and Ito (1975).

2.3.1 Source

The enzyme has been detected in a variety of organisms including fungi, bacteria, and insects, among which fungal deacetylases are widely explored (Tsigos et al., 2000; Meens et al., 2001; Zhao et al., 2010a) (Table 2). The physiological role of CDAs in microbes is primarily concerned with the production of chitosan, a cell wall component along with the pathogenesis of plant pathogenic fungi.

Table. 2 Characteristic features of chitin deacetylase from microorganisms

Organism		M.W (kDa)	pI	Opt. pH	Opt. temp.	Acetate Inhibition	Activation/ Inhibition	Min. DP	References
Mucor rouxii ATCC 24905	Intra	75	3	4.5	50 ^{0}C	Yes	-	4	Kafetzopoulos et al., 1993
Aspergillus nidulans CECT 2544	Extra	27	2.75	7.0	50 ^{0}C	Yes	-	2	Alfonso et al., 1995
Colletotrichum lindemuthianum DSM 63144	Extra	150	-	8.5	50 ^{0}C	No	Co^{+2}	2	Tsigos et al., 1995
Colletotrichum lindemuthianum ATCC 56676	Extra	□ 31.5	3.7	11.5-12.0	60 ^{0}C	Yes	Co^{+2}	4	Tokuyasu et al., 1996
Vibrio alginolyticus H-8	Extra DA1 DA2	 48 46	 3.3 3.5	 8.5-9 8.0-8.5	 45 ^{0}C 40 ^{0}C	-	 Ag^+, Hg^{+2}	 2	Ohishi et al., 1997

Thermus caldophilus	Intra	45	-	7.5	80 ^{0}C	-	Mn^{+2}, Co^{+2}, Fe^{+3} (Ac.); Cu^{+2} (In.)	6	Shin et al., 1999
Metarhizium anisopliae	Extra	70, 37, 26	2.6, 3.8, 4.1	8.5-8.8	-	No	-	-	Nahar et al., 2004
Mucor circinelloides	Intra	-	-	4.5	50 ^{0}C	-	-	-	Amorim et al., 2005
Scopulariopsis brevicaulis	Extra	55	-	7.5	55 ^{0}C	-	-	2	Cai et al., 2006
Mortierella sp. DY-52	Extra	50, 59	-	5.5	60 ^{0}C	-	Co^{+2}, Ca^{+2}	2	Kim et al., 2008
Absidia corymbifera DY-9	Extra	-	-	6.5	55 ^{0}C	Yes	Co^{+2}, Ca^{+2}, Mg^{+2} (Ac.)	2	Zhao et al., 2010

(Ac., Activation; In., Inhibition; DP, Degree of polymerization)

2.3.1.1 Deacetylases of fungal origin

Various studies have been taken up for CDA production from fungi mainly from *Mucor rouxii* (Davis and Bartnicki-Garcia, 1984; Kafetzopoulos et al., 1993a), *Colletotrichum lindemuthianum* (Tsigos and Bouriotis, 1995; Tokuyasu et al., 1996; Shrestha et al., 2004), *Absidia coerulea* (Gao et al., 1995), *Aspergillus nidulans* (Alfonso et al., 1995), *Gongronella butleri* (Maw et al., 2002 a,b), *Metarhizium anisopliae* (Nahar et al., 2004), *Rhizopus nigricans* (Jeraj et al., 2006), *Scopulariopsis brevicaulis* (Cai et al., 2006), *Mortierella* sp. DY-52 (Kim et al., 2008), *Rhizopus circinans* (Gauthier et al., 2008), *Flammulina velutipes* (Yamada et al., 2008), *Absidia corymbifera* (Zhao et al., 2010b), etc. Fungi are known to produce both extra and intracellular deacetylases and these might be secreted during different periods owing to their specific biological roles. For instance, an extracellular CDA from *C. lindemuthianum* was exclusively secreted during fungal hyphae penetration into plants to modify the chitin that could be recognized by a plant resistance system (Tsigos and Bouriotis, 1995). In contrast, an intracellular CDA from *M. rouxii* was produced during fungal cell wall development (Davis and Bartnicki-Garcia, 1984).

2.3.1.2 Deacetylases of yeast origin

Apart from these fungal strains, some yeast species viz. *Saccharomyces cerevisiae* (Martinou et al., 2002) and *Schizosaccharomyces pombe* (Matsuo et al., 2005) are also known to produce CDAs during sporulation, which may be involved for the synthesis of spore wall.

2.3.1.3 Deacetylases of bacterial origin

Among the bacterial strains, members belonging to family Vibrionaceae, widely distributed in all oceanic and estuarine waters, are considered as the major CDA producers (Ferguson and Gooday, 1996), where they are involved in the chitin metabolism (Hunt et al., 2008). Most of the *Vibrio* strains are known to produce chitin oligosaccharide deacetylase (COD), which in turn produces a unique inducer for chitinase production for chitin catabolism. COD from *V. parahemolyticus* and *V. cholerae* was purified and characterized by Kadokura et al. (2007 a,b) and

Li et al. (2007), respectively. Hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1 also possesses a deacetylase, involved in their chitin catabolic pathway (Tanaka et al., 2003). CDA from *Bacillus amyloliquefaciens* was isolated and characterized by Zhou et al. (2010).

2.3.1.4 *Deacetylases of insect origin*

Occurrence of CDAs is not only restricted to microbial population but also detected in insect species, including *Trichoplusia ni* (Guo et al., 2005), *Drosophila melanogaster* (Luschnig et al., 2006; Wang et al., 2006a), *Helicoverpa armigera* (Campbell et al., 2008), *Anopheles gambiae, Apis mellifera, Tribolium castaneum* (Dixit et al., 2008) and *Mamestra configurata* (Toprak et al., 2008). Most of the reported insect CDAs are associated with the midgut peritrophic membrane and are evenly distributed throughout the entire length of peritrophic membrane, as shown for the CDAs from *T. ni* (Guo et al., 2005), *H. armigera* (Campbell et al., 2008), and *M. configurata* (Toprak et al., 2008). In addition, the presence of these enzymes in the midgut tissue of larvae was detected only during the feeding period, the larvae when had stopped feeding in their later stages, this protein was detected to be absent in the midgut tissue (Guo et al., 2005). Although the involvement of CDA in peritrophic membrane is viewed as a common feature of insects, the occurrence of CDAs in insect is not restricted to peritrophic membrane. For instance, in *D. melanogaster*, two CDA-like proteins, CDA1 and CDA2 (serpentine and vermiform) were found to be associated with the tracheal extracellular matrix ((Luschnig et al., 2006).

2.3.2 Classification

CDAs (EC 3.5.1.41) are members of carbohydrate esterase family 4 (CE-4s) as defined in the CAZY database (http://afmb.cnrs-mrs.fr / ~ cazy/ CAZY). According to Henrissat classification, the family also includes rhizobial NodB chitooligosaccharide deacetylases, peptidoglycan N-acetylglucosamine deacetylases (EC 3.5.1.104), acetyl xylan esterases (EC 3.1.1.72) and xylanases A, C, D, E (EC 3.5.1.8) (Coutinho and Henrissat, 1999). Members of this family share a conserved region in their primary structure named the "NodB homology domain"or "polysaccharide

deacetylase domain" (Caufrier et al., 2003; Gauthier et al., 2008). All five members of this family catalyze the hydrolysis of either *N*-linked acetyl groups from *N*-acetylglucosamine residues or *O*-linked acetyl groups from *O*-acetylxylose residues of their substrates viz. chitin, NodB factors, peptidoglycan and acetyl xylan.

2.3.3 Catalytic mechanism

Mode of catalysis of CDAs were studied both on chitin polymers and oligomers using HPLC, FAB-MS and solid-state NMR spectroscopy by several groups. Tokuyasu et al. (1997) studied the deacetylation of chitooligosaccharides (dp 2-4) by CDA purified from *Colletotrichum lindemuthianum*. Using FAB-MS and ^{1}H NMR, they concluded that *N, N', N'', N'''*-tetraacetylchitotetraose and *N, N', N''*- triacetylchitotriose were converted into fully deacetylated corresponding chitosan oligomers. But *N, N'*- diacetylchitobiose was deacetylated on either of the glucosamine residues to yield an unique compound i.e. 2-acetamido-4-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose [GlcN-GlcNAc]. Similarly, catalytic action of *Mucor rouxii* CDA on water-soluble partially deacetylated chitosans (dp- 30; F_A- 0.681) was studied by Martinou et al. (1998) and concluded that the enzyme deacetylates following multiple-attack mechanism with a degree of multiple attack of at least three. The polarity of CDA was preferentially towards the reducing end and it follows an endo-type mechanism with no preferential attack at any sequence in the chitosan chain. Deacetylation reaction was not detected at the non-reducing end of the chain. The relative rate of enzymatic deacetylation increased linearly with the increasing F_A of the chitosans. Deacetylation of *N*-acetylchitooligosaccharides (dp 2-7) by *Mucor rouxii* ATCC 24905 CDA was studied by Tsigos et al. (1999) using an exo-splitting system. The extent of deacetylation depends on the length of the substrate. The enzyme could not effectively deacetylate chitin oligomers with a degree of polymerization less than three. Only Tetra-*N*- tetraacetylchitotetraose and penta-*N*- pentaacetylchitopentaose were fully deacetylated by the enzyme while the reducing end residues of *N*- acetylchitotriose, *N*- acetylchitohexaose, *N*- acetylchitoheptaose always remained

intact. The enzyme initially removes an acetyl group from the non-reducing end residue and further catalyzes the hydrolysis of the next acetamido group in progressive fashion. This report was in contrast with the findings of Martinou et al. (1998), in which the enzyme was observed to be more active towards reducing end.

2.3.4 Substrate specificity

Substrate specificity of *M. rouxii* CDA was first evaluated by Araki and Ito (1975) and observed that enzyme was active on glycol chitin and chitooligomers with degree of polymerization greater than two. Deacetylase activity of the enzyme depends on the number of monosaccharide units in the substrate. Caufrier et al. (2003) had analyzed the activity of CDA from *M. rouxii* and acetyl xylan esterase from *Streptomyces lividans* on acetyl xylan, peptidoglycan and soluble chitin as the substrates. The enzymes were observed to be active on acetyl xylan and soluble chitin while inactive on peptidoglycan, which means that not only chitin but also acetyl xylan could be handled by CDA, as both the enzymes viz. CDA and acetyl xylan esterase have a similar catalytic domain which is different from that of peptidoglycan deacetylase. Structural analysis together with sequence alignment studies suggested that one disulfide bond, tethering the *N*-terminal and *C*-terminal ends, is conserved in *M. rouxii* CDA, *C. lindemuthianum* CDA and *S. lividans* acetyl xylan esterase while absent from the homologous bacterial *Streptococcus pneumoniae* peptidoglycan deacetylase and *Bacillus subtilis* peptidoglycan deacetylase (Blair et al., 2005, 2006; Taylor et al., 2006).

2.3.5 Multiplicity

Multiplicity corresponds to existence of multiple forms of the similar enzymes or isozymes, which catalyzes the same reaction but differ in their amino acid sequence and physicochemical properties, such as molecular weight, isoelectric point, kinetic constants etc. Such multiple forms of CDAs had been observed in many fungal and bacterial species. In *Uromyces viciae-fabae*, five isoforms of CDA (12.7 to 48.1 kDa) were produced during penetration of fungus

through leaf stomata (Deising and Siegrist, 1995). Three CDA isoforms were also reported by Trudel and Asselin (1990) in *Mucor racemosus* with molecular mass of 26, 30 and 64 kDa. Similarly, *C. lindemuthianum*, *M. anisopliae* and *R. nigricans* are also known to produce two, three and four CDA isozymes respectively. Among bacterial species, *V. alginolyticus* H-8 produced two extracellular CDA isoforms (Ohishi et al., 1997). Possible reasons for this multiplicity may be due to gene duplication, differential m-RNA processing, post translational modifications such as glycosylation and auto aggregation. Multiple CDAs can also be the product from different alleles of the same gene i.e. allozymes.

2.3.6 Structure-function relationship

The structure of CDA from *C. lindemuthianum* (*Cl*CDA, Blair et al., 2006) is the first and till now the only structure among CDAs to be known. It consists of a single catalytic domain, similar to the deformed $(\beta/\alpha)_8$ fold in other CE-4 family members. The active site cleft of the protein is formed from the *C*-terminal ends of β- strands 2,4,5,7 and 8 of the $(\beta/\alpha)_8$ barrel and includes five distinct sequence motifs conserved in the family (Blair et al., 2006). Two intramolecular disulfide linkages are known to stabilize the structure. Sequence alignment suggests that one disulfide (Cys38-Cys237), tethering the *N*- and *C*-terminal ends of the structure is conserved in the fungal CDA from *M. rouxii* and other CE-4 family members (Fig. 2). *Cl*CDA is a metalloenzyme using a His-His-Asp zinc binding triad with a nearby aspartic acid and histidine acting as catalytic base and acid, respectively (Blair et al., 2004, 2005).

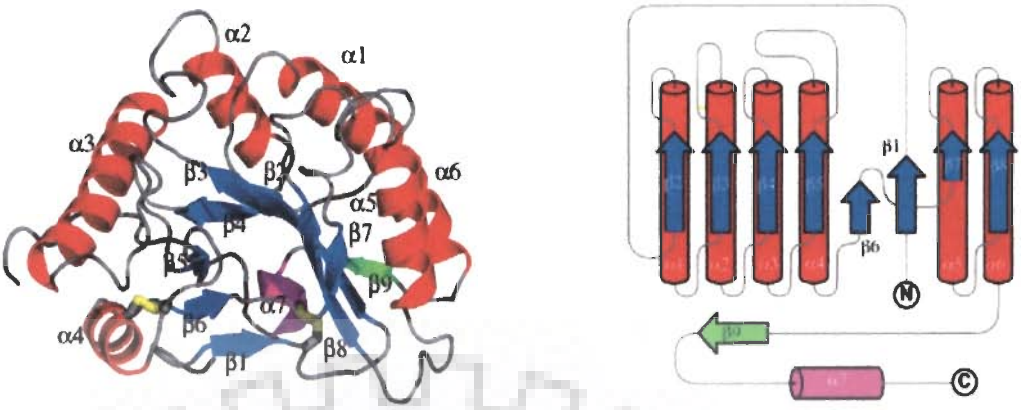


Fig. 2 Structure of CDA from *Colletotrichum lindemuthianum* (Blair et al., 2006)

2.4 Approaches to enhance the production level

Higher levels of enzyme activity is a prerequisite for economical and commercial production of a particular product through microbial routes. The level of the molecule or product of interest in naturally occurring strains, in most cases, is too low for commercial exploitation. Multiple approaches could be recruited for achieving improvement in the levels of production i.e. genetic manipulation and process optimization.

2.4.1 Genetic manipulation

2.4.1.1 *Mutagenesis*

Mutagenesis of a wild type strain is widely used for achieving improved levels of production. Mutagens, either chemical or physical, confer genetic changes and resultant mutants are then screened to select the one with improved productivity. About 1.6 fold enhanced production of CDA from *Gongronella butleri* was achieved by UV mutagenesis (Maw et al., 2002b). Chemical mutagens have so far not been utilized for improving the levels of CDA production. Attempts have also been made to improve yield of chitinase (Lim et al., 1991; Vaidya et al., 2003; Gohel et al., 2004) and chitosanase (Su et al., 2006) employing mutagenesis. Among physical mutagens, UV irradiation is successfully employed for obtaining improved yields of an array of metabolites (Chand et al., 2005; Rangel et al., 2006a, b; Li et al., 2010). Chemical mutagenesis was performed

using various alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine, ethyl methane sulfonate and intercalating agents like ethidium bromide, acridine orange for achieving increased levels of commercially important enzymes and metabolites. Induced mutagenesis was performed using various physical and chemical mutagens for obtaining increased levels of xylanase (Bakalova et al., 2002), glucose oxidase (Khattab and Bazaraa, 2005), tannase (Purohit et al., 2006), , glucoamylase (Kumar and Satyanarayana, 2009), fructofuranosidase fructohydrolase (Ali and Ashiq, 2010) and cellulase (Adsul et al., 2007; Fang et al., 2009; Li et al., 2010; Vu et al., 2011). Mutational strategies are then supplemented with derivations of nutritional, conditional and other necessary factors for achieving increase in the yields for producing amino acids, enzymes, vitamins, solvents and organic acids. As the regulatory mechanisms are genetically controlled, mutations may profoundly affect the production of secondary metabolites. Mutagenesis had led into a 100–1000 fold enhanced production of antibiotics from their initial discovery to the present time. These, therefore, affect into considerable increase in fermentation productivity and therefore leading to the decreased costs for production (Demain, 2000).

2.4.1.2 ***Gene cloning***

Genetic engineering has been a good alternative of traditional approaches for attempting the improvement in the levels of production (Govindappa et al., 2008). Till date CDA genes from various sources have been isolated, cloned and expressed into suitable homologous as well as heterologous hosts. The choice of a suitable host vector system is a major factor in order to achieve higher degree of success. A deacetylase encoding gene DA1 was cloned from *Vibrio alginolyticus* H-8 and sequenced using shot-gun method with pUC118 by Ohishi et al., (2000). The open reading frame of the gene starts at base 256 and ending at the base 1536. This 1281 nucleotides long sequence encoded 427 amino acids long enzyme. A putative Shine-Dalgarno (SD) sequence, ACGA was found upstream to the start codon-ATG.

Two CDA genes (CDA1 and CDA2) of *S. cerevisiae* were cloned and expressed into the plamid pSK and sequenced (Mishra et al., 1997). Both these deacetylases are expressed constitutively during sporulation, since spore wall of the yeast contains chitosan which along with the dityrosine layer have a role in spore protection in extreme conditions. CDA1 and CDA2 deficient mutants are found to be more sensitive towards chemical and environmental challenges. Kafetzopoulos et al. (1993b) isolated, sequenced and characterized a cDNA for CDA from *M. rouxii* ATCC 24905. CDA gene from *C. lindemuthianum* ATCC 56676 was over expressed in *E. coli* cells as a fusion protein using an expression vector pQE60 (Tokuyasu et al., 1999). The CDA ORF consists of two regions; one from the strat codon (ATG) encoding a deduced preprodomain of 27 amino acids and the other encoding a mature CDA of 221 amino acids. *R. nigricans* B 154 CDA was cloned by Jeraj et al. (2006) using yeast expression vector pFL61in *E. coli* DH5α. Sequence analysis divulged an open reading frame of 1341 nucleotides encoding a complete 447 amino acid protein. CDA gene from *C. lindemuthianum* UPS9 was isolated and cloned in *P. pastoris* as a tagged protein with six added terminal histidine residues. The specific activity of the purified protein was 72 Umg^{-1} (Shrestha et al., 2004). A 75 kDa CDA from *R. circinans* was cloned in *P. pastoris* expression system using cDNA library and 4.8 times increment was achieved in the production level (Gauthier et al., 2008). *Fv-pda*, a gene encoding CDA was isolated from *F. velutipes* during fruiting body development and expressed in *P. pastoris* (Yamada et al., 2008). The recombinant FV-PDA was observed to effectively catalyze the deacetylation of chitin oligomers (dimer to pentamer), glycol chitin and colloidal chitin.

2.4.2 Medium design and process optimization

Besides manipulating organism, selecting a suitable fermentation system and further alterations in the medium constituents and culturing conditions is a major tool for achieving improved levels of production. Among the various fermentation systems, submerged and solid-state were mainly utilized for enzyme production using microbial sources (Papinutti and Forchiassin,

2007; Marin-cervantes et al., 2008; Acikel et al., 2010; Sanchez-Otero et al., 2011). In recent years, there has been a spurt in the activities towards utilization of agro-industrial residual materials such as molasses (Makkar and Cameotra, 1997) cassava bagasse (Pandey et al., 2000), sugarcane bagasse (Kumar et al., 2003), coconut oil cake (Ramachandran et al., 2004), barley bran (Gomez et al., 2005), groundnut shells and seeds (Couto and Sanoraman, 2006) as well as the horticultural materials viz. chest nut shell, grape seeds, kiwi fruit waste and mandarin peels (Rosales et al., 2005) for production of various industrially important enzymes and metabolites under solid-state fermentation.

Medium components mainly carbon and nitrogen sources and other macro and micro nutrients are significant prerequisite for obtaining optimal growth and higher product yield. Culture conditions viz. pH, temperature, agitation, aeration, moisture content, inoculum age and inoculum size had also been observed to significantly affect enzyme production levels. Over the past decades, optimization of production process of several industrially important enzymes and metabolites had been studied by several groups both using one-factor-at-a-time and statistical approaches. The conventional approach of optimizing one-factor-at-a-time is laborious, time consuming and cannot provide the information on mutual interactions of the variables on the desired outcome. On the other hand, statistical experimental designs provide a systematic and efficient plan for experimentation to achieve certain goals so that many factors can be simultaneously analyzed (Bas and Boyaci 2007). Statistical data analysis allows visualization of the interactions among several experimental variables, leading to the prediction of data in areas not directly covered by experimentation. The statistical programs create several classes of RSM designs (Khuri and Cornell, 1987). Among these CCD is preferred as it convincingly provides information on experimental variable effects and overall experimental error in a minimum number of required experiments (Myers and Montgomery, 1995; Montgomery 2000, 2004). Statistical approaches have been successfully employed for optimization of medium constituents and process variables for production of industrially important

enzymes (Kumari et al., 2009; Barbosa et al., 2010; Karan et al., 2011; Rossa et al., 2011) and metabolites (Prakash et al., 2005, 2008; Desai et al., 2008; Shinde et al., 2010).

2.5 Biological roles of CDA

2.5.1 Chitin catabolism- deacetylases from marine bacteria

Marine ecosystems contain huge quantities of chitin, with an annual production of $>10^{11}$ metric tons. This bulk amount of chitin is recycled by the members of the family Vibrionaceae. Many *Vibrio* species that live in aquatic environments are capable of using chitin as the sole carbon source. Studies of the nonpathogenic marine organism *Vibrio furnissii* have shown that chitin utilization is a complex process involving at least three steps: chitin sensing, attachment, and degradation (Bassler et al., 1989, 1991). Pruzzo et al. (2008) stated that interactions between *V. cholerae* and chitin occur at multiple hierarchical levels in the environment and include cell metabolic and physiological responses, e.g., chemotaxis, cell multiplication, induction of competence, biofilm formation, commensal and symbiotic relationship with higher organisms, cycling of nutrients, as well as pathogenicity for humans and aquatic animals. Microarray expression profiling and mutational studies of *V. cholerae* showed that three sets of differentially regulated genes i.e. a $(GlcNAc)_2$ catabolic operon, two extracellular chitinases, a chitoporin, makes it able to utilize chitin (Meibom et al., 2004). Induction of *V. cholerae* with $(GlcNH_2)_2$ or crab shells resulted into the production of chitin oligosaccharide deacetylase (COD). The *cod* gene was cloned, overproduced, and purified to apparent homogeneity by Li et al. (2007). It hydrolyzes the *N*-acetyl group attached to the penultimate GlcNAc unit and found to be highly conserved among *Vibrio* species and Photobacteria.

V. alginolyticus produces a deacetylase specific for $(GlcNAc)_2$, but inactive with higher oligosaccharides (Ohishi et al., 2000). The COD enzymatic products, GlcNAc-$GlcNH_2$-$(GlcNAc)_n$, closely resemble those obtained by hydrolysis of the chito-oligosaccharides with Nod B: $GlcNH_2$-$(GlcNAc)_{3-4}$ that are key intermediates in the biosynthesis of Nod factors, critically important in

communications between the symbiotic nitrogen fixing bacteria and plants. Conceivably, the oligomers generated by deacetylases play equally important roles in cellular communications. COD is considered as an essential part of the chitin catabolic cascade of marine bacteria (Jung et al., 2008) due to its ability to produce heterodisaccahride- GlcNAc-GlcN, a unique inducer for chitinase production. Chitin utilization among several bacteria viz. *V. parahaemolyticus* KN1699 (Kadokura et al., 2007), *V. furnissii* (Bassler et al., 1991), *T. kodakaraensis* KOD1 (Tanaka et al., 2003), *Serratia marcescens* (Watanabe et al., 1997), *S. lividans* (Miyashita et al., 2000), and *Streptomyces coelicolor* (Saito et al., 2000, 2007) is found to be associated with the expression of COD. Hirano et al. (2009) had also observed that the heterodisaccharide is an inducer of the production of the two hydrolases, particularly chitinase in chitin-decomposing *Vibrio* strains harboring the carbohydrate esterase family 4 COD gene; however, such an increase in chitinase production was not observed in chitinolytic *Vibrio* strains that did not possess the COD gene.

2.5.2 Fungal deacetylases

Fungi are considered as the major producers of CDAs. Expression of CDA among fungal strains is thought to be related to various stages of growth to execute different functions.

2.5.2.1 Cell wall synthesis

In *M. rouxii* and *A. coerulea*, CDA was localized near the periplasmic space in the mycelia and contributed to formation of chitosan in the cell wall from nascent chitin synthesized by the action of chitin synthetase (Davis and Bartnicki-Garcia, 1984; Ruiz-Herrera and Martinez-Espinoza, 1999). Even though enzymology and cytology of chitin biosynthesis in fungi has been extensively studied, very little information exists on the correlation between CDA and chitosan biosynthesis (Hunt et al., 2008).

2.5.2.2 Formation of spore wall

S. cerevisiae requires chitin as an essential component for vegetative growth, however for spore wall formation both chitin synthesis and chitin deacetylation are necessary. Chitin is

synthesized by three chitin synthases Chs1, Chs2 and Chs3 in *S. cerevisiae*, among them Chs3 plays a major role. Conversion of chitin to chitosan by either Cda1 or Cda2 had allowed the second layered structure of the spore wall next to the outer dityrosine layer. The chitosan based structure is important for spores to retain its structural rigidity and resistance to various stresses (Mishra et al., 1997, Christodoulidou et al., 1999). Cda2p is the predominant deacetylase and performs most of the deacetylation task, while Cda1p which probably contributes to a fine tuning process, are indispensable for proper ascospore wall assembly (Martinou et al., 2002, 2003). It was reported that chitosan forms a layer of the ascospore cell wall in *S. cerevisiae* and is suggested to be in the bridges between individual spores. The results demonstrated that the interspore bridges can maintain a physical connnection between spores after they are released from the ascus (Coluccio and Neiman, 2004).

In addition, a *cda1*$^{+}$ encoded CDA in a fission yeast *S. pombe* was identified and analyzed (Matsuo et al., 2005). It was found that spore formation of a *cda1*$^{+}$ disruptant was abnormal and expression of *cda1* mRNA increased during sporulation process, suggesting CDA in *S. pombe* is required for proper spore formation.

Four CDAs viz. Cda1, Cda2, Cda3 and Fpd1 have been identified from *C. neoformans*, responsible for chitosan synthesis which is an important component of the vegetative cell wall and helps to maintain cell integrity and aids in bud separation. Among the four CDAs, Cda1, Cda2 and Cda3 account for all of the chitosan produced during vegetative growth in culture, but the function for Fpd1 remains undetermined (Baker et al., 2007).

2.5.2.3 Plant-pathogen interaction

CDA plays dual role in pathogenesis of plant pathogenic fungi viz. wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* and the broad bean rust fungus *U. fabae*, and the causative agents of anthracnose, *C. graminicola* and *C. lindemuthianum*. First, it protects penetrating fungal hyphae from being lyzed by secretory plant chitinases by transforming the superficial cell wall chitin into

chitosan and secondly, it diminishes the activity of chitin oligomers. Fungal plant pathogens, when colonizing their host plant tissues, encounter an elaborate defense system consisting of chemically and physically performed resistance factors and of induced resistance reactions. Hydrolases, i.e. chitinases and β-1,3-glucanases, represent standard antifungal enzymes found in most plants. The endo-type chitinases from plants degrade fungal chitin into chitin oligomers, which elicit plant-defense mechanisms (callose formation, lignification and synthesis of coumarin derivatives) whereas their deacetylated forms do not. CDA deacetylate the chitin oligomers and diminish their elicitor activity (Kauss et al., 1983; Walker-Simmons et al., 1984; Vander et al., 1998).

A successful fungal pathogen could evade plant antimicrobial hydrolases by enzymatic modification or affinity modulation. Based on the studies of cell wall composition of invasive fungal hyphae, it was suggested that exposed fungal chitin polymers are partially deacetylated during the infection and initial growth within the host for escaping from plant antimicrobial hydrolases (Gueddari et al., 2002). Based on the studies of catalytic mechanism of *C. lindemuthianm* CDA, it was assumed that the partially deacetylated product from chitin by the CDA should be a poor substrate for both chitinases and chitosanases (Hekmat et al., 2003). One avirulence protein (Avr4), containing an invertebrate chitin-binding domain (CBM14), is believed to mask and protect fungal cell wall chitin against hydrolysis by plant chitinases accumulated during infection by affinity modulation (van den Burg et al., 2006).

2.5.3 Insect deacetylases

Comparatively fewer studies have been performed on the CDAs from insect origin. In *D. melanogaster*, two CDA-like proteins, CDA1 and CDA2 (serpentine and vermiform), were found to be associated with the tracheal extracellular matrix and limited tube elongation, presumably by deacetylating the terminal *N*-acetyl-D-glucosamine that is extended to form the chitin chain (Luschnig et al., 2006; Wang et al., 2006). Deacetylation increases the solubility and decreases the

density of chitin fibrils *in vitro* and therefore may influence the structure and orientation of chitin fibrils in the cuticle.

A CDA, *Mc*CDA1, was identified from *M. configurata* peritrophic matrix (Campbell et al., 2008). This protein may be involved in altering the physical and chemical properties of the chitin in the peritrophic matrix by deacetylating *N*-acetyl-D-glucosamine. This would not only alter chitin fibril structure but also affect the binding of peritrophic matrix proteins, their integrity and porosity. The expression of *McCDA1* and *TnPM-P42* (Guo et al., 2005) was restricted to the midgut. In addition, of the nine *T. castaneum* CDA genes, *TcCDA-6*, *-7*, *-8* and *-9* were exclusively expressed in the gut (Dixit et al., 2008; Arakane et al., 2009), suggesting that a subclass of CDA play a role in gut physiology. They also speculated that Group V CDA may be involved in insect immunity or may alleviate the inhibitory effect of chitooligosaccharides on the activity of gut chitinases needed for the moulting process. Recent analysis on peritrophic matrix proteins indicated that midgut contains, CDA-like and mucin-like proteins and the former one may participate in immobilization of digestive enzymes, actively protect the gut from parasite invasion and intercept toxins such as lectins (Campbell et al., 2008).

2.6 CHITIN DEACETYLASE : FUNCTIONAL ASPECTS

The ability to act on carbon-nitrogen bonds of aminopolysaccharides, makes CDA a candidate of interest for various biological applications. Their major role is in the biological production of chitosan, a versatile biopolymer with a range of properties and applications. Apart from this, the enzyme also has some role in fungal cell wall formation and as a biocontrol agent.

2.6.1 Bioconversion to chitosan

A major objective for developing CDA has been to replace the harsh chemical process for conversion of chitin into chitosan, which moreover is not an environment friendly one. The enzymatic deacetylation would be able to provide chitosans with defined levels of deacetylation, as like the chemical process it does not proceed in a random fashion and permit the partially

deacetylated chitosans obtained from the chemical process to undergo to desired degree of deacetylation. Deacetylation potential of fungal CDAs towards chitinous substrates and partially deacetylated chitin substrates was studied by various research groups. The enzymatic deacetylation of various chitinous substrates was investigated by Aye et al. (2006) using the CDA isolated from *R. oryzae*. Chitin was observed to be a very poor substrate for the enzyme, but re-precipitated chitin was moderately better. *F. velutipes* CDA catalyzes deacetylation of *N*-acetyl-chitooligomers, from dimer to pentamer, glycol chitin and colloidal chitin (Yamada et al., 2008). Chitosan with lower degree of deacetylation (28 and 42%) can be further deacetylated with *M. rouxii* deacetylase (Martinou et al., 1995, 1997 a,b). Yield and rate of deacetylation was observed to be more with amorphous chitin substrates. Martinou et al. (2002) had attempted deacetylation of glycol chitin, chitin-50 and *N*-acetylchitooligosaccharides using cobalt activated CDA from *S. cerevisiae* and concluded that the enzyme requires at least two *N*-acetyl-D-glucosamine residues for catalysis, exhibiting maximum activity on hexa-*N*-acetylchitohexaose. CDAs from *C. lindemuthianum* (Kauss and Bausch, 1988; Tsigos and Bouriotis, 1995; Tokuyasu et al., 1997), *A. nidulans* (Alfonso et al., 1995), *S. brevicaulis* (Cai et al., 2006), *Mortierella* sp. (Kim et al., 2008) also exhibited similar deacetylation kinetics towards chitin and its oligomers. Crude CDA from *C. lindemuthianum* was active on partially deacetylated chitin (chitin with 50, 65, 70, and 82% degree of deacetylation) (Shrestha et al., 2004).

Crystallinity and insolubility of chitin makes it a poor substrate for enzymatic deacetylation. Pretreatment of chitin either by physical or chemical means prior to enzymatic reaction was necessary to increase the substrate accessibility to the enzyme (Martinou et al., 1997). Nearly 90% deacetylation of superfine chitin generated using $CaCl_2.2H_2O$/ methanol solvent system was achieved using CDA from *A. coerulea* and *C. lindemuthianum* (Win and Stevens, 2001). Attempts were made to decrystallize the chitin using physical treatment viz. sonication and heating. But chemical treatment was observed to be more effective than the physical ones. Beaney et al. (2007)

also tried to modify chitin either physically or chemically followed by deacetylating the same with extracellular deacetylase from *C. lindemuthianum.* Modifications of the chitin affected the degree of deacetylation to various extents and it was found that the dissolution and drying method used in modifying the chitin had significant impact on the final efficiency of the enzymatic deacetylation reaction. The degree of crystallinity of the chitin must be reduced to enable enzymes to access the internal polysaccharide structure. Jaworska et al. (2009) found that immobilization of CDA to diethylaminoethyl cellulose *via* divinyl sulfone led to high activity and stability towards various chitin and chitosans.

2.6.2 ***Biocontrol agent***

The fungal cell wall for the biological control of human fungal pathogen is the crucial target for antifungal therapies as it contributes to cell structure and integrity. It is needed for the localization or attachment of known virulence factors viz. polysaccharide capsule, melanin and it is critical for host-pathogen interactions. The chitin and the chitosan produced by the enzymatic removal of acetyl groups from nascent chitin is an important component of the cell walls of certain fungi and helps in maintaining cell integrity. Thus, CDAs and the chitosan may prove to be excellent antifungal targets (Brosson et al., 2005; Das et al., 2006; Baker et al., 2007). The inhibition of CDA therefore in turn would result into hydrolysis of fungal cell wall by plant chitinases, thus the control of the plant pathogenic fungi becomes feasible (Tokuyasu et al., 1996). Similarly, CDA could also be a versatile tool in the biological control of insect pests. For instance, CDA appeared to be initiate the pathogenesis of *M. anisopliae* (a kind of insect-pathogenic fungus) by softening the insect cuticle to aid the mycelial penetration. CDA herein may have a dual role in modifying the insect cuticular chitin for easy penetration of fungal pathogen as well as in altering its own cell walls for defense from insect chitinase (Nahar et al., 2004).

2.7 CHITOSAN : APPLICATIONS

2.7.1 Biomedical applications

The properties such as biodegradability, biocompatibility, mucoadhesion, high charge density and low toxicity makes chitosan molecule suitable for use in a wide range of biomedical and pharmaceutical applications.

2.7.1.1 Drug delivery system

Biodegradable and biocompatible nature of chitosan confers it as an ideal matrix for formulation of controlled drug delivery systems such as tablets, films, microspheres, hydrogels and nanoparticles (Chandy and Sharma, 1990; Onishi et al., 1997; Hirano, 1999, Alves and Mano, 2008; Park et al., 2003a, 2010). The reactive amino groups in the backbone of chitosan make it possible to chemically conjugate various biological molecules such as different ligands and antibodies, which may improve targeting efficiency of the drug to the site of action (Dufes et al., 2000; Hejazi and Amiji, 2003). Suitability of chitosan for drug delivery applications depends on its degree of deacetylation and molecular weight for developing sustained drug delivery systems, prolonging the duration of drug activity, improving therapeutic efficiency and reducing side effects (Illum, 1998; Jiang et al., 2004). Degree of deacetylation controls the degree of crystallinity and hydrophobicity in chitosan which in turn controls the loading and release characteristics of chitosan matrices. The bioadhesive properties of chitosan molecules reduces the rate of drug clearance thereby increasing their bioavailability (Soane et al., 1999).

Chitosan polymers and oligomers enhance the absorption of hydrophilic drug molecules across the epithelial layers. Investigations have indicated that low molecular weight chitosan is more preferred for drug delivery applications due to their increased solubility (Chiou et al., 2001; Desai and Park, 2006 a,b; Gupta and Jabrail, 2007). The polymers are used both for oral and parenteral drug delivery. It can also be used for nasal drug delivery because it in non-toxic and forms a gel like layer in aqueous environment (Sinha et al, 2004). Chitosan molecules based

microspheres, gels, nanoparticles, films, etc. are extensively investigated by various reserach groups for sustainable delivery of an array of drugs viz., antibiotics, antihypertensive agents, anticancer agents, proteins, peptide drugs and vaccines (Sinha et al., 2004; Kofuji et al., 2005; Ren et al., 2005; Saito et al., 2006; Wu et al., 2006; Jayakumar et al., 2010) .

2.7.1.2 Gene delivery

Cationic character of chitosan confers it an ability to interact with negatively charged molecules i.e. DNA and makes it a suitable non-viral vector for gene delivery. The DNA gets entrapped in the chitosan microspheres and DNA packaging is considered stable due to its interaction with chitosan moieties. It offers several advantages as compared to viral vectors viz. does not produce endogenous recombination, oncogenic effects or immunological reactions, minimal cytotoxicity and low cost preparations (Borchard, 2001; Liu and Yao, 2002). The system was first developed by Mumper et al. (1995). The molecular weight of chitosan is a key parameter to be considered in the preparation of chitosan-plasmid DNA complexes since transfection efficiency to a greater extent depends on to the molecular weight of the chitosans. High molecular weight chitosan renders very stable complexes but the transfection efficiency lower. Recent studies suggested the use of low molecular weight chitosans (MacLaughlin et al., 1998; Richardson et al., 1999; Sato et al., 2001; Romoren et al., 2003) and oligomers (Thanou et al., 2002; Koping-Hoggard et al., 2001) in gene delivery vectors to obtain higher transfection efficiency. Several combinations of molecular weight and degree of deacetylation of chitosan were investigated by Lavertu et al. (2006). They observed that chitosan with 10 kDa molecular weight and degree of deacetylation of 92 and 80 % results into high transfection efficiency.

2.7.1.3 Wound healing

Chitosan and chito-oligosaccharides (degree of polymerization, 2-7) are observed to stimulate fibroblast production by affecting fibroblast growth factor which in turn induces the formation of collagen fibers and thereby accelerating the wound healing. Key factors in the

rebuilding of tissues exerted by chitosans are an enhanced vascularization and a continuous supply of chitooligomers to the wound that stimulate correct deposition, assembly, and orientation of collagen fibrils and are incorporated into the extracellular matrix components (Howling et al., 2001; Sezer et al., 2007). The chitooligomers needed are released by the hydrolytic action of lysozyme and *N*-acetyl-α-D-glucosaminidase. A positively charged chitosan mixed with negatively charged heparin produces a stable complex that can be prepared in the form of beads, gels, fibers or films. Heparin that is ionically linked to chitosan stimulates re-epithelialization phase of wound healing (Kojima et al, 2004). Deters et al. (2008) had studied the mucin-stimulating effect of glucosamine oligosaccharides (degree of polymerization 3 and 5). Tegasorb, is one such commercially available dressing which contains chitosan particles that swell, absorbing exudate and producing a soft gel; a layer of waterproof Tegaderm film; for leg ulcers, sacral wounds, chronic wounds; reportedly superior to Comfeel and Granuflex.

2.7.1.4 Tissue engineering

Chitosan scaffolds are the new backbone of regenerative tissue engineering due to their biodegradability, porosity and low immunogenicity. Several studies have been conducted so far regarding utilization of chitosan scaffolds (Madihally and Matthew, 1999; Chatelet et al., 2001; Elder et al., 2004; Amaral et al., 2005; Zhang et al., 2007; Duarte et al., 2010; Ragetly et al., 2010). The degree of deacetylation and molecular weight were found to be critical factors for scaffold formation depending on specific cellular function. Studies conducted by Tigh et al. (2007) had further affirmed the utilization of chitosan species with relatively lower degree of deacetylation (75-85 %) for fabrication of chitosan scaffold with regular structure and uniform pores because it influences cell intrusion, proliferation and function. Mechanical strength, biodegradability, cell attachment and viability to scaffolds were observed to be dependent on degree of deacetylation but swelling ratio was independent. Chitin and chitosan tubes for nerve regeneration were synthesized by Freier et al. (2005). The compressive strength of these tubes was found to increase with

increasing degree of deacetylation. Nwe et al. (2009) synthesized scaffolds using 87 % deacetylated chitosan from *G. butleri* mycelium, which was found to have superior biological and mechanical properties than the chitosan derived from crab and shrimp shells. Injectable thermo-responsive chitosan hydrogels are currently receiving a great deal of interest for tissue engineering.

Apart from the above mentioned uses, chitosan molecules are also used for burn wound healing (Degim et al., 2002; Alemdaroglu et al., 2006) and bone healing (Jung et al., 2007b; Wang et al., 2010; Muzzarelli, 2011). Chitosan membranes are also used as artificial kidney membranes due to their high tensile strength and suitable permeability. It is also used in dentistry (Ezoddini-Ardakani et al., 2011). No allergic reactions nor any infections were detected when chitosan was used in dental application. Some of the chitosan derivatives like chitosan sulphates were observed as good anti-coagulants (Hirano, 1999; Vongchan et al., 2002; Vikhoreva et al., 2005). Chitosan fibres are explored as haemostatic material and also possess good anti-thrombogenic properties. It has also been utilized for the manufacturing of ocular bandage lenses which are used as protective devices for acutely or chronically traumatic eyes (Sinha et al., 2004).

2.7.2 Applications in Food Industry

2.7.2.1 Food Preservation

Antimicrobial activity of chitosan polymers and oligomers denotes these as ideal food preservative. Further, the use of biopolymer for preservation reduces the harmful efects of chemical preservatives on food and human health. Chitosan is approved as a food additive in Japan and Korea since 1983 and 1995, respectively (Weiner, 1992; KFDA, 1995). Chitosan has wide spectrum of activity and induces high killing rate against Gram-positive and Gram-negative bacteria (Franklin and Snow, 1981; Takemono et al., 1989). Antimicrobial activity of chitosan has been demonstrated against many bacteria i.e. *E.coli*, *S. aureus*, *L. monocytogenes*, *B. subtilis*, *P. aeruginosa* and filamentous fungi i.e. *A. niger*, *F. oxysporum*, *A. solani* (Fujita et al., 2004; Chen et al., 2006; Deng et al., 2007; Hayashi et al., 2007a,b; Ma et al., 2008; Kong et al., 2010).

One of the reasons for the antimicrobial character of chitosan is its positively charged amino group which interacts with negatively charged microbial cell membranes, leading therefore to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Shahidi et al., 1999). Chitosan also acts as a chelating agent that selectively binds to trace metals and thereby inhibiting the production of toxins and microbial growth (Cuero et al., 1991). It also activates several defence processes in the host tissue (El Ghaouth et al., 1992), acts as a water binding agent, and inhibits various enzymes. Binding of low molecular weight and water-soluble chitosan with DNA interferes with the synthesis of mRNA and proteins and hence microbial growth (Sudarshan et al., 1992). High molecular weight and solid chitosan including larger size nanopaticles interact with cell surface instead and alter cell permeability (Leuba and Stossel, 1985), or form an impermeable layer around the cell, thus blocking the transport of essential solutes into the cell (Choi et al., 2001; Eaton et al., 2008). Moreover, the antibacterial activity of chitosan is related to its degree of deacetylation, concentration and the pH of the medium (Liu et al., 2001).

2.7.2.2 Nutraceutical

Chitosan has been used in multiple nutritional suplemets due to its hypolipidemic and anti-cholesterol effect. Its polycationic character promotes binding to negatively charged lipids, hence reducing their gastrointestinal uptake (Deuchi et al., 1995) and lowering serum cholesterol (Ormrod et al., 1998). This natural "fat blocker" dissolves in the stomach and converts to a gel which "traps" fat, thereby preventing its absorption and subsequent storage. Liu et al. (2008) have reported that rats fed diets containing the deacetylated chitosan significantly lowered plasma cholesterol and LDL, and increased HDL level. *In vivo* absorption of different molecular weight chitosan in mice was studied by Zeng et al. (2008) and found that absorption of chitosan and at the same time its water solubility increases with its decreasing molecular weight. Zhang et al. (2011) had studied the hypolipidemic effect of chitosan nanoparticles in hyperlipidemic rats.

The efficacy of water insoluble, high molecular weight chitosan (650 kDa), approved by the Japanese Ministry of Health, Labour and Welfare as functional food, for preventing the increase in bodyweight and white adipose tissue weight, hyperlipidaemia and fatty liver was studied by Han et al. (1999). The effect of chitosan on its cholesterol lowering action was observed to be independent of its viscosity. However, the lipid oxidation by the chitosans of higher viscosity was greater as compared to the samples with lower viscosity (Chiang et al., 2000). Studies on animal systems showed that chitosan can reduce the serum cholesterol level when administered to (1-1.5 $gday^{-1}$) higher amounts (Shahidi et al., 1999). Several chitosan based fibre formulations are manufactured at large scale in recent years to control obesity eg. Fat Zapper (HeadStart, Florida, USA).

2.7.2.3 Edible Films

The film-forming ability of chitosan molecules makes them as an ideal alternative of the conventional packaging films (Tsai & Su, 1999; Park & Zhao, 2004; Suyatma et al., 2005). Many authors have investigated chitosan coatings for their ability to enhance the quality and extend the storage life of food products (Darmadji & Izumimoto, 1994; Rhoades & Rastall, 2000; Rabea et al., 2003; Sathivel et al., 2007; Fisk et al., 2008). As compared with other bio-based food packaging materials, chitosan has the advantage of its being antimicrobial (Chen et al., 2002; Jeon et al., 2002a; Möller et al., 2004). Antimicrobial efficacy of konjac glucomannan edible film was observed to be improved by incorporating chitosan and nisin (Li et al., 2006). Incorporation of chitosan and lauric acid into starch based film enhanced its antimicrobial activity against *B. subtilis* and *E. coli* (Salleh et al., 2007). Recently, a chitosan-starch film has been prepared using microwave treatment which could be of potential significance for food packaging (Tripathi et al., 2008 a, b).

2.7.3 Wastewater Treatment

Chitosan and its derivatives have extreme potential as biosorbents for removal of heavy metal ions i.e. Al^{+3}, Hg^{+2}, Ag^{+}, Pb^{+2} and Cd^{+2} etc. (Udaybhaskar, 1990; Deans & Dixon, 1992; Pesic

et al., 1994; Bassi et al., 2000; Varma et al., 2004; Alves and Mano, 2008; Rangel-Mendez et al., 2009) from contaminated sites. Polymer chain of chitosan consists of several –OH and $–NH_2$ groups, which donate their single-pair of electrons to empty d-trajectories of metal ions and chelate into a steady complex (–N–M–O–) (Zhang, 1979). Chitosan has binding capacity to about 1 $mmolg^{-1}$ for heavy and toxic metals, with the exception of chromium, and is more efficient in scavenging Ni^{+2} from industrial waste . Chitosan has also been effectively used for removal of Hg^{+2} from hard waters at neutral pH (Muzzarelli & Rocchetti, 1974) and also the arsenic concentration in ground water can also be substantially lowered (Elson et al., 1980). The maximum adsorption capacities of chitosan for Cd^{+2}, Cr^{+3} and Hg^{+2} were shown to be 558, 92, 1123 mg/g of chitosan, respectively, which were higher than that of other materials studied (Bailey et al., 1999).

Apart from removal of metal ions, chitosan species are extensively utilized as flocculating agents for removal of organic contaminants as well as solid suspending substances (Ishii et al., 1995, Bolto, 1995). The active amino groups ($–NH_2$) in the chitosan molecule can be protonated with H^+ in water into a cationic polyelectrolyte (Jaafari et al., 2004) for static attractions and adsorption. It has already been used in organic matter removal from wastewaters especially those resulting from food processing, thus affecting into reduced chemical oxygen demand. Glutaraldehyde cross-linked chitosans have been used for recovery of cadmium (Rorrer et al., 1993), molybdenum (Guibal et al., 2000) and platinum (Jaworska et al., 2003). Various chitosan derivatives had been formulated by several research groups in order to increase their metal sorption capacity. Zeng et al. (2007) studied superiority of a novel composite chitosan flocculant, made from chitosan, polyaluminium chloride and silicate over conventional ones. The use of chitosan based material to remove anionic dyes has been recently reviewed by Crini and Badot (2008).

2.7.4 Antioxidant

Chitosan (Xie et al., 2001; Park et al., 2004; Xing et al., 2005) and chitooligosaccharides (Park et al., 2003b; Je et al., 2004; Yang et al., 2006) have been observed to have significant

scavenging capacity against different radical species, the results being comparable to those obtained with commercial antioxidants. Chitosan from crab shells and shiitake stipes were observed to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide radical and alkyl radical (Yen et al., 2007, 2008). Ferrous ion chelating ability of chitosan makes them as an antioxidant to increase shelf life of lipid containing foods (Peng, 1998).

2.7.5 Immobilization of biocatalysts

Enzymes and whole cells can be immobilized on various inert and reactive supports to reuse these for multiple cycles with minimum change in catalytic activity (Ray and Chattopadhyay, 2006; Nigam et al., 2007; Riaz et al., 2009). Recently various research groups utilized chitosan to immobilize various industrially important enzymes viz. urease (DeGroot et al., 2001), tannase (Boadi and Neufeld, 2001), carbonic anhydrase (Simsek-Ege et al., 2002), lipase (Amorim et al., 2003) and laccase (Gary Delanoya, 2007). Enzymes can be immobilized on chitosan matrices either by adsorption or covalent binding or by encapsulation. Loading and release kinetics of enzymes depends on molecular weight and degree of deacetylation of chitosans and was observed to vary depending on the catalyst and its application (Alsarra et al., 2002; Taqieddin and Amiji, 2004).

2.7.6 Immunomodulation and antitumor activity

The immune-stimulating activity of chitosan has been reported for several decades. Nishimura et al. (1984) had first reported that chitosan (70% deacetylated) acts as an immune regulator and activate macrophages and natural killer cells and improve the delayed-type hypersensitive reaction, increase cytotoxicity and induce mitosis in cells producing interleukins and interferons. Stimulation of cytolytic T-lymphocytes by chitosan and chito-oligosaccharides are responsible for its anti-tumor potential (Suzuki et al., 1986; Tsukada et al., 1990; Ueno et al., 2001; Jeon et al., 2002b; Kim et al., 2005). Immunity-enhancing effect of chitosan can also be attributed to its ability to augment antibody response (Chang et al., 2004).

2.8 PATENTS ON CHITOSAN

Various patents had also been filed for the production and application of the chitosan, recent ones are listed below (Table 3),

Table. 3 Patents during last five years for chitosan production and application

S. No.	Patent No	Date	Title
1.	US0237811	11.10.2007	Chitosan wound dressing
2.	US0254007	01.11.2007	Chitosan/nanocrystalline hydroxyapatite composite microsphere-based scaffolds
3.	US0254294	01.11.2007	Method for storing DNA by using chitosan, and products using the methods
4.	US0281904	06.12.2007	Chitosan-derivative compounds and methods of controlling microbial populations
5.	US0214494	04.09.2008	Method of drug delivery by carbon nanotube-chitosan nanocomplexes
6.	US0242850	02.10.2008	Method of producing chitosan scaffold having high tensile strength and chitosan scaffold produced using the method
7.	US0241229	02.10.2008	Preparation method of an anti-microbial wound dressing and the use thereof
8.	US0254125	16.10.2008	Processing of chitosan and chitosan derivatives
9.	US0258839	15.10.2009	Compositions comprising chitosan suitable for comprehensive therapeutic treatment or comprehensive prevention of the metabolic syndrome
10.	US0275745	05.11.2009	Chitosan manufacturing process
11.	US0291252	18.11.2010	Method of producing ultra thin chitosan fibers and non-woven fabrics
12.	US0316739	16.12.2010	Chitosan gel for dermatological use, production method and use
13.	US0021745	27.01.2011	Ultra-small chitosan nanoparticles useful as bioimaging agents
14.	US0064664	17.03.2011	Methods and compositions involving chitosan nanoparticles
15.	US0129528	02.06.2011	Nanoparticles of chitosan and hyaluronan for the administration of active molecules

CHAPTER III

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and other materials

Various chemicals used for the study were of highest purity, analytical grade and procured from different national and international manufacturers. Chitin from crab shell, glycol chitosan, CM-sepharose, DEAE-sepharose, *N*-acetylglucosamine, chitooligosaccharides (chitobiose, chitotriose and chitopentaose), 3-methyl-2-benzothiazolinone hydrazone hydrocholoride (MBTH) and calcoflour white M2R were purchased from Sigma-Aldrich, USA. Standard protein molecular weight markers were procured from Bangalore Genei, India. Agro-horticultural residual materials viz. sugarcane bagasse, red gram husk, black gram husk, cotton hull, tea waste, wheat bran, rice bran, groundnut shell, corn residues and mustard oil cake were procured from the local market of Roorkee, India. All other chemicals were of analytical grade and purchased from standard commercial manufacturers and were of the highest grade available.

3.1.2 Microorganisms

Penicillium oxalicum SAE_M-51 was developed following mixed mutagenesis (physical and chemical) of the wild type strain *Penicillium oxalicum* SA-1 (ITCC 6965) which was isolated from the residual materials of sea food processing industry (Saubhagya Seafoods, Porbandar, Gujarat, India) and identified by the Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi. The strain was maintained on PDA (2% dextrose, 2% agar, 20% potato extract) slants, stored at 4°C and sub-cultured periodically.

3.2 METHODS

3.2.1 Isolation of strains

A total of 18 different fungal strains were isolated from the residual materials of a sea food processing industry, insect cuticle, decaying snail shell and degrading fish waste. Briefly, samples from the mentioned sites were collected and mixed with two parts of double distilled water. The suspension was centrifuged (5000 g, 10 min) at room temperature. The clear supernatant following serial dilution was inoculated onto potato dextrose agar plates. Growth was observed after 48 hours of incubation at 30 ^{0}C. On the basis of distinct morphological features, 18 pure fungal cultures were isolated. The cultures were transferred to PDA slants, incubated at 30 ^{0}C for 5 days and further stored at 4 ^{0}C for future use.

3.2.2 Screening of CDA producing strains

Primary screening of the isolated strains for CDA activity was carried out on plates containing 4-nitroacetanilide as described by Srinivasan and Vadake (1998). The screening medium contained (gl^{-1}): glucose, 10; peptone, 5; yeast extract, 3; 4-nitroacetanilide, 0.5; agar, 20 and was sterilized by autoclaving (15 psi, 15 min) and inoculated with isolated pure cultures by point inoculation method. Plates were then incubated at 30 ^{0}C for 5-6 days. Enzymatic deacetylation of 4-nitroacetanilide had resulted in the production of yellow colored 4-nitroaniline in the plate. Control plates did not show yellow color development. All fungal isolates were further screened quantitatively using UV-visible spectrophotometry for their CDA production ability. Isolates were grown in YPG medium which contained (gl^{-1}): glucose, 10; peptone, 5; yeast extract, 3 (pH 6.0) for 144 hrs (200 rpm) and culture supernatant was used for the estimation of extracellular enzyme activity.

3.3 MUTAGENESIS AND SCREENING

P. oxalicum SA-1 was genetically modified through mutagenesis to obtain improved levels of enzyme production. The schematic representation of the step-wise mutagenesis and screening is shown in Fig. 3.

3.3.1 Single-step mutagenesis

3.3.1.1 *Physical mutagenesis*

Physical mutagenesis was performed using microwave irradiation according to Xia et al. (2007). Spore suspension (3.6 x 10^6 spores ml^{-1}) of five days old culture of *P. oxalicum* SA-1 was prepared in sterile saline (0.85 %) and was exposed to microwave irradiation (2450 MHz, 650 W) for different time periods ranging from 10 to 60 sec. Following serial dilutions, treated spore suspensions were inoculated on potato dextrose agar plates to check their survivability. A parallel control was also maintained except that microwave exposure was not performed.

3.3.1.2 *Chemical mutagenesis*

3.3.1.2.1 *Ethidium bromide treatment*

Mutagenesis of *P. oxalicum* SA-1 was also carried out using ethidium bromide (EtBr) as described by Chand et al. (2005). For mutagenesis, fungal spores (3.6 x 10^6 spores ml^{-1}) obtained from five days old culture, were suspended in sterile saline (0.85 %) and treated with ethidium bromide for different time periods (200 µg ml^{-1}, 10-60 min) followed by washing with distilled water. Survivability and frequency of mutants was determined on PDA plates. Control spore suspension was also treated identically excluding EtBr.

3.3.1.2.2 *Ethyl methane sulfonate treatment*

Mutagenesis of *P. oxalicum* SA-1 was also carried out using ethyl methane sulfonate (EMS) as described by Chand et al. (2005). Briefly, a spore suspension of five days old grown culture of *P. oxalicum* SA-1 was prepared in sterile saline solution (0.85 %). The spores (3.6 x 10^6 spores ml^{-1}) were treated with 200 mM of EMS in saline solution for different time durations (10-120 min). Following treatment, spores were washed twice with distilled water, plated on PDA medium to determine survivability and frequency of mutants. A control spore suspension was also identically treated, excluding the use of EMS.

3.3.2 Mixed mutagenesis

Among the mutants that were obtained from Ist stage of mutation, strain SAE-50, obtained following EtBr treatment had shown comparatively greater increase in CDA activity and was selected for second stage of mutagenesis to determine if further improvement in enzyme production levels can be achieved. SAE-50 was successively treated with EtBr, EMS and was also subjected to microwave irradiation in three different sets of experiments with simultaneous determination of enzyme activity.

3.3.3 Mutant isolation and screening

Rapid screening of mutants for their enhanced CDA production ability was performed on YPG agar medium with 4-nitroacetanilide as an indicator compound as described earlier. The colonies showing yellow color development were selected to get their pure culture and further subjected to quantitative estimation of CDA activity spectrophotometrically.

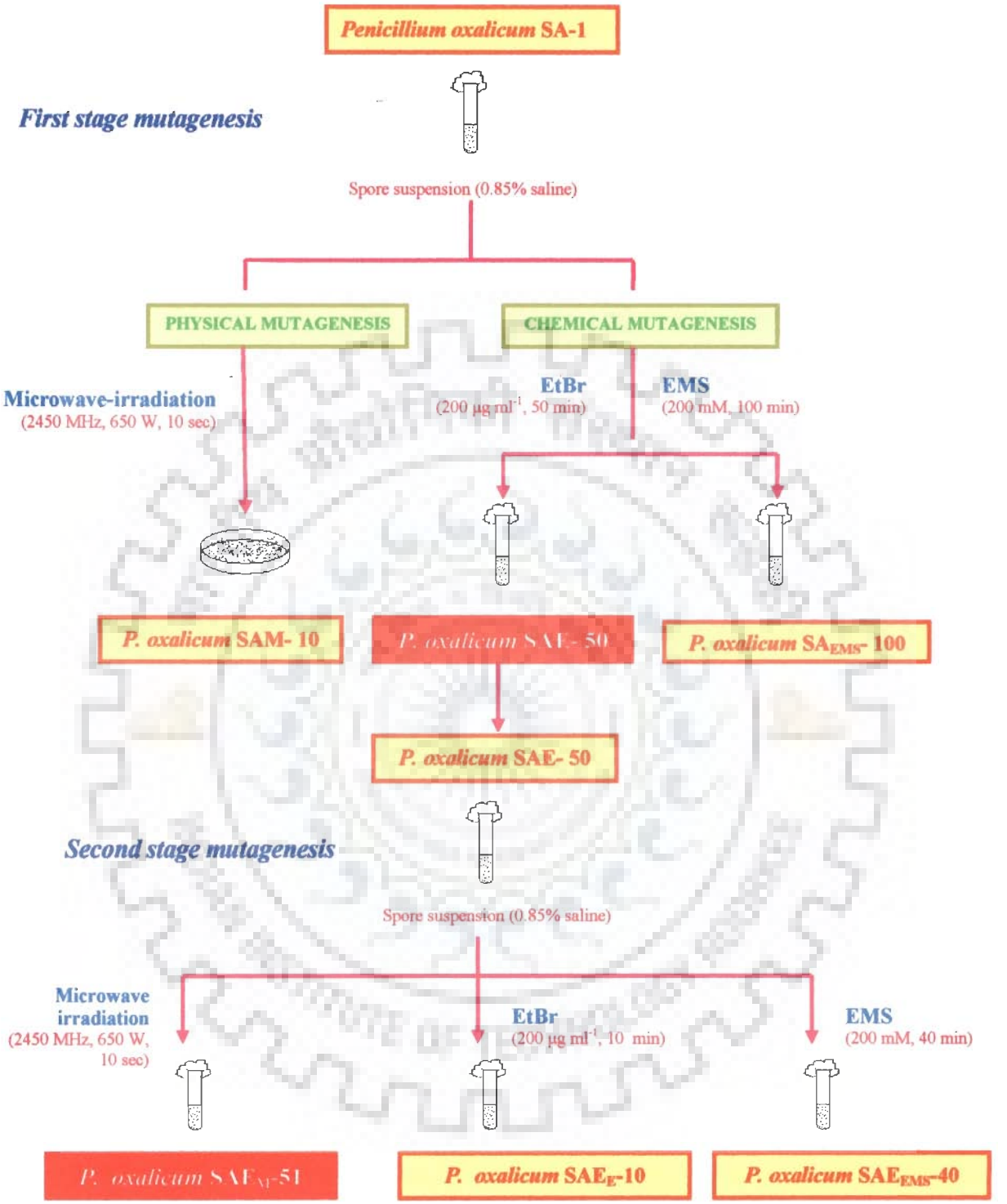


Fig. 3 Mutagenesis scheme for *P. oxalicum* SA-1

3.4 FERMENTATION

The preliminary studies of fermentation for CDA production were carried out as described (Nahar et al., 2004). The composition of the medium used for CDA production was (gl^{-1}): Glucose, 10; yeast extract, 3.0; peptone, 5.0; KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5 and $CaCl_2.2H_2O$, 0.5. The pH of the medium was adjusted to 6.0 and then autoclaved (121 ^{0}C, 15-20 min, 15 psi).

3.4.1 Submerged fermentation

Submerged fermentation was carried out in 50 ml of defined medium in Erlenmeyer flasks (250 ml). Medium was inoculated with mutant *P. oxalicum* SAE_M-51 following incubation at 30°C in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (200 rpm). The fermentation was carried out for 8 days and samples for the enzyme activity were taken intermittently. Following completion of the process, broth was centrifuged (8000 g, 10 min) and used for estimation of enzyme activity.

3.4.2 Surface fermentation

Medium (50 ml) following inoculation with mutant *P. oxalicum* SAE_M-51 as mentioned above, were kept in an incubator at 30 ^{0}C under static conditions. The fungal mycelia were allowed to grow on the surface of the medium in the form of a mycelial mat with occasional shaking to maintain the pH of the medium. The fermentation was carried out for 8 days and samples for the enzyme activity measurement were withdrawn at desired time intervals. Following completion of fermentation, broth was centrifuged (8000 g, 10 min) and used for enzyme assays.

3.4.3 Solid-state fermentation

Different agro-horticultural residual materials i.e. sugarcane bagasse, red gram husk, black gram husk, cotton hull, tea waste, wheat bran, rice bran, groundnut shell, corn residues and mustard oil cake were washed thoroughly in the double distilled water to remove the residual dust, pulverized and utilized as the substrates. Oven dried solid substrate (5 g) was taken in Erlenmeyer flask (250 ml) and moistened with fermentation medium so as to maintain the

appropriate moisture level. Slurry of the fermentation medium containing solid support was autoclaved and inoculated with *P. oxalicum* SAE_M-51 and incubated statically at 30 ^{0}C for 8 days. Waste fermentation gases generated, were removed by connecting the fermentation flasks to a water suction pump and the fresh air (1.5-2.0 l min^{-1}) was circulated over the mycelia.

Following completion of the process, enzyme was extracted from the fermentation system with appropriate volume of assay buffer. The extraction of the enzyme was carried out on a rotary shaker (200 rpm) at 4 ^{0}C for 1 hr. The slurry was squeezed through cheese cloth and the extract was clarified by centrifugation (12,000 g, 4 ^{0}C, 15 min) to remove the suspended particles and clear supernatant thus obtained was used as enzyme source.

3.4.4 Statistical optimization for CDA production

3.4.4.1 ***Plackett-Burman design***

Extent of effect of eleven variables (including eight nutritional viz. glucose, peptone, yeast extract, K_2HPO_4 : KH_2PO_4 (1:3), $(NH_4)_2SO_4$, $MgSO_4$, NaCl and $CaCl_2$ and three dummy variables) on CDA production by *P. oxalicum* SAE_M-51 under submerged condition was evaluated by Plackett-Burman design (PBD) in twelve experiments (N = k+1; k is the number of variables) (Plackett and Burman, 1946). Effect of each variable was studied at two levels, high and low, denoted by (+1) and (-1) signs, respectively (Table 4). The difference between the two values was taken large enough to ensure that the peak area for highest enzyme production is included. The Plackett-Burman experimental design matrix for all the eight nutritional variables along with three dummy variables is given in Table 5. All the experiments were conducted in triplicates. The statistical software package "Design Expert Version 7.0", Stat-Ease Inc., Minneapolis, USA was used for analyzing the experimental data.

3.4.4.2 ***Central composite design***

Optimization of the most significant nutritional variables, identified by PBD and four independent culture variables viz. pH, temperature, inoculum age and size on submerged fermentation was carried out by central composite design (CCD) for enhancing CDA

production. All the variables were studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$, where $\alpha = 2^{k/4}$, k is the number of variables) (Tables 6, 7) in a set of 30 experiments that including eight axial points, sixteen factorial points and six center points (Tables 8, 9).

For solid-state fermentation, three variables i.e. amount of substrate, moisture content and inoculum size at five different levels (Table 10) were optimized in a set of 20 experiments (Table 11). The same software package as used for PBD was used for analyzing the experimental data. All experiments were conducted in triplicates and the mean value of CDA activity (UI^{-1}) was taken as the response (Y). Quadratic regression analyses of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The behavior of the system was explained by the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i,j=1}^{n} \beta_{ij} X_{ij}$$

where Y is the predicted response, β_0 is intercept, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} squared coefficient, β_{ij} is the ij^{th} interaction coefficient. Optimum levels of the independent variables were determined by solving the regression equation and analyzing the response surface contour plots.

3.4.4.3 Validation of the experimental model

Validation of the model and regression equation was performed under the conditions predicted by the model. CDA production was carried out under submerged/ solid-state conditions. Samples were drawn at desired intervals and analyzed for CDA production as described earlier.

Table 4 Range of different nutritional variables for the Plackett-Burman design

Variables	Medium components	Levels (gl^{-1})	
		-1	+1
X_1	Yeast extract	1.50	4.50
X_2	Peptone	3.00	7.00
X_3	Glucose	6.00	14.00
X_4	$(NH_4)_2SO_4$	0.80	2.00
X_5	$MgSO_4 . 7H_2O$	0.40	1.00
X_6	K_2HPO_4:KH_2PO_4 (1:3)	2.00	6.00
X_7	NaCl	0.30	0.70
X_8	$CaCl_2. 2H_2O$	0.30	0.70

Table 5 **Plackett-Burman experimental design matrix for analysis of major nutritional variables for CDA production under submerged fermentation**

Experiment No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Table 6 **Range of concentrations of four independent nutritional variables used in CCD**

Variable	Component	Levels (gl^{-1})				
		$-\alpha$	-1	0	+1	$+\alpha$
X_1	Yeast extract	0.00	1.50	3.00	4.50	6.00
X_2	Peptone	1.00	3.00	5.00	7.00	9.00
X_4	$(NH_4)_2SO_4$	0.20	0.80	1.40	2.00	2.60
X_5	$MgSO_4.7H_2O$	0.10	0.40	0.70	1.00	1.30

$\alpha = 2^{k/4}$, where k = 4 represents no. of variables, k = 4, $\alpha = 2$

Table 7 Experimental design matrix for analysis of nutritional variables for CDA production under submerged condition using CCD

Run	X_1	X_2	X_4	X_5
1	+1	+1	+ α	+1
2	0	0	0	0
3	0	0	- α	0
4	-1	-1	-1	-1
5	-1	+1	-1	-1
6	-1	-1	+1	+1
7	0	0	+ α	0
8	+1	-1	-1	+1
9	0	0	0	0
10	+α	0	0	0
11	0	0	0	0
12	0	0	0	- α
13	-1	+1	+1	-1
14	0	0	0	0
15	-1	-1	+1	-1
16	+1	-1	+1	+1
17	0	0	0	+ α
18	0	0	0	0
19	+1	+1	-1	-1
20	+1	0	-1	-1
21	+1	+1	-1	+1
22	-1	+1	-1	+1
23	+1	+1	+1	-1
24	0	+ α	0	0
25	- α	0	0	0
26	-1	+1	+1	+1
27	0	- α	0	0
28	-1	-1	-1	+1
29	+1	-1	+1	-1
30	0	0	0	0

Table 8 Concentration ranges and corresponding coded levels of variables used in CCD to evaluate culture parameters

Variable	Component	Range studied	Coded levels				
			-α	-1	0	+1	+α
X_1	pH	4.00-8.00	4.00	5.00	6.00	7.00	8.00
X_2	Temperature (^{0}C)	20.00-40.00	20.00	25.00	30.00	35.00	40.00
X_3	Inoculum age (hour)	72.00-196.00	72.00	96.00	120.00	144.00	168.00
X_4	Inoculum size (%, v/v)	1.00-13.00	1.00	4.00	7.00	10.00	13.00

$\alpha = 2^{k/4}$, $k = 4$, $\alpha = 2$

Table 9 Central Composite design matrix for derivation of culture conditions for chitin deacetylase production under submerged condition

Run	X_1	X_2	X_3	X_4
1	-1	-1	-1	-1
2	- 1	- 1	-1	+1
3	-1	-1	+1	-1
4	-1	-1	+1	+1
5	-1	+1	-1	-1
6	-1	+1	-1	+1
7	-1	+1	+1	-1
8	-1	+1	+1	+1
9	+1	-1	-1	-1
10	+1	-1	-1	+1
11	+1	-1	+1	-1
12	+1	-1	+1	+1
13	+1	+1	-1	-1
14	+1	+1	-1	+1
15	+1	+1	+1	-1
16	+1	+1	+1	+1
17	+ α	0	0	0
18	- α	0	0	0
19	0	+ α	0	0
20	0	- α	0	0
21	0	0	+ α	0
22	0	0	- α	0
23	0	0	0	+ α
24	0	0	0	- α
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0

Table 10 Ranges of concentration of independent variables used in CCD for enhanced production of CDA under solid-state fermentation

Variable	Component	Range studied	Levels				
			-α	-1	0	+1	+α
X_1	Amount of substrate (g)	2.00-10.00	2.00	4.00	6.00	8.00	10.00
X_2	Moisture content (%)	40.00-120.00	40.00	60.00	80.00	100.00	120.00
X_3	Inoculum size (ml)	1.00-13.00	1.00	4.00	7.00	10.00	13.00

$\alpha = 2^{k/4}$, k = 3, α = 1.638

Table 11 Experimental design matrix for analysis of CDA production under solid-state fermentation using CCD

Run	X_1	X_2	X_3
1	-1	-1	-1
2	0	0	-α
3	-1	-1	+1
4	+1	+1	-1
5	+1	-1	+1
6	0	0	0
7	+α	0	0
8	+1	-1	-1
9	0	0	0
10	0	0	0
11	-1	+1	+1
12	-α	0	0
13	+1	+1	+1
14	0	+α	0
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	+α
19	0	-α	0
20	-1	+1	-1

3.5 Purification of CDA

The crude extracellular enzyme preparation was subjected to three-step purification comprised of ultrafiltration, cation and the anion exchange chromatography.

3.5.1 *Ultrafiltration*

The culture broth was centrifuged at 8,000 g for 10 min. The cell free crude enzyme supernatant thus obtained was ultrafiltered using Amicon ultra-centrifugal filter unit (10 kDa molecular weight cut-off, Millipore). Concentrated CDA obtained from ultrafiltration was assayed for CDA activity and protein content as described above and used for further purification.

3.5.2 *Ion exchange chromatography*

Concentrated enzyme thus obtained was loaded on to a borate buffer (100 mM, pH 9.0) equilibrated CM- sepharose (Sigma-Aldrich Co., USA) column. The same buffer with a sodium chloride gradient (50 - 500 mM) was used for elution of protein with a flow rate of 1.0 ml min^{-1}. Fractions (2 ml) were collected and analyzed for CDA activity and the protein content as described above. CDA active fractions were pooled and concentrated, and then purified using DEAE- sepharose (Sigma-Aldrich Co., USA) column as described above. The active fractions were pooled and stored for further studies.

3.6 BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

3.6.1 Determination of optimal pH, temperature and stability

The optimal pH for purified CDA was detected by analyzing its activity over a pH range of 4.0-11.0 using the following buffers: 100 mM citrate buffer (4.0-6.0), 100 mM borate buffer (7.0-9.0) and 100 mM carbonate-bicarbonate buffer (10.0-11.0). The stability at varying pH of the enzyme was determined by pre-incubating the purified enzyme in the buffers described above for 24 hrs at 4 ^{0}C and subsequently analyzing the residual activity under standard assay conditions.

The optimum temperature was determined by assaying the CDA activity at various temperatures (50^0 - 80 ^{0}C) for 30 min. The thermostability of enzyme was determined by pre-incubating the enzyme at various temperatures ranging from 50^0 - 80 ^{0}C. Aliquots were withdrawn at various time intervals, cooled and assayed under standard conditions.

3.6.2 Thermodynamics of enzyme denaturation

The activation energy ($E_{a,d}$) of the purified CDA was calculated from the slope of Arrhenius plot ($\ln k_d$ vs 1/T). Free energy (ΔG_d^*), enthalpy (ΔH_d^*) and entropy (ΔS_d^*) of activation for denaturation of the enzyme was calculated using following equations (i), (ii), (iii), respectively. Half-life ($t_{1/2}$) of the enzyme was calculated according to equation (iv).

$$\Delta G_d^* = -RT \ln (k_d h / k_b T) \quad \text{(i)}$$

$$\Delta H_d^* = E_{a,d} - RT \quad \text{(ii)}$$

$$\Delta S_d^* = (\Delta H_d^* - \Delta G_d^*) / T \quad \text{(iii)}$$

$$t_{1/2} = \ln 2 / k_d \quad \text{(iv)}$$

where, R is the gas constant, h is the Planck constant, k_b is the Boltzmann constant, T is the absolute temperature and k_d is the first-order rate constant calculated from the slope of the regression line obtained by plotting ln relative activity (%) vs time at different temperatures.

3.6.3 Determination of V_{max}, K_m, K_{cat} and K_{cat} / K_m values

The kinetic parameters of the purified CDA were determined by measuring the enzyme activity at different concentrations of *N*-acetyl glucosamine, chitin oligomers (chitobiose, chitotriose, chitopentaose) and glycol chitin. The kinetic constants were then estimated using linear regression plots of Linweaver and Burk.

3.6.4 Effect of various metal ions and EDTA on CDA activity

Effect of various mono and bi-valent metal ions (K^+, NH_4^+, Ca^{+2}, Cd^{+2}, Co^{+2}, Cu^{+2}, Zn^{+2}, Mn^{+2}, Mg^{+2}, Fe^{+2}, Ni^{+2}, Li^{+2}, Bi^{+2}, Ba^{+2}, Mo^{+2}, Pb^{+2}) and EDTA on the CDA activity was determined by pre-incubating the purified enzyme in the 100 mM borate buffer (pH 9.0) added

with an ion (1 mM). After 1 hr of incubation, residual activity was measured under standard conditions. The activity assayed in the absence of metal ions was recorded as 100 %.

3.6.5 Acetate inhibition studies

Effect of acetate on CDA activity was determined by assaying the enzyme activity in the presence of various concentrations of acetate (10-100 mM) under standard assay conditions.

3.6.6 Sodium-dodecylsulphate polyacrylamide gel electrophoresis

SDS-PAGE of the samples was performed according to Laemmli (1970). Polyacrylamide gels (10%) of 1.5 mm thickness were prepared for electrophoretic analysis.

3.6.6.1 *Reagents*

Solution A: Acrylamide solution 29.0 % (w/v) containing 1.0 % (w/v) bisacrylamide (N, N'-methylene-bisacrylamide)

Solution B: Resolving buffer, 1.5 M Tris-buffer, pH 8.8

Solution C: Stacking buffer, 1.0 M Tris-buffer, pH 6.8

Solution D: SDS 10 % (w/v)

Solution E: Ammonium persulfate 10 % (w/v) (freshly prepared)

Solution F: TEMED (N, N, N', N'-tetramethylethylenediamine)

Electrophoresis buffer, 0.02 M Tris, 0.2 M glycine, pH 8.3

Preparation of resolving and stacking gels

Reagents	Resolving gel (10 %) (ml)	Stacking gel (4 %) (ml)
Solution A	3.3	0.67
Solution B	2.5	-
Solution C	-	0.5
Solution D	0.1	0.04
Solution E	0.1	0.04
Solution F	0.004	0.004
Water	4.0	2.7
Total Volume (ml)	10.0	4.0

3.6.6.2 *Casting of gel*

A sandwich of glass plates (8 x 7 cm) was assembled by plastic spacers (1.5 mm) and the plates were held together by plastic clamps. The base of the gel mould was sealed. Resolving gel mixture was prepared by mixing all the components except ammonium persulfate and TEMED. The mixture was degassed for 15 min and ammonium persulfate and TEMED were added to it. The mixture was gently mixed and poured carefully between the plates, leaving the required space at the top for pouring the stacking gel. After polymerization of the resolving gel, the assembly was tilted to pour off the top layer, washed with sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly degassed and layered on the resolving gel; comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

3.6.6.3 *Sample preparation*

Fractions containing proteins were concentrated using Amicon ultra-centrifugal filter unit (10 kDa molecular weight cut-off, Millipore) and added with SDS-PAGE sample buffer containing Tris-HCl (0.125 M, pH 6.8), 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) β-mercaptoethanol, 0.004 % (w/v) bromophenol blue. Samples were heated in a boiling water bath for 3-5 min and loaded onto gel for electrophoresis.

3.6.6.4 *Electrophoresis*

Samples (appropriate concentration of the purified protein) were gently loaded on the gel using a microsyringe. Electrophoresis was carried out at constant voltage (100 V) until the tracking dye reached about 1 cm above the bottom of the gel. After electrophoresis, the gel was removed and stained overnight with gentle shaking in Coomassie brilliant blue R-250 (0.1 %) in methanol, glacial acetic acid and water in a ratio of 5:2:5 (v/v/v) at room temperature. The gel was destained by using the methanol: acetic acid: water (30:10:60) solution.

3.6.6.5 *Zymogram analysis*

Native polyacrylamide gel electrophoresis (PAGE) using 10 % gel was performed for visualization of enzyme activities in situ as described (Trudel and Asselin, 1990). After electrophoresis, gel was incubated in borate buffer (100 mM, pH 9.0) for 5 min at room temperature. The gel was then put on a clean glass plate and was covered with a 7.5 % (w/v) polyacrylamide overlay gel containing 0.1% glycol chitin. Appropriate contact between the two gels was ensured by gently rolling a test tube on top of the overlay gel which was still wet with buffer. Gels were incubated at 50 ^{0}C for 4-5 hrs in plastic containers under moist conditions. After incubation, gels were transferred in a fresh solution of 0.01 % (w/v) Calcofluor white M2R in 0.5M tris-HCl (pH 9.0). After 5 min, gels were removed and washed with several changes in distilled water for at least 2 hrs. Protein exhibiting CDA activity was visualized under UV light. The band with CDA activity appeared as bluish-white bands and was more fluorescent than the background fluorescence. Chitosan generated after enzymatic deacetylation was depolymerized by exposure to nitrous acid for 10 min, gel was then washed with distilled water and stained with Calcofluor white M2R as described previously.

3.6.7 Circular dichroism spectroscopy

Circular dichroism (CD) spectral measurements were performed on a Spectropolarimeter (Chirascan, Applied Photophysics, UK). The entire instrument, including the sample chamber, was constantly flushed with N_2 gas during the operation. Far-UV CD spectra (190–240 nm), were recorded in 1 cm path length quartz cell at a protein concentration of 1.0 mg ml^{-1} in buffers (50 mM) of different pH values (HCl–KCl, pH 2; glycine–HCl, pH 4; sodium phosphate, pH 6–8; and glycine–NaOH, pH 9–12). Buffer scans were recorded under the same conditions and subtracted from the protein spectra before further analysis. The results of all the CD measurements were expressed as mean residue ellipticity $[\theta]_\lambda$ in deg cm^2 dmol^{-1} using the relation: $[\theta]_\lambda = \theta_\lambda M_0 / 10cl$, where θ_λ is the observed ellipticity in millidegrees at wavelength λ, M_0 is the mean residue weight of the protein (M_0 = 110), c is the protein concentration (mg cm^{-3})

and *l* is the path length (cm). The percentage of different secondary structures (α- helix, β- sheet, turn and random coil) were estimated using K2D2 software (Greenfield, 2007).

3.6.8 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF/ToF) analysis

For determination of partial internal sequence through mass spectrometry, the purified protein was first electrophoresed using SDS-PAGE (10 %) and was carefully excised. The excised protein band was analyzed by MALDI-ToF/ToF (Bruker Daltonics Ultraflex ToF/ToF) at proteomics facility of The Center for Genomic Application (TCGA), Okhla Industrial Estate, New Delhi, India. The peptide fragments obtained were analyzed with the Flex Analysis Software and database homology search for protein identification was carried out using short sequence BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information).

3.7 ANALYTICAL METHODS

3.7.1 Estimation of CDA activity

CDA activity was estimated using acetylated ethylene glycol chitosan as the substrate which was prepared as described by Araki and Ito (1975). Briefly, ethylene glycol chitosan (EGC, 40 mg) was treated with a solution containing sodium bicarbonate (400 mg) and acetic anhydride (4.5 ml) and kept at 4 ^{0}C for 24 hrs. Following incubation, 200 µl of acetic anhydride was added and the mixture was further incubated at 4 ^{0}C for 24 hrs. It was then dialyzed against deionized water and acetylated ethylene glycol chitosan (1 mg ml^{-1}) thus recovered was used as a substrate for assay of CDA.

The CDA was assayed as described (Kauss and Bausch, 1988). Briefly, the assay mixture in a total volume of 250 µl contained 100 µl of acetylated ethylene glycol chitosan (1 mg ml^{-1}) and 100 µl of 50 mM sodium tetraborate buffer (pH 8.5) along with suitable aliquot of the purified enzyme. The mixture was incubated at 37 ^{0}C for 30 min with constant shaking. The reaction was terminated by the addition of 250 µl of potassium hydrogen sulfate (5 %). For color

development, 250 µl of 5 % sodium nitrite was added, allowed to stand for 15 min and then followed by addition of 250 µl of 12.5 % ammonium sulfamate. After 5 min, 250 µl of freshly prepared 3-methyl-2-benzothiazolinonehydrazone hydrochloride (0.5 %) was added and the mixture was heated in boiling water bath for 3 min. The tubes were cooled and 250 µl of 0.5 % ferric chloride was added. Absorbance was read at 650 nm after 30 min. One unit of enzyme was defined as the activity that released 1 µmol of acetate per minute.

3.7.2 Estimation of sugar consumption

Sugar was estimated by the dinitrosaliyclic (DNS) acid method (Miller, 1959). One ml of broth supernatant was added with 1.0 ml of DNS reagent. The tubes were placed in boiling water bath for 5 min, allowed to cool and the absorbance was read at 546 nm.

3.7.3 Protein estimation

Protein was estimated as described by Bradford (1976) using bovine serum albumin as a standard.

3.7.4 Biomass estimation

Growth was measured by determining the dry cell weight. Dry biomass was obtained by filtering the samples and drying the mycelia to constant weight at 75 ^{0}C (Oliveira et al., 2006).

3.7.5 Analysis of kinetic parameters

Kinetic parameters for product formation and substrate consumption were analyzed using the batch fermentation process (Pirt, 1975).

3.7.6 Determination of acetic acid concentration

Acetic acid concentration was determined with analytical kit procured from Boehringer Mannheim GmbH (Germany) (Cat. No 148261). Briefly, 1 ml of the assay solution 1 (L-malic acid, $MgCl_2$, triethanolamine buffer (pH 8.4)) and 200 µl of the solution 2 (ATP, coenzyme A, NAD) was mixed with appropriate volume of the sample solution. The absorbance (A_0) was then recorded at 365 nm. Next, 10 µl of solution 3 (L-malate dehydrogenase, citrate synthase) was added to the reaction mixture and absorbance (A_1) was recorded. Then 20 µl of solution 4

(acetyl-CoA synthetase) was added to the reaction mixture and read the absorbance (A_2) at 365 nm. The acetic acid concentration was then determined after calculating the absorbance differences.

3.8 ENZYMATIC DEACETYLATION OF CHITIN

3.8.1 Enzyme production

The mutant *P. oxalicum* SAE_M-51 was used for CDA production under solid-state fermentation. The enzyme was further purified using ion exchange chromatography before using for deacetylation.

3.8.2 Deacetylation of chitin

Crystallinity of chitin is an important barrier to its enzymatic deacetylation. A two-stage chemical and enzymatic deacetylation process was followed to achieve higher degree of deacetylation (DDA). The commercial crab shell chitin, procured from Sigma-Aldrich Co., USA, was subjected to different chemical treatments, as described below in order to decrease its crystallinity and also to improve the substrate accessibility.

3.8.2.1 *Phosphoric acid treated chitin (PA-CT)*

Commercial chitin was treated with phosphoric acid (45 %) at room temperature for 40 min and filtered through cheese cloth. Chitin was then precipitated by adding 6 M NaOH to pH 8.0. Afterwards, the resultant precipitate was washed several times with distilled water to attain neutral pH and freeze-dried.

3.8.2.2 *Amorphous chitin (Am-CT)*

Chitin was treated with a cold solution consisting of NaOH (20 %) and sodium dodecyl sulphate (SDS, 0.2 %). It was then allowed to swell for an hour at 4 ^{0}C. Chitin slurry was kept overnight at -20 ^{0}C and neutralized with 6 N HCl. Precipitate was then collected by filtration, successively washed with ethanol, water, ethanol and acetone and freeze-dried.

3.8.2.3 *Colloidal chitin (Co-CT)*

Crab shell chitin was allowed to react with HCl (20 %) with constant agitation for 20 min at room temperature. Solution was filtered with glass wool in prechilled distilled water with constant mixing and allowed to settle. A dense white precipitate was formed, which was centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. Precipitate was washed several times with cold distilled water until the pH reached to neutrality. Supernatant was discarded and colloidal chitin was freeze dried overnight following freezing of the sample in liquid nitrogen.

3.8.2.4 *Superfine chitin (SF-CT)*

Chitin was dissolved in methanol (100 ml) with calcium chloride dihydrate (60 %, w/v) overnight. Dissolved chitin was precipitated using 1 % calcium citrate solution. The precipitate was then dialysed for about 12 hrs and the resultant chitin was dried overnight at 50 ^{0}C in a vaccum oven. Superfine chitin thus obtained was then treated with formic acid (25 %) at room temperature and dried overnight.

3.8.2.5 Enzymatic deacetylation

The deacetylation potential of *P. oxalicum* SAE_M-51 CDA for the above chitin samples was evaluated by treating the chitin samples with purified enzyme preparation. Commercial crab shell chitin and the above mentioned chemically modified chitin substrates were treated with purified CDA for 24 hrs at 50 ^{0}C. The reaction was then analyzed for the amount of acetate released and the degree of deacetylation of the product. The higher is the amount of acetate released the more effective would be the enzymatic deacetylation. Among the aforementioned substrates, the one that was observed to have maximum level of deacetylation was selected for further studies to derive the enzyme dose and substrate amount using response surface methodology (Tables 12, 13) to achieve maximum deacetylation.

Table 12 Range of concentrations of variables analyzed for analysis of enzymatic deacetylation of chitin using CCD

Variable	Component	Levels (gl^{-1})				
		-α	**-1**	**0**	**+1**	**+α**
X_1	Amount of substrate (g)	1.00	2.00	3.00	4.00	5.00
X_2	Enzyme dose (U)	20	40	60	80	100

$\alpha = 2^{k/4}$, k = 2, α = 1.414

Table 13 Central composite design matrix for analysis of enzymatic deacetylation of chitin

Run	X_1	X_2
1	-1	-1
2	+1	-1
3	-1	+1
4	+1	+1
5	- α	0
6	+ α	0
7	0	- α
8	0	+ α
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0

3.9 CHARACTERIZATION OF CHITINOUS SUBSTRATES

3.9.1 Scanning electron microscopy

The detailed morphological studies of fungal strains were carried out using scanning electron microscopy (SEM, Leo 435 VP, UK). The samples were taken and subjected for fixation using 3 % (v/v) glutaraldehyde and 2 % (v/v) formaldehyde (4:1) for 24 hrs. Following primary fixation, these were washed thrice with double distilled water and then treated with the alcohol gradients of 30 %, 50 %, 70 %, 80 %, 90 % and 100 % for dehydration. Samples were kept for 15 min each up to 70 % alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with 100 % alcohol, samples were air dried and examined under SEM using a gold shadowing technique (Gabriel, 1982). Electron photomicrographs were taken at desired magnifications.

3.9.2 Fourier-transform infrared spectroscopy

The degree of deacetylation of treated and untreated chitin samples were determined using fourier transform infrared spectroscopy (FTIR). An aliquot of the sample was mixed with potassium bromide, compressed into pellets and used to record an infrared spectrum using FT-IR spectrophotometer (Perkin-Elmer 1600). The absorbances at 1655 cm^{-1} (amide-I) and at 3450 cm^{-1} (OH group) were used to calculate the DDA according to the following equation (Khan et al., 2000).

DDA (%) = $[1-(A_{1655}/A_{3450})/ 1.33] \times 100$.

Factor 1.33 represents the ratio of A_{1655}/A_{3450} for fully N-acetylated chitosan.

3.9.3 Potentiometric titration

The degree of deacetylation of chitosan obtained following treatment was determined using potentiometric titration. Substrate (0.025 g) was dissolved in HCl solution (0.1 M, 25 ml) and excess HCl was back titrated with NaOH solution (0.05 M). pH meter was used for pH measurements under continuous stirring. The differential and integral titration curves were drawn between solution pH and volume of alkali added and a curve with two inflection points

was obtained. The differential volume of NaOH solution between first and second neutralization point corresponds to the acid consumed for salification of the amine groups of chitosan and allows the determination of DDA of chitosan using following equation.

DDA (%) = $(203Q/ 1 + 42Q)$

$Q = N\triangle V/ m$

where, m is the weight of chitosan sample, N is the strength of NaOH solution used in titration and $\triangle V$ is the volume of NaOH solution between the two inflection points.

3.9.4 Elemental analysis

The degree of deacetylation of the treated and untreated chitin samples was further verified by elemental analysis using EDAX (Quanta 200F, FEI, Netherlands) according to the following equation (Kassai et al., 2000)

DDA (%) = $(1- (C/N) - 5.145/ 6.816 - 5.145) \times 100$

where, C/ N is the percent ration of carbon and nitrogen.

3.9.5 Thermal analysis

The thermal properties of treated and untreated chitin samples were measured by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) (Perkin Elmer, Pyris Diamond, USA). For the TGA and DSC analyses, the samples (10.0 mg) were placed in an aluminum cup and hermetically sealed. Afterwards, samples were heated from 25^0 - 600 ^{0}C at a heating rate of 20 ^{0}C min^{-1} under nitrogen atmosphere (10 ml min^{-1}) (Mao et al., 2004). Enthalpy ($\triangle$ H), onset (T_0), peak (T_p) and completeion (T_c) temperatures were computed automatically.

3.9.6 X-ray diffraction

The X-ray diffraction measurement was utilized to determine the crystallinity of treated and untreated chitin samples and their patterns were recorded using a X-ray diffractometer (Bruker AXS D8) with Cu radiation (40 kV, 30 mA). The 2θ angle was scanned between 5^0 and 40^0 with scanning speed of 0.20 s^{-1}. The crystalline index (%) of the samples was determined by using following equations (Focher et al., 1990).

$CrI_{020} = [(I_{020} - I_{am})/ I_{020}] \times 100$

$CrI_{110} = [(I_{110} - I_{am})/ I_{110}] \times 100$

where, I_{020} is the maximum intensity at $2\theta \approx 9^0$, I_{am} is the intensity of amorphous diffraction at $2\theta \approx 16^0$ and I_{110} is the maximum intensity at $2\theta \approx 20^0$.

CHAPTER IV

RESULTS

Chapter 4

RESULTS

4.1 ISOLATION AND SCREENING OF A POTENT MICROBIAL STRAIN

Overall eighteen fungal strains were isolated from different sites viz. residual materials from sea food processing industry, insect cuticle, decaying snail shell and degrading fish waste. Fungal colonies were isolated on the basis of morphological appearance i.e. mycelial and spore color (Table 14). These strains were primarily screened qualitatively for their CDA production ability on the basis of yellow color development on 4-nitroacetanilide containing YPG agar plates. All the colonies were further screened quantitatively for CDA production (Table 15). Out of eighteen strains, six isolates i.e. SA-1, SA-2, SA-4, SA-5, SB-2 and SD-1 had shown notable levels of CDA activity and were selected for further analysis. Among selected strains, maximum CDA activity was detected with strain SA-1 (Table 15). Therefore, strain SA-1 was finally selected for further study and was identified as *Penicillium oxalicum* (ITCC 6965) from Indian Agriculture Research Institute, New Delhi, India. The strain was maintained by periodical transfers on potato dextrose agar (PDA) slants at 4^0C for future studies.

Table 14 Morphological analysis of isolated fungal strains

Isolated strain	Mycelial morphology	Spore color	Source
SA-1	White mycelium, turning to brown possibly due to pigmentation	Green	Sea food waste
SA-2	Light pink mycelium, turning to brown at later stages	-	Sea food waste
SA-3	White cottony mycelium, oily	-	Sea food waste
SA-4	White mycelium	Green	Sea food waste
SA-5	White mycelium	Black	Sea food waste
SB-1	Grey flattened mycelium	-	Insect exoskeleton
SB-2	White mycelium	Green	Insect exoskeleton
SB-3	Aerial white mycelium	Brown-black	Insect exoskeleton
SB-4	Creamish mycelium with round edged colony	Black	Insect exoskeleton
SC-1	White aerial mycelium	Brown-black	Decaying snail shell
SC-2	Light yellow aerial mycelium	Black	Decaying snail shell
SC-3	White mycelial growth with irregular margins	Dark green	Decaying snail shell
SC-4	Grey mycelium	Grey	Decaying snail shell
SC-5	Round edged colony with white mycelium	Green	Decaying snail shell
SC-6	Grey mycelium with white periphery and concentric circles	Grey	Decaying snail shell
SD-1	White mycelium showing spreading growth	-	Degrading fish waste
SD-2	Compact cottony white mycelium	-	Degrading fish waste
SD-3	White mycelium with slight pink color	-	Degrading fish waste

Table 15 Chitin deacetylase production from the selected fungal isolates

S.No.	Potent Strains	CDA Activity (UI^{-1})
1.	SA-1	103.78 ± 1.98
2.	SA-2	97.86 ± 1.44
3.	SA-4	93.88 ± 1.79
4.	SA-5	76.74 ± 2.26
5.	SB-2	87.29 ± 2.02
6.	SD-1	96.97 ± 1.93

(Values are mean of three replicates ± SE)

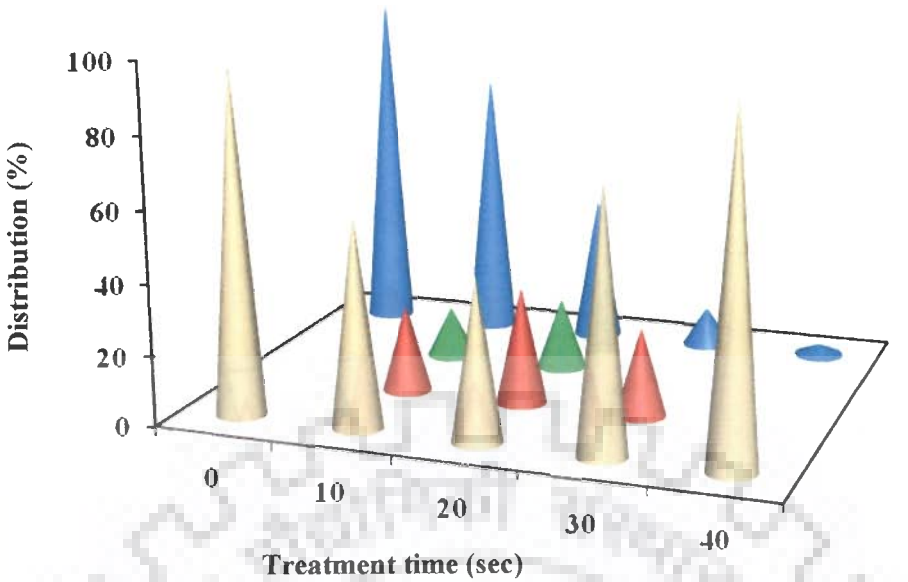
4.2 MUTAGENESIS OF *P. OXALICUM* SA-1 FOR ENHANCED CDA PRODUCTION

Fungal strain, SA-1 isolated from residual materials of sea food processing industry was subjected to physical (microwave irradiation), chemical (EtBr and EMS) and mixed mutagenic treatments to find out if an enhancement in the levels of CDA production can be achieved. Spore suspensions of wild type *P. oxalicum* SA-1 were irradiated with microwaves and/or treated with chemical mutagens followed by determining the survivability and percentage distribution of positive, negative and the corresponding colonies.

Following microwave irradiation (10 sec), a mutant SAM-10 was obtained that had shown maximum increase in enzyme production (148.54 ± 1.53 UI^{-1}). Following this exposure, a total of 25% colonies had increased level of enzyme production as compared to the parental strain and were termed as positive mutants, whereas 14 % of the colonies had lesser enzyme production and denoted as negative mutants; rest 61 % had similar level of enzyme production to that of the parental strain i.e. *P. oxalicum* SA-1, hence termed as the corresponding mutants (Fig. 4 a). The parental strain was also subjected to EtBr (200 μg ml^{-1}) and EMS (200 mM) treatments. SAE-50 mutant obtained following 50 minutes of treatment with EtBr was observed

to have maximum levels of CDA production (181.82 ± 1.21 Ul^{-1}) and 11%, 33% and 56% respectively were negative, positive and unaltered mutants (Fig. 4 b) whereas mutant SA_{EMS}-100 obtained following the EMS treatment (200 mM, 100 min) resulted into 159.92 ± 1.18 Ul^{-1} of CDA (Table 16). EtBr or EMS treatment for extended period, led to decreased number of corresponding mutants (Fig. 4 b, c).

Among the selected positive mutants, SAE-50 which was observed to have maximal level of enzyme production was subjected for second stage mutagenesis using microwave irradiation and chemical mutagens to find out if a further improvement in CDA production ability of the strain can be achieved (Fig. 4 d, e, f). Among the various mutants obtained following second stage mutagenesis, the mutagenized strain SAE_M-51 obtained following microwave irradiation had maximum increase in enzyme activity (210.71 ± 1.65 Ul^{-1}) (Table 16). A total of 18 %, 7 % and 75 % of positive, negative and corresponding mutants, respectively were obtained under these conditions (Fig. 4 d). Thus about 2.0 fold increase in the enzyme production was obtained as compared to the wild type *P. oxalicum* SA-1 strain.


Distribution (%)
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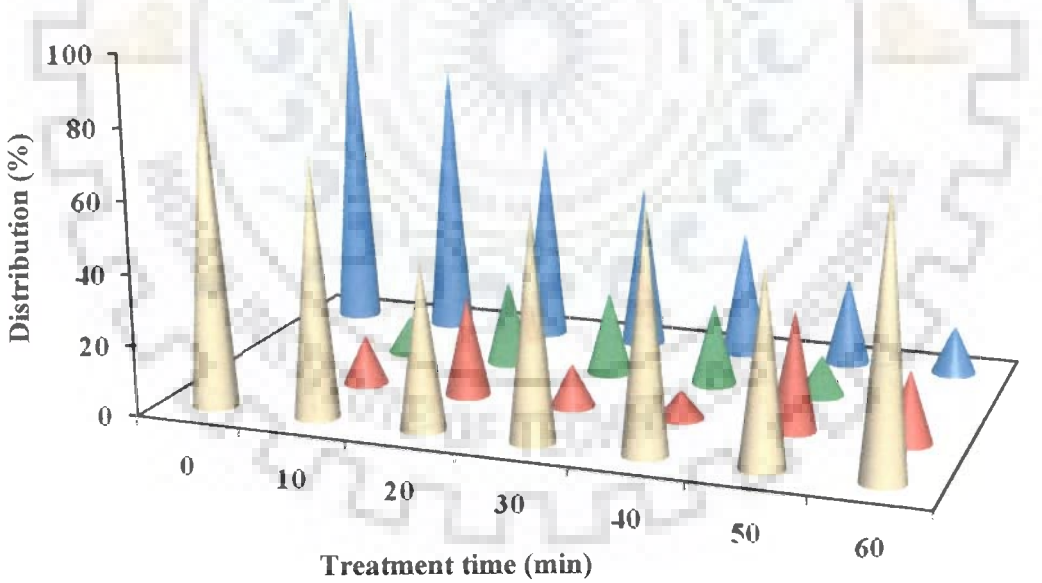
40
20
0
0
10
20
30
40
Treatment time (sec)

(a)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30
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50
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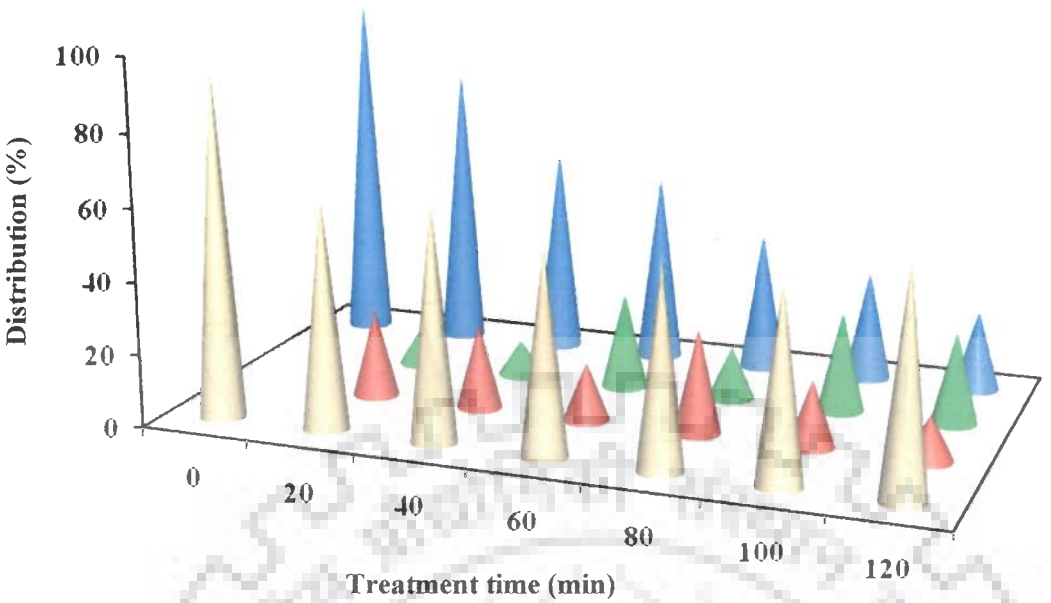
Treatment time (min)

(b)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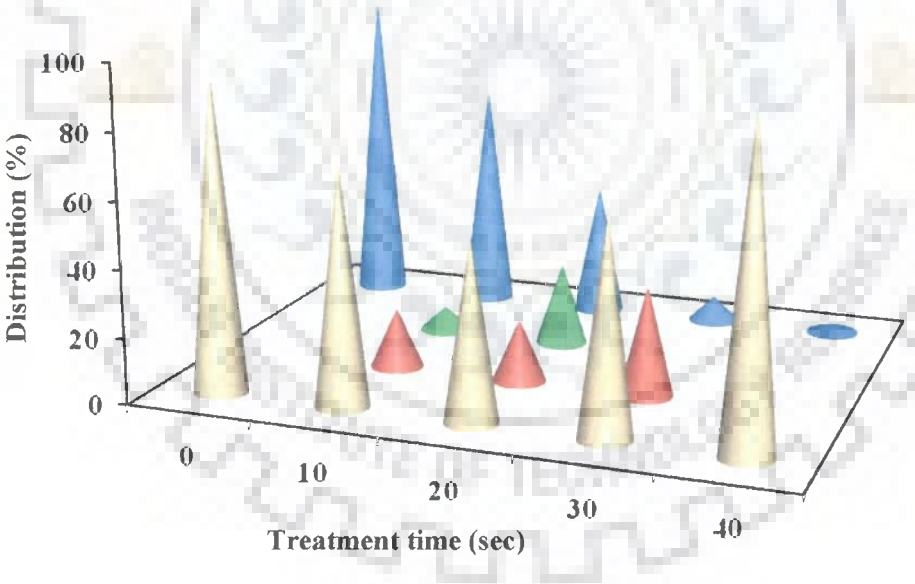
100
120
Treatment time (min)

(c)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30
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Treatment time (sec)

(d)

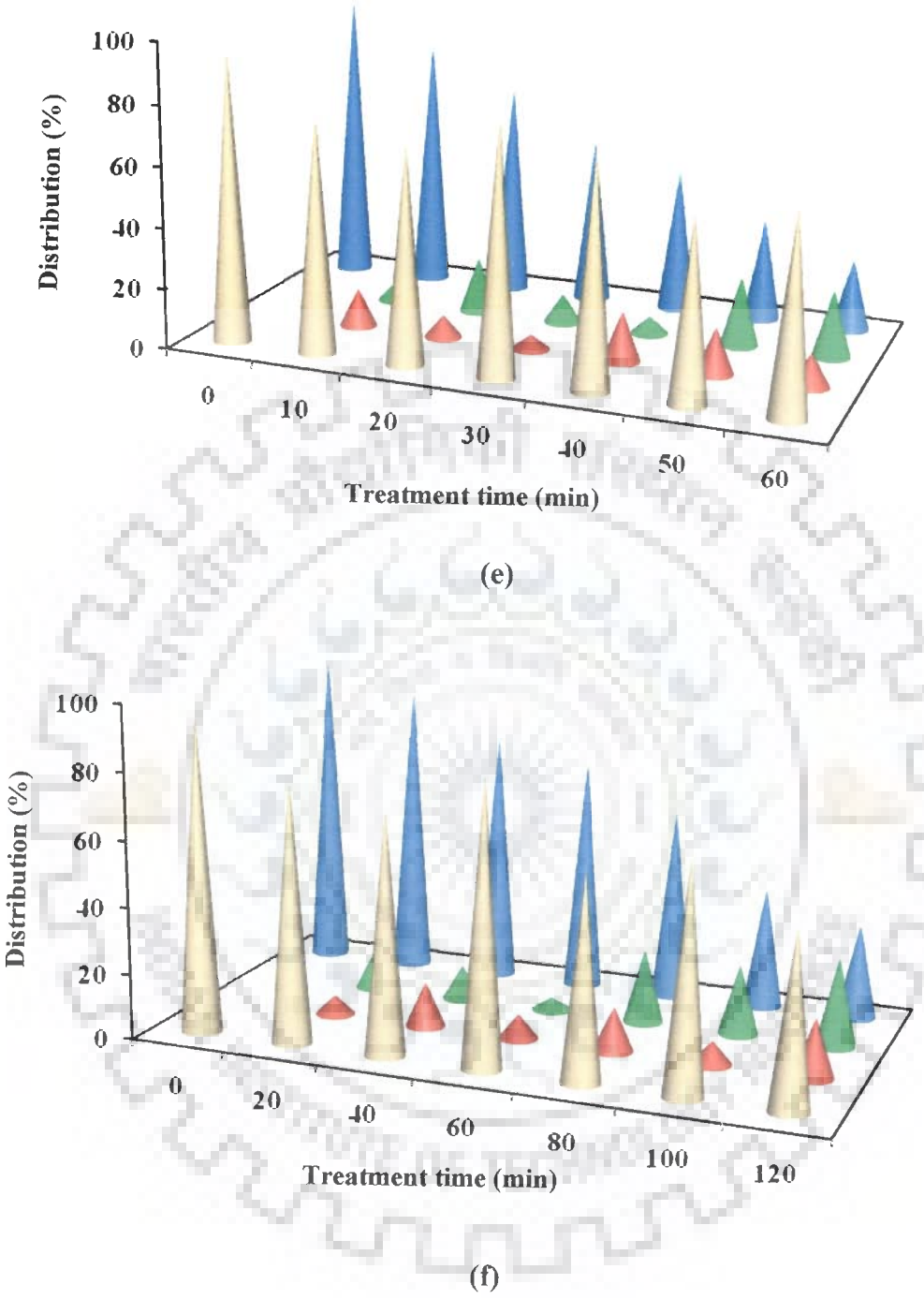


Fig. 4 Mutagenesis of *P. oxalicum* SAE_M-51 strain using (a) Microwave irradiation; (b) Ethidium bromide (EtBr); (c) Ethyl methane sulfonate (EMS); (d) EtBr + microwave irradiation; (e) EtBr + EtBr; (f) EtBr + EMS treatments

(, , , ; represent corresponding, positive, negative mutants and survivability)

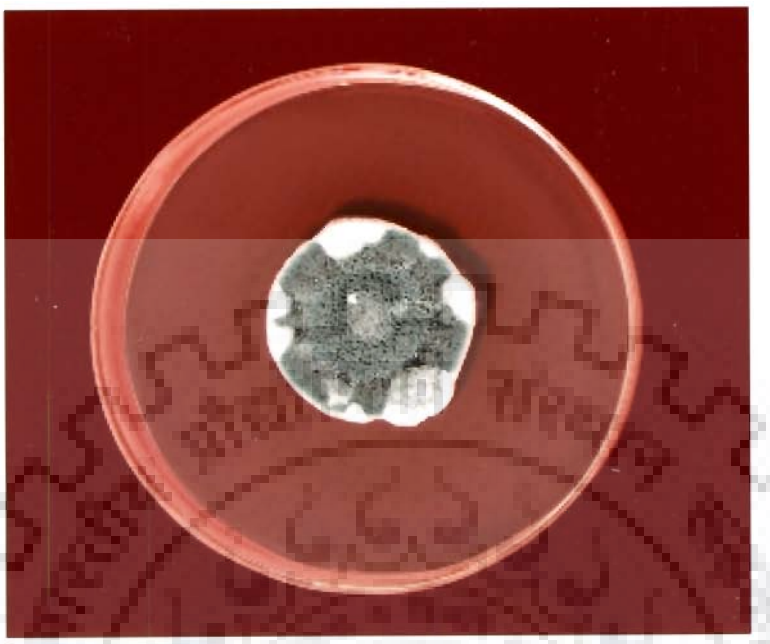
Table 16 Selected mutants obtained following mutagenesis of wild type strain *P. oxalicum* SA-1

S. No.	Mutagen	Strain	Chitin deacetylase (UI^{-1})	Fold increase
1.	None	SA-1	108.26 ± 1.98	-
2.	Microwave	SAM-10	148.54 ± 1.53	1.44
3.	EtBr	SAE-50	181.82 ± 1.21	1.76
4.	EMS	SA_{EMS}-100	159.92 ± 1.18	1.55
5.	EtBr + Microwave	SAE_M-51	210.71 ± 1.65	2.00
6.	EtBr + EtBr	SAE_E-10	190.10 ± 3.76	1.85
7.	EtBr + EMS	SAE_{EMS}-40	183.06 ± 2.04	1.77

(Values are mean of three replicates ± SE)

4.2.1 *Morphological analysis*

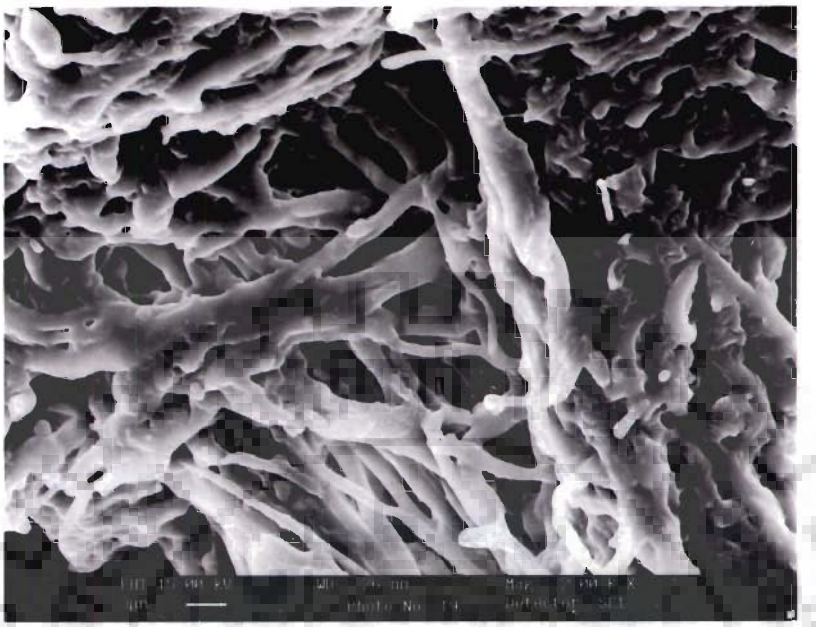
Both wild type and mutant strains could easily be distinguished on the basis of their morphology. Mycelia of wild type strain SA-1 were compact and white colored as compared to the mycelia of the mutant strain SAE_M-51 that appeared loose (Fig. 5 a, b). However, the spores produced by the parental strain appeared dark grey unlike that of mutant which was light grey in color. Scanning electron microscopy had demonstrated that mutant strain had elongated, flattened and smooth mycelia as compared to the mycelia of wild type that appeared to be irregular, rough surfaced and coiled (Fig. 6 a, b). Characteristic differences in spore morphology were also observed. Spores of the wild type were crescent shaped as compared to the oval and irregular appearing spores of the mutant strain (Fig. 7 a, b).



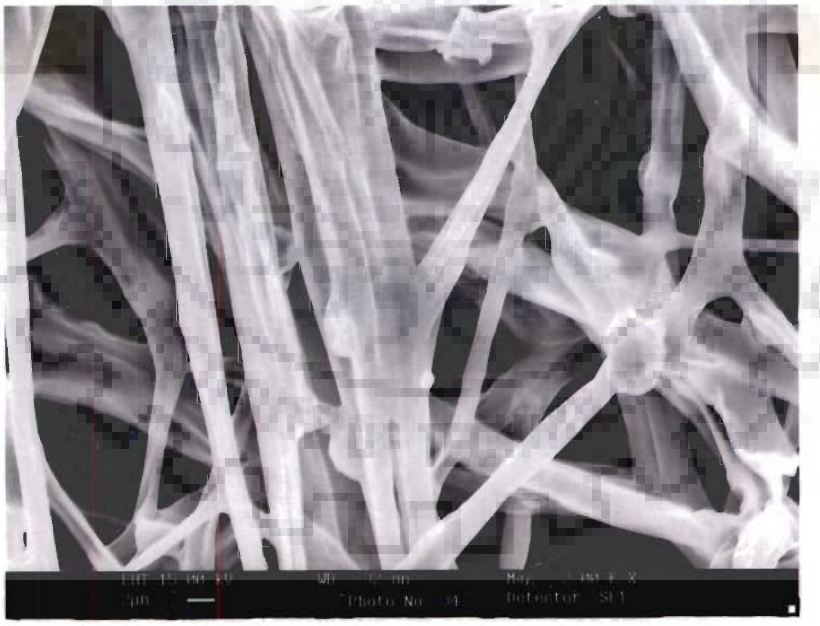
(a)

(b)

Fig. 5 Morphological features of wild type *P. oxalicum* SA-1 (a) and mutant *P. oxalicum* SAE_M-51 (b) strains

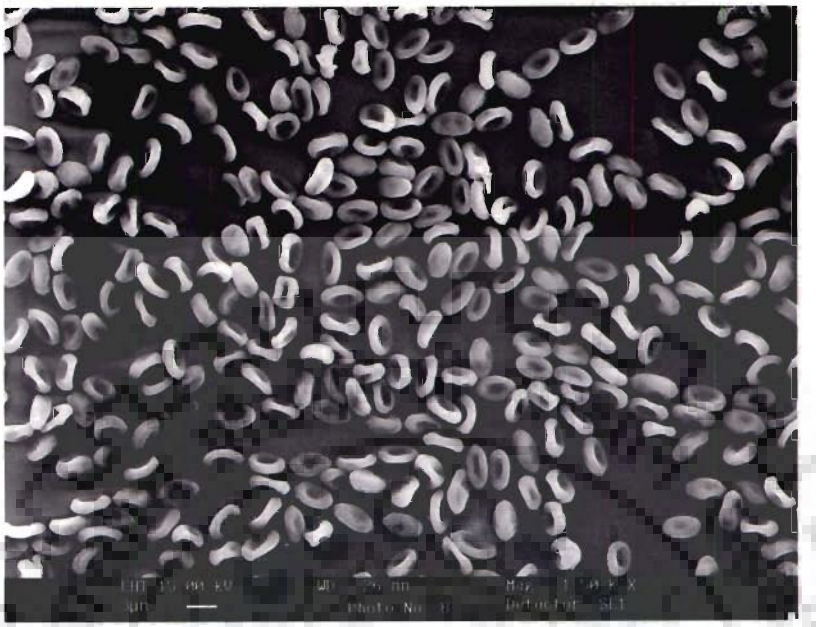


(a)

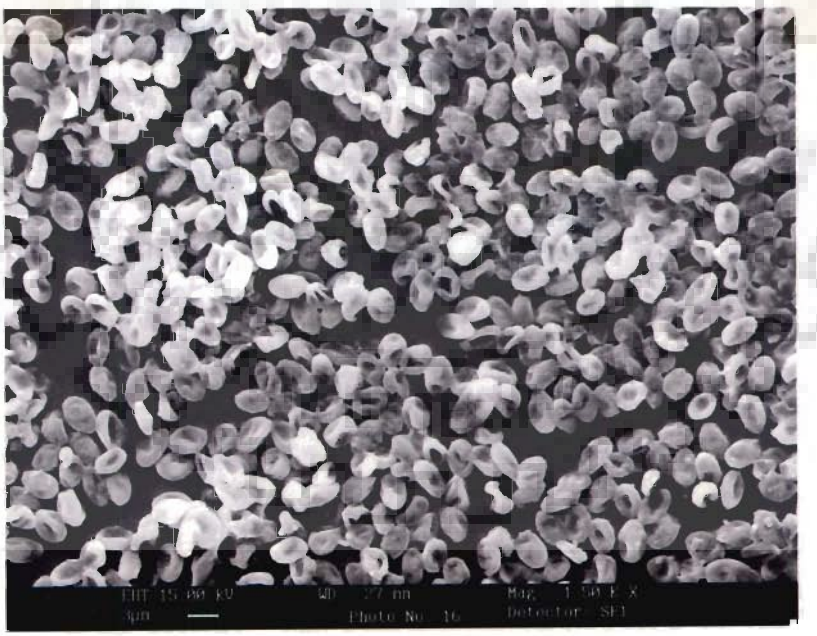


(b)

Fig. 6 Scanning electron microscopy of mycelia of wild type *P. oxalicum* SA-1 (a) and mutant *P. oxalicum* SAE_M-51 (b) strains



(a)



(b)

Fig. 7 Scanning electron microscopy of spores of *P. oxalicum* SA-1 (a) and mutant *P. oxalicum* SAE_M-51 (b) strains

4.2.1 *Evaluation of kinetic parameters*

Comparison of enzyme productivity (Q_p) and substrate consumption rate (Q_s) for extracellular CDA production had demonstrated improvement in product yield by the mutant strain (Q_p = 1.46 ± 0.82 $UI^{-1}h^{-1}$; Q_s = 0.05 ± 0.03 g $l^{-1}h^{-1}$) as compared to the parental strain. Comparison of other parameters had denoted improved levels of extracellular CDA production (Table 17). Although the wild type strain had higher growth yield ($Y_{x/s}$ = 2.05 ± 0.74 g g^{-1}) than the mutant strain, the later had demonstrated higher product yield. Further, both strains when analyzed for specific rate constants for product formation (q_p) and substrate utilization (q_s), the mutant strain *P. oxalicum* SAE_M-51 had improved values (≥1.5 fold) for q_p and q_s. Therefore, analysis of kinetic variables had indicated that mutant strain showed ~2.0 fold improved values for Q_p, Q_s, $Y_{p/x}$, $Y_{p/s}$, q_p, q_s over the parental strain.

Table 17 Analysis of kinetic variables for CDA production by wild type *P. oxalicum* SA-1 and mutant *P. oxalicum* SAE_M-51 strains

Kinetic variables	Chitin deacetylase		
	***P. oxalicum* SA-1**	***P.oxalicum* SAE_M-51**	**Fold increase**
Specific growth rate μ (h^{-1})	0.27 ± 0.08	0.34 ± 0.12	
Product formation parameters			
$Y_{p/s}$ (U g^{-1})	25.77 ± 1.60	29.68 ± 2.12	1.2
$Y_{p/x}$ (U g^{-1})	12.59 ± 1.10	16.59 ± 1.80	1.3
Q_p (U $l^{-1}h^{-1}$)	0.75 ± 0.53	1.46 ± 0.82	1.9
q_p (U $g^{-1}h^{-1}$)	3.36 ± 0.75	5.70 ± 1.13	1.7
Substrate consumption parameters			
$Y_{x/s}$ (g g^{-1})	2.05 ± 0.74	1.81 ± 0.67	0.89
Q_s (g $l^{-1}h^{-1}$)	0.03 ± 0.02	0.05 ± 0.03	1.6
Q_x (g $l^{-1}h^{-1}$)	0.06 ± 0.04	0.09 ± 0.04	1.5
q_s (g $g^{-1}h^{-1}$)	0.13 ± 0.24	0.18 ± 0.14	1.5

4.3 CDA PRODUCTION UNDER SUBMERGED FERMENTATION

4.3.1 Induction of enzyme production

Chitin, its variant colloidal chitin and glucose were evaluated for their impact on CDA production. Chitin in the production medium when replaced with colloidal chitin or glucose had denoted that glucose had resulted into the maximum CDA levels (221.57 ± 1.59 Ul^{-1}) followed by colloidal chitin (157.11 ± 1.92 Ul^{-1}) and chitin (142.62 ± 2.07 Ul^{-1}) after 144 hrs of incubation (Fig. 8 a, b). However, period required for maximum levels of production was prolonged in the presence of these chitinous substrates.

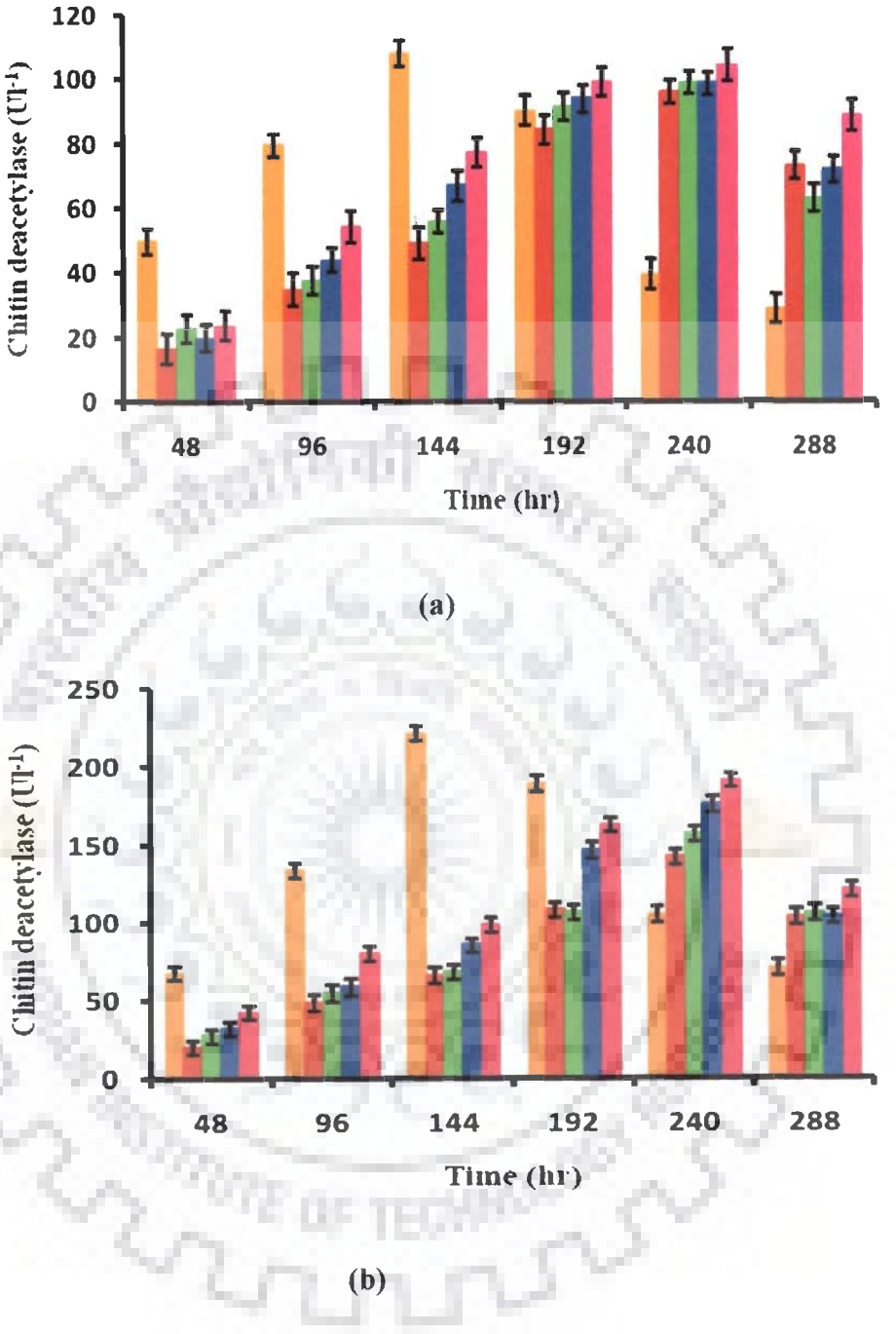


Fig. 8 Inducibility of CDA production by wild type *P. oxalicum* SA-1 (a) and mutant *P. oxalicum* SAE_M-51 (b) using 1% each of glucose, chitin, colloidal chitin; chitin + glucose (0.8 + 0.2 %), colloidal chitin + glucose (0.8 + 0.2 %) as the carbon source. (Symbols: , glucose; , chitin; , colloidal chitin; , chitin + glucose; , colloidal chitin + glucose)

4.3.2 Optimization of medium constituents

4.3.2.1 *Plackett–Burman design*

Plackett-Burman design (PBD) is a powerful tool for identifying factors, which had significant influence on CDA production. 9-factor-12-run experiments were conducted and significant variation in CDA production was observed (197.41 – 410.10 Ul^{-1}) (Table 18) that highlights the importance of optimizing nutritional factors for attaining higher enzyme titers by mutant *P. oxalicum* SAE_M-51 under submerged condition.

The results of regression analyses and ANOVA on PBD experiments are shown in Tables 19 and 20. From the regression analyses, it is clear that among the eight variables screened, four variables (yeast-extract, peptone, ammonium sulfate and magnesium sulfate) are critically significant in terms of enzyme production. High model '*F- value*' of 10.37 makes the model highly significant. This corresponds to model *p*-value (>*F*) is 0.0402, which states that there are 4.02% chances that the model *F*-value this large (10.37) can occur due to noise only. Again, coefficient of determination (R^2) of 0.9651 indicates that the model is able to explain 96.51% variability of the data, only a poor fraction of data remains unexplained by the model. A very low coefficient of variance (CV) value (6.75%) also supports the reliability of the model. Based on individual probability of failure value (Prob > *F*), the variables corresponds to a probability less than 0.1 were considered significant. Hence, four variables; yeast-extract (X_1), peptone (X_2), ammonium sulfate (X_4) and magnesium sulfate (X_5) having the lowest probability of failure values were selected for further optimization.

Table 18 Plackett-Burman experimental design matrix for screening of important nutritional variables for CDA production under submerged fermentation

Experiment No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	Chitin deacetylase (UI^{-1})
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	410.10 ± 2.2
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	297.13 ± 2.4
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	295.05 ± 1.2
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	332.34 ± 1.7
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	248.60 ± 2.7
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	235.12 ± 1.1
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	270.12 ± 2.5
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	327.21 ± 1.9
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	320.05 ± 1.6
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	261.06 ± 1.9
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	299.48 ± 2.7
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	197.41 ± 2.6

(N.B. Matrix indicates the concentration of each variable for a particular experiment in terms of +1 or -1 level. Values are mean of three replicates ± SE)

Table 19 Result of multiple regression analyses of Plackett-Burman design for screening of important nutritional variables for CDA production under submerged fermentation

Variables	Medium components (gl^{-1})	Coefficient	*F*- value	*P*-value
-	Intercept	291.14	-	-
X_1	Yeast extract	29.20	26.49	0.0142
X_2	Peptone	33.51	34.90	0.0097
X_3	Glucose	-4.24	0.56	0.5086
X_4	$(NH_4)_2SO_4$	14.39	6.43	0.0850
X_5	$MgSO_4 . 7H_2O$	18.49	10.63	0.0471
X_6	PO_4^{-3}	11.06	3.80	0.1462
X_7	NaCl	0.50	0.076	0.9358
X_8	$CaCl_2. 2H_2O$	-2.08	0.13	0.7387

Table 20 ANOVA for quadratic model for screening of critical nutritional variables for CDA production under submerged fermentation

Source	SS	DF	MS	*F*-value	*P*-value (Prob>*F*)
Model	32028.11	8	4003.51	10.37	0.0402
Residual	1158.22	3	386.07	-	-
Total	33186.33	11	-	-	-

R^2: 0.9651; Adj R^2: 0.8720; C.V.: 6.75 %; Adeq. precision: 12.534

4.3.2.2 *Central composite design*

To fully explore the sub-region of the response surface in the neighborhood of the optimum, an experimental design with more than two levels of each factor is required, so that a second order approximation to the response surface can be developed. A CCD with five coded levels was used for four variables (k=4) that were initially screened by Plackett-Burman technique. The CCD design and the corresponding experimental data are shown in Table 21. By applying quadratic regression analyses on the experimental data, results of the CCD design were fitted in a second-order polynomial equation and in coded form the equation emerged as follows (equation 1).

$$Y= 408.83 + 9.75\ X_1 + 45.42\ X_2 + 9.83\ X_4 + 1.25\ X_5 + 18.67\ X_1^2 - 13.21\ X_2^2 + 2.04\ X_4^2 + 10.04X_5^2 - 5.75\ X_1X_2 - 4.37\ X_1X_4 + 41.75\ X_1X_5 + 25\ X_2X_4 + 0.62\ X_2X_5 - 2.75\ X_4X_5 \quad (1)$$

Where, Y represents CDA production (Ul^{-1}) and X_1, X_2, X_4 and X_5 are coded values of yeast extract, peptone, ammonium sulfate and magnesium sulfate, respectively.

Results of regression analyses and ANOVA are shown respectively in Tables 22 and 23. ANOVA for CDA production indicated the '*F- value*' to be 3.29, which implied that the second-order model is very adequate in approximating the response surface of the experimental design. Corresponding probability of failure value (>*F*), tells that there is only 1.43 % chances that this value could occur due to noise. Model terms having values of probability of failure (Prob > *F*) less than 0.05 are considered very significant, whereas those greater than 0.10 are insignificant. As per the regression analyses done on this present model, the linear effect of peptone and the interaction effect of yeast extract and magnesium sulfate are very significant (Table 22). Again, the quadratic effect of yeast extract and interaction effect of peptone and ammonium sulfate are critically significant for the production of CDA by *P. oxalicum* SAE_M-51 (Table 22). Coefficient of determination (R^2) for CDA production was calculated to be 0.7545, which can explain up to

75.45% variability of the response. The low coefficient of variance value (11.64%) also supports the claim that the model is good enough to optimize the variables. The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to the noise. Adequate precision is a measure of the signal (response) to noise (deviation) ratio and a value greater than 4 is generally desirable. The 'adequate precision' value of 8.15 indicated an adequate signal and suggested that the model can be used to navigate the design space. The "lack of fit test" compares the "residual error" to the "pure error" from replicated design points. Further, very high *F*-value (771.58) for lack-of-fit test strengthens the confidence in the model.

Three dimensional response surface curves and the corresponding contour plots were plotted to determine the optimum level of each variable and the effect of their interactions on CDA production against any two independent variables, while keeping other variables at their respective 'O' levels. CDA production was observed to be significantly affected by peptone concentration in the culture medium (*p*-value: 0.0004). However, parallel nature of the contours indicated that enzyme production was not affected by its interaction with yeast extract, ammonium sulfate and magnesium sulfate (Fig. 9-11). The strong interaction between yeast extract and magnesium sulfate is evident from the response surface contour plot between them (Fig. 12). Again, quadratic effect of yeast extract on the CDA production is evident from bi-folding nature of the interaction plots between yeast extract and ammonium sulfate (Fig. 13). Fig. 14 depicts the interaction between ammonium sulfate and magnesium sulfate. Again, the parallel nature of the contours along the two axes suggested that the two parameters were quite independent of each other. The response surface contour plots generated from the interactions of different variables studied using CCD, clearly emphasized that CDA production by *P. oxalicum* SAE_M-51 was greatly influenced by the variables studied.

The optimum coded values calculated from the model equation for yeast extract, peptone, ammonium sulfate and magnesium sulfate were enumerated to be 0.1101, -0.1230,

-1.9078 and -0.5486, respectively. The corresponding uncoded optimum values are (gl^{-1}): 3.165, 4.75, 0.255 and 0.5359 for yeast extract, peptone, ammonium sulfate and magnesium sulfate respectively.

Table 21 Central composite design matrix for derivation of nutritional variables for CDA production under submerged fermentation

Run	X_1	X_2	X_4	X_5	Chitin deacetylase (UI^{-1})	
					Observed	Predicted
1	+1	+1	+ α	+1	516.74 ± 3.9	547.12
2	0	0	0	0	409.53 ± 2.3	408.83
3	0	0	- α	0	427.15 ± 2.6	397.33
4	-1	-1	-1	-1	412.84 ± 3.1	414.62
5	-1	+1	-1	-1	434.42 ± 2.8	465.71
6	-1	-1	+1	+1	356.71 ± 2.4	310.79
7	0	0	+ α	0	410.43 ± 2.1	436.67
8	+1	-1	-1	+1	432.67 ± 3.8	461.12
9	0	0	0	0	411.60 ± 2.9	408.83
10	+α	0	0	0	497.25 ± 3.2	503.00
11	0	0	0	0	411.75 ± 1.9	408.83
12	0	0	0	- α	456.83 ± 2.6	446.50
13	-1	+1	+1	-1	545.14 ± 3.5	549.62
14	0	0	0	0	409.35 ± 2.4	408.83
15	-1	-1	+1	-1	419.64 ± 2.5	398.54
16	+1	-1	+1	+1	479.39 ± 3.6	416.54
17	0	0	0	+ α	445.72 ± 1.7	451.50
18	0	0	0	0	407.16 ± 2.6	408.83
19	+1	+1	-1	-1	320.49 ± 1.6	398.96
20	+1	0	-1	-1	445.62 ± 2.5	370.87
21	+1	+1	-1	+1	502.74 ± 4.2	491.71
22	-1	+1	-1	+1	394.38 ± 2.1	391.46
23	+1	+1	+1	-1	503.93 ± 2.5	465.38
24	0	+ α	0	0	515.41 ± 3.4	446.83
25	- α	0	0	0	473.74 ± 2.8	464.00
26	-1	+1	+1	+1	421.18 ± 2.1	464.38
27	0	- α	0	0	200.86 ± 1.7	265.17
28	-1	-1	-1	+1	331.18 ± 2.4	337.87
29	+1	-1	+1	-1	301.74 ± 3.2	337.29
30	0	0	0	0	406.53 ± 2.8	408.83

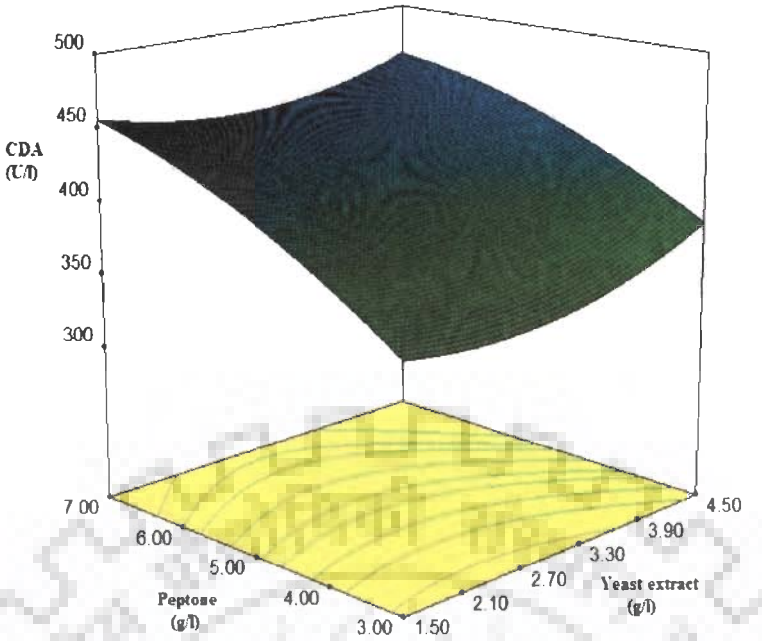
Table 22 Result of regression analyses of CCD for derivation of nutritional variables for CDA production using CCD under submerged fermentation

Variables	Coefficient	Standard error	*F*- value	*P*-value
Intercept	408.83	20.13	-	-
X_1	9.75	10.07	0.97	0.3481
X_2	45.42	10.07	4.51	0.0004
X_4	9.83	10.07	0.98	0.3441
X_5	1.25	10.07	0.12	0.9028
X_1^2	18.67	9.42	1.98	0.0660
X_2^2	-13.21	9.42	-1.40	0.1810
X_4^2	2.04	9.42	0.22	0.8312
X_5^2	10.04	9.42	1.07	0.3030
X_1X_2	-5.75	12.33	-0.47	0.6476
X_1X_4	-4.37	12.33	-0.35	0.7276
X_1X_5	41.75	12.33	3.39	0.0041
X_2X_4	25.00	12.33	2.03	0.0607
X_2X_5	0.62	12.33	0.051	0.9602
X_4X_5	-2.75	12.33	-0.22	0.8265

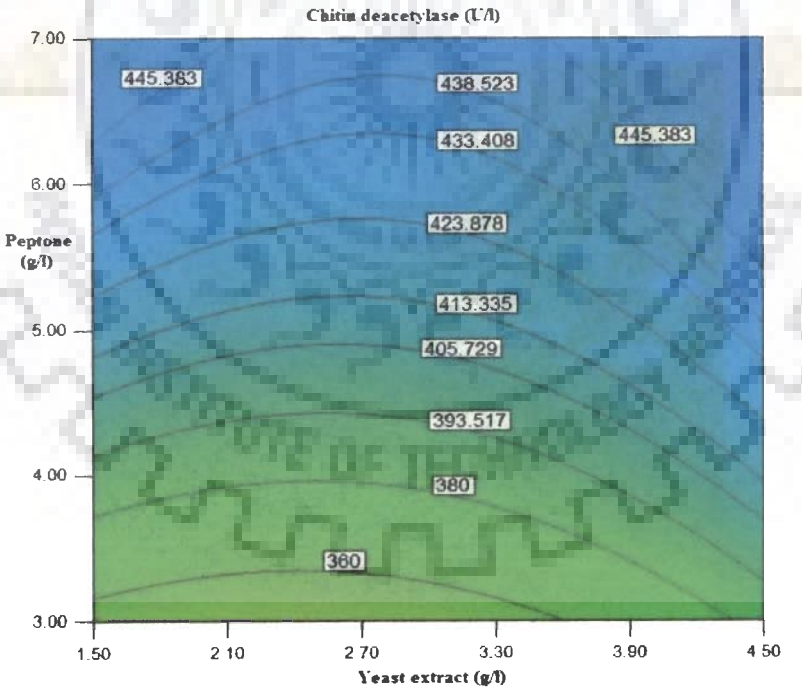
Table 23 ANOVA for quadratic model for derivation of nutritional variables for CDA production using CCD under submerged fermentation

Source	SS	DF	MS	*F*-value	*P*-value (Prob>*F*)
Model	1.120E+005	14	8000.35	3.29	0.0143
Residual	36439.16	15	2429.28	-	-
Lack of fit	36415.56	10	3641.56	771.58	□0.0001
Pure error	23.60	5	4.72	-	-
Total	1.484E+005	29	-	-	-

R^2: 0.7545; C.V.: 11.64 %; Adeq. precision: 8.150

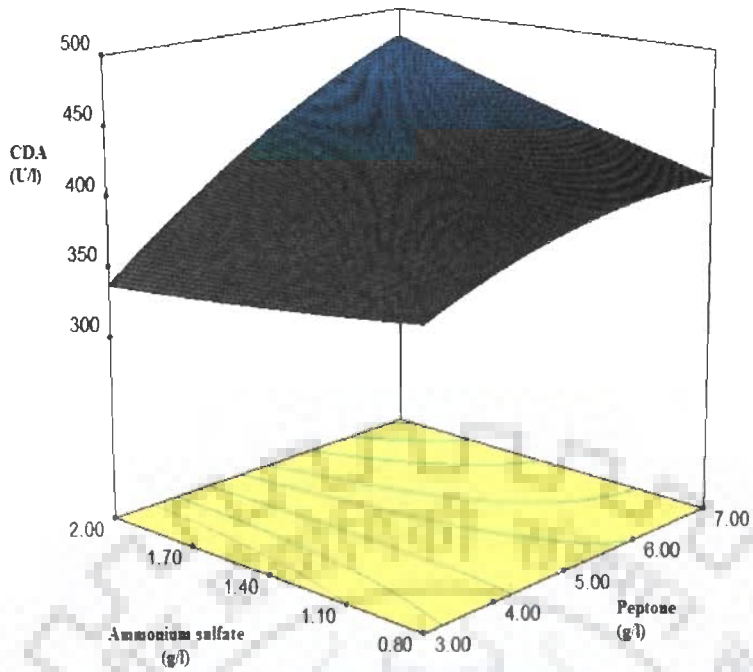


(a)



(b)

Fig. 9 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of yeast extract and peptone



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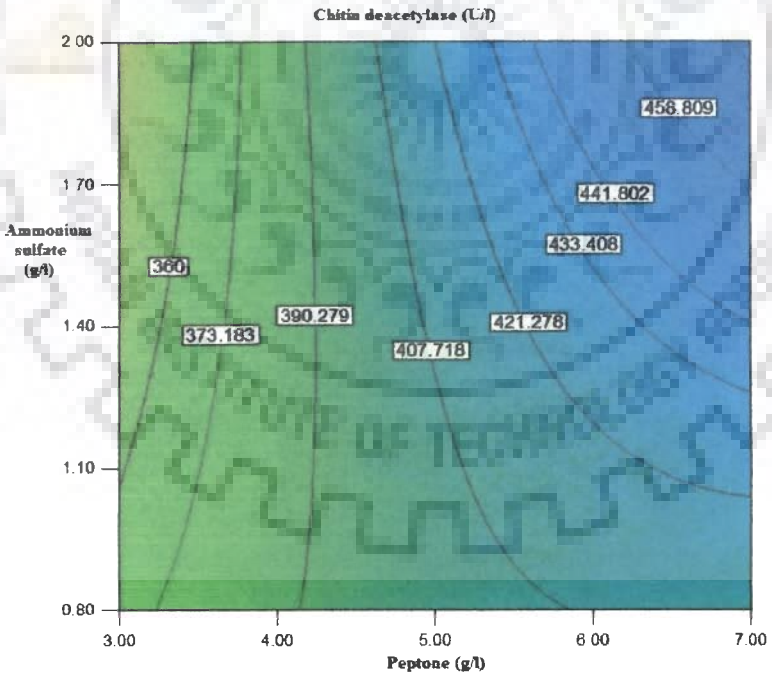
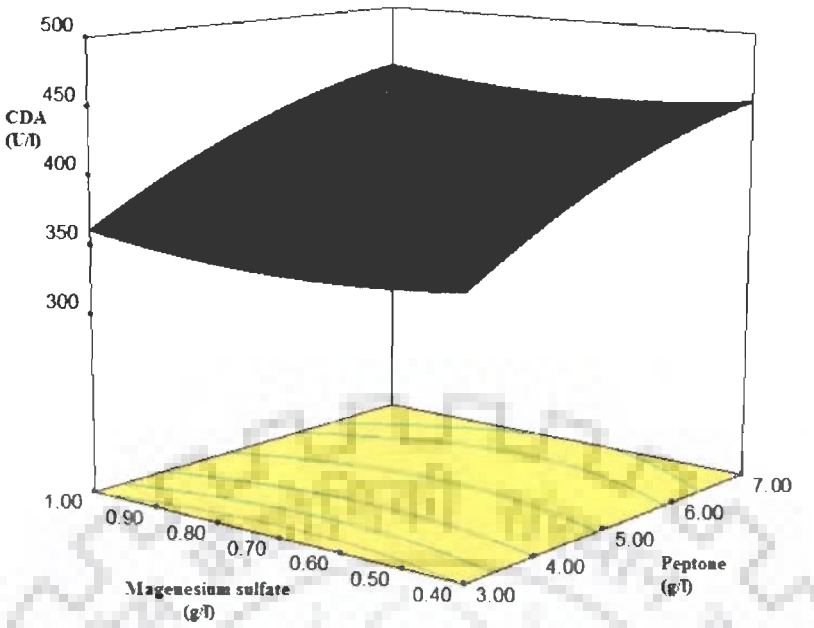
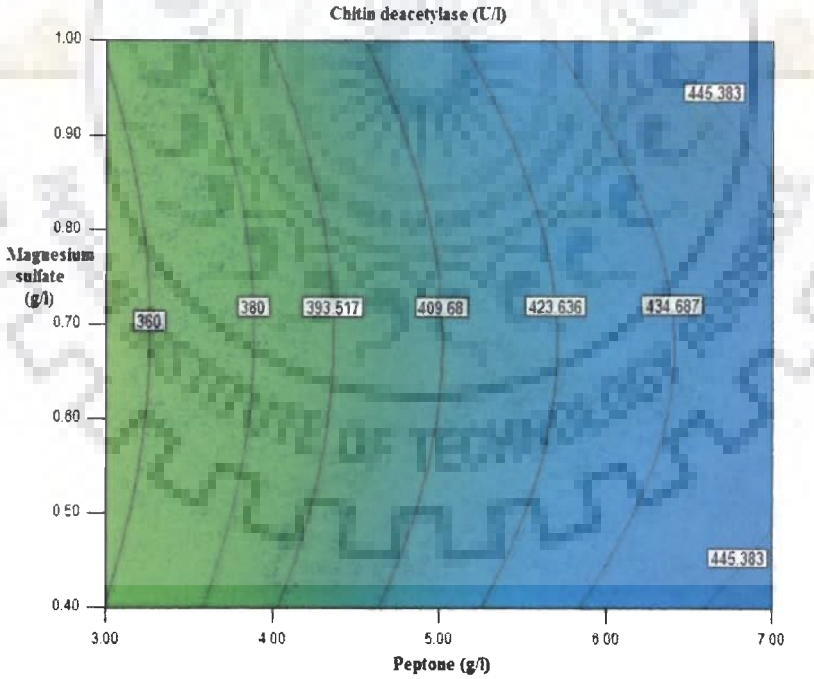


(b)

Fig. 10 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of peptone and ammonium sulfate

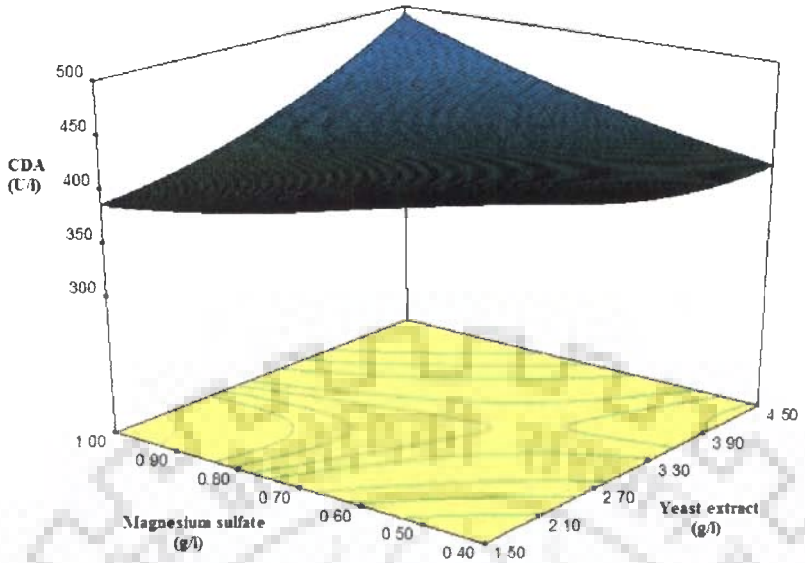


(a)

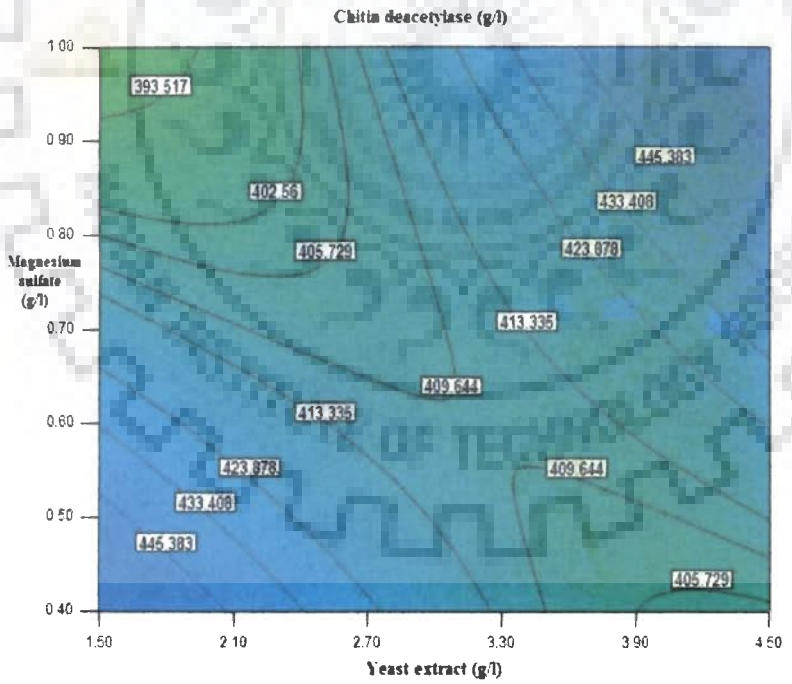


(b)

Fig. 11 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of peptone and magnesium sulfate

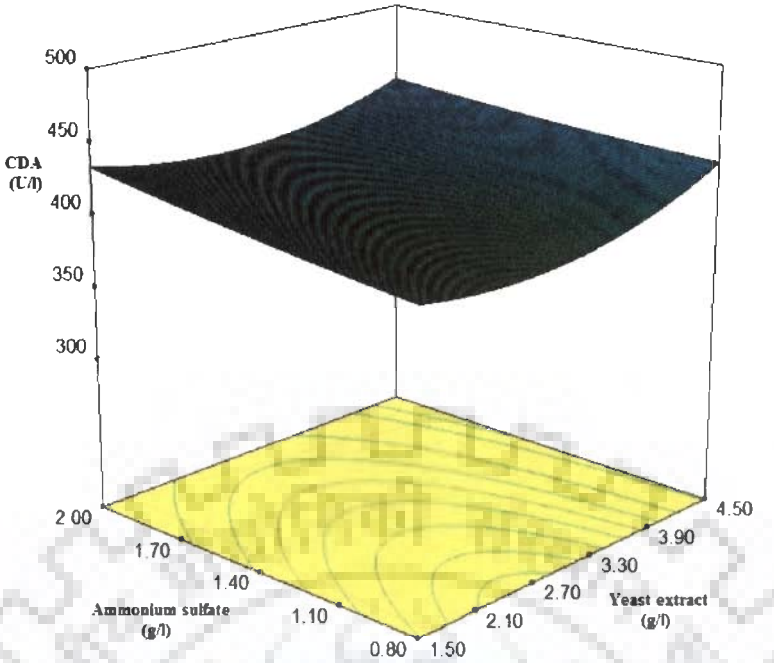


(a)

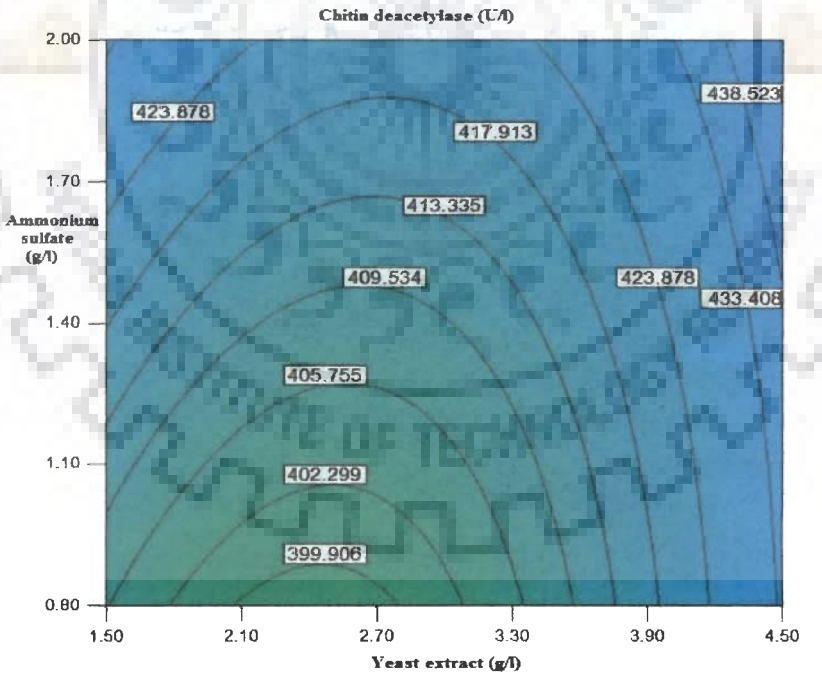


(b)

Fig. 12 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of yeast extract and magnesium sulfate

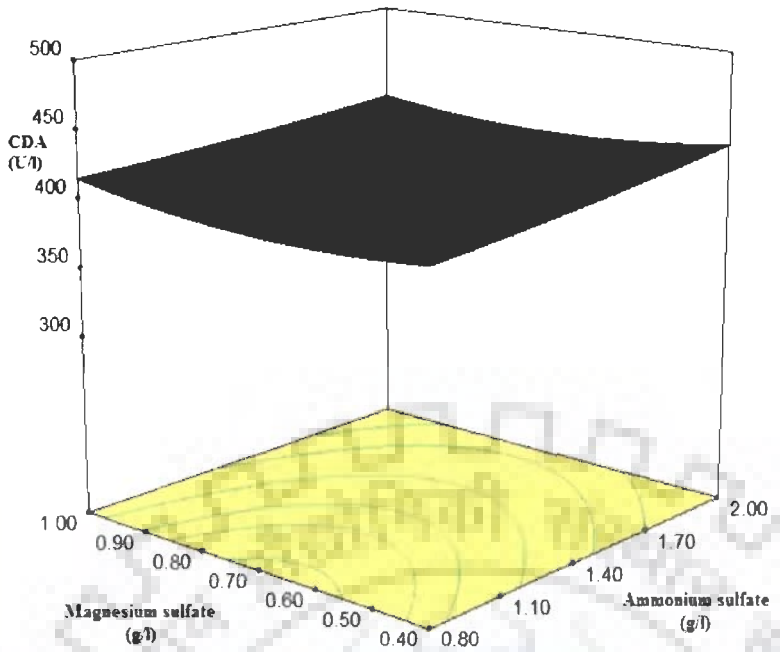


(a)

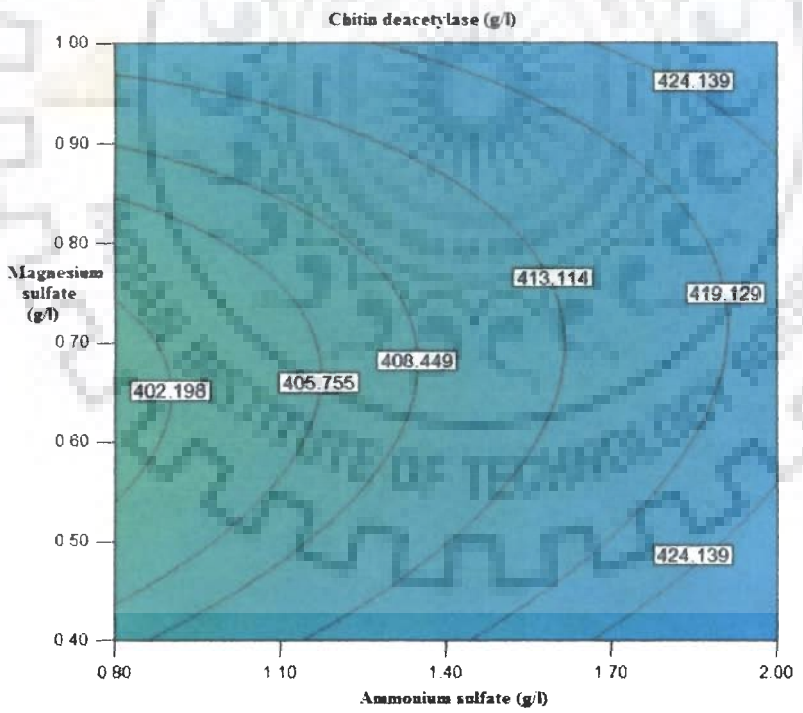


(b)

Fig. 13 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of yeast extract and ammonium sulfate



(a)



(b)

Fig. 14 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of ammonium sulfate and magnesium sulfate

4.3.2.3 *Validation of the experimental model*

Verification of the predicted value was done with experiments that were performed using derived medium as well as the basal medium and the result is shown in Fig. 15. The predicted response for CDA production (399.08 Ul^{-1}) was very close to the actual response (414.7 ± 6.32 Ul^{-1}). The excellent correlation between predicted and experimental values justifies the validity of the response model.

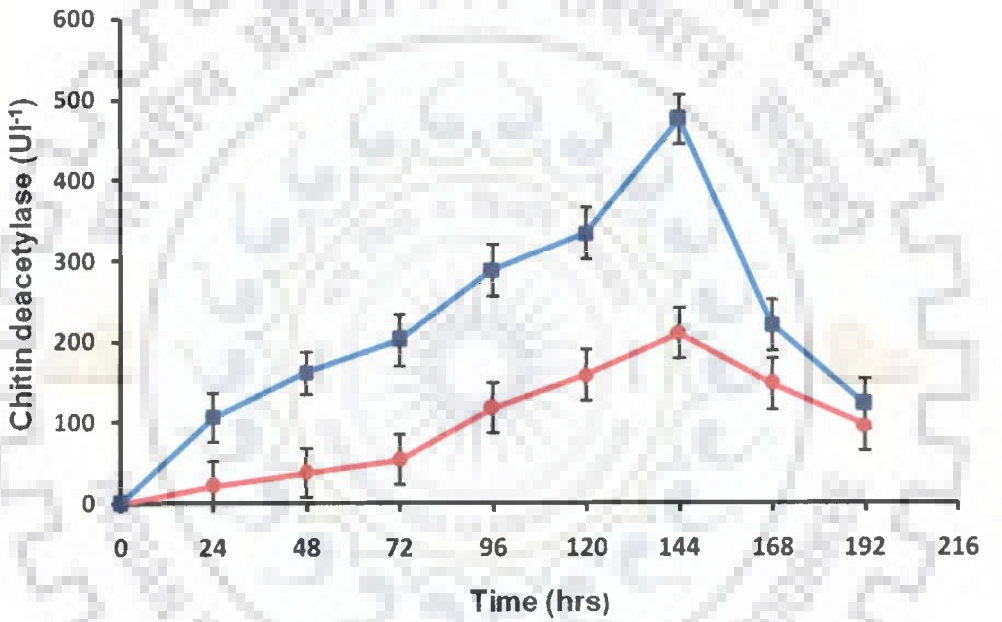


Fig. 15 Chitin deacetylase production at varying time intervals by mutant *P. oxalicum* SAE_M- 51 under submerged fermentation

(■ , derived medium;● ,basal medium)

4.3.3 Optimization of culture conditions

4.3.3.1 *Central composite design*

The effect of four culture variables viz. pH, temperature, inoculum age and inoculum size on CDA production was determined by CCD. The experimental design matrix of the variables in coded units is given in Table 24 along with the predicted and experimental values of response. The results obtained by CCD were analyzed using regression analyses and ANOVA (Tables 25 and 26). The experimental outcomes of the CCD were fitted with a second-order polynomial function that could explain the CDA production as a function of the four variables studied (equation 2):

$$Y = 545.17 + 19.04 X_1 + 7.79 X_2 - 16.38 X_3 + 11.21 X_4 + 6.72 X_1^2 - 4.66 X_2^2 - 12.66 X_3^2 + 6.47 X_4^2 + 2.81 X_1X_2 + 4.06 X_1X_3 - 0.44 X_1X_4 - 4.44 X_2X_3 - 5.69 X_2X_4 + 5.06 X_3X_4 \quad (2)$$

where Y represents CDA production (UI^{-1}) and X_1, X_2, X_3 and X_4 are coded values of pH, temperature, inoculum age and inoculum size respectively.

The statistical significance of the response surface quadratic model was governed by *F*-test and the ANOVA. The Fisher's *F*-test for the model with a very low probability value ($p < 0.0001$) had indicated the significance of the model. ANOVA for CDA production indicated the '*F* value' to be 24.61, which implied that the model is highly significant in approximating the response surface of the experimental design. Corresponding probability of failure value, tells that only 0.01 % chances are there that this value could occur due to noise. The goodness of fit of the model was examined by coefficient of determination (R^2). Coefficient of determination (R^2) for CDA production was calculated to be 0.9583, which can explain up to 95.83% variability of the response. The 'adequate precision' value of 22.56 had indicated an adequate signal and suggested that the model can be used to navigate the design space. The lower value of coefficient of variance (1.72%) indicated that experiments are conducted with

very high precision. The *F*- value (23.67) for lack of fit test implies that it is highly significant and there is only 0.14% chance that this large value could occur due to noise. The significance of each coefficient on the response and also the interaction strength between each independent variable was determined by *p*-values, the smaller the *p*-values, the bigger the significance of the corresponding coefficient (Table 25). Model terms having values of 'Prob > *F*' less than 0.05 were considered significant, whereas those greater than 0.10 are insignificant. According to the present model, all the linear and squared terms of pH, temperature, inoculum age and inoculum size significantly affected CDA production. In addition, interactions of inoculum size with temperature and inoculum age had also been observed to have notable effect on CDA production. However, interaction between pH and other variables like temperature, inoculum size and age of inoculum was insignificant.

Table 24 Central composite design matrix for derivation of culture conditions for CDA production under submerged fermentation

Run	X_1	X_2	X_3	X_4	Chitin deacetylase (UI^{-1})	
					Observed	Predicted
1	-1	-1	-1	-1	527.85 ± 3.2	520.75
2	- 1	- 1	-1	+1	543.16 ± 4.1	545.17
3	-1	-1	+1	-1	475.32 ± 2.8	478.63
4	-1	-1	+1	+1	528.73 ± 2.5	523.42
5	-1	+1	-1	-1	559.26 ± 3.4	550.96
6	-1	+1	-1	+1	558.14 ± 2.1	552.75
7	-1	+1	+1	-1	490.25 ± 3.1	491.08
8	-1	+1	+1	+1	516.35 ± 2.7	513.13
9	+1	-1	-1	-1	539.03 ± 1.8	545.96
10	+1	-1	-1	+1	568.34 ± 2.3	568.75
11	+1	-1	+1	-1	513.23 ± 3.4	520.08
12	+1	-1	+1	+1	551.76 ± 2.9	563.13
13	+1	+1	-1	-1	581.28 ± 3.3	587.42
14	+1	+1	-1	+1	587.87 ± 4.1	587.46
15	+1	+1	+1	-1	542.84 ± 3.8	545.17
16	+1	+1	+1	+1	556.54 ± 3.4	564.08
17	+ α	0	0	0	629.74 ± 2.7	610.13
18	- α	0	0	0	521.71 ± 2.5	533.96
19	0	+ α	0	0	540.65 ± 2.4	542.13
20	0	- α	0	0	519.85 ± 3.2	510.96
21	0	0	+ α	0	472.16 ± 1.7	461.79
22	0	0	- α	0	523.37 ± 2.4	527.29
23	0	0	0	+ α	596.39 ± 3.5	593.46
24	0	0	0	- α	552.75 ± 2.6	548.63
25	0	0	0	0	546.24 ± 2.3	545.17
26	0	0	0	0	542.08 ± 3.1	545.17
27	0	0	0	0	543.61 ± 1.6	545.17
28	0	0	0	0	545.29 ± 3.5	545.17
29	0	0	0	0	547.66 ± 2.4	545.17
30	0	0	0	0	548.15 ± 2.6	545.17

Table 25 Model coefficients estimated by regression analyses derivation of culture conditions for CDA production using CCD under submerged fermentation

Variables	*F*- value	*P*-value
Intercept	-	-
X_1	100.65	<0.0001
X_2	16.85	0.0009
X_3	74.43	<0.0001
X_4	34.87	<0.0001
X_1^2	14.32	0.0018
X_2^2	6.88	0.0192
X_3^2	50.82	<0.0001
X_4^2	13.27	0.0024
X_1X_2	1.46	0.2450
X_1X_3	3.05	0.1010
X_1X_4	0.035	0.8532
X_2X_3	3.64	0.0756
X_2X_4	5.99	0.0272
X_3X_4	4.74	0.0458

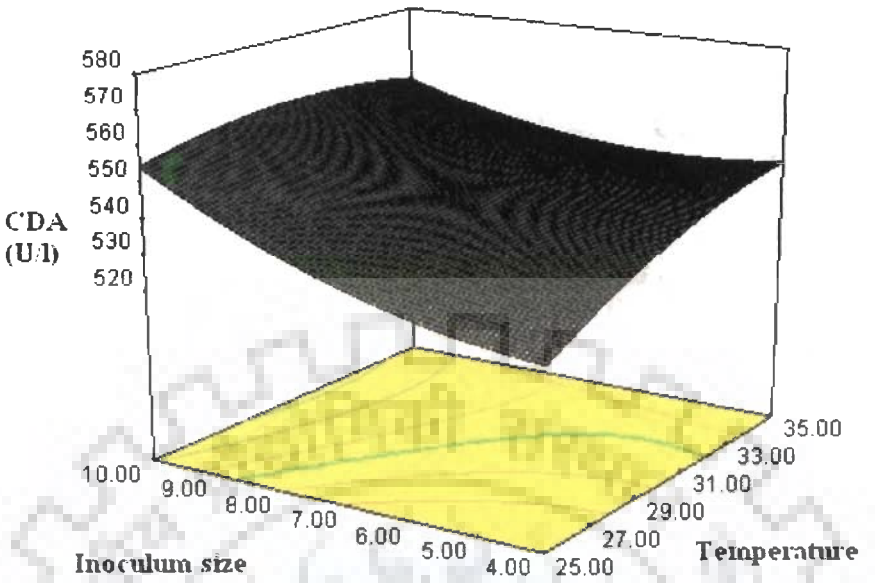
Table 26 ANOVA for quadratic model for derivation of culture conditions for CDA production using CCD under submerged fermentation

Source	SS	DF	MS	*F*-value	*P*-value (Prob>*F*)
Model	29794.55	14	2128.18	24.61	<0.0001
Residual	1296.92	15	86.46	-	-
Lack of fit	1270.08	10	127.01	23.67	0.0014
Pure error	26.83	5	5.37	-	-
Total	31091.47	29	-	-	-

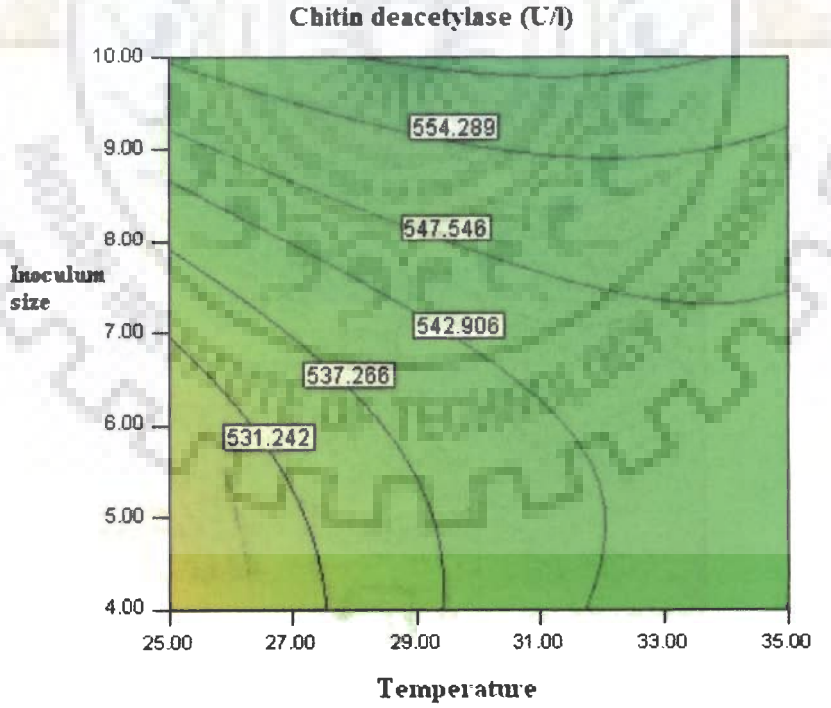
R^2: 0.9583; Adj R^2: 0.9194; C.V.: 1.72 %; Adeq. precision: 22.560

4.3.3.2 *Determination of optimum conditions*

The optimum level of each variable and the effect of their interactions on CDA production were studied from response surface and contour plots, generated for the pair-wise combination of any two independent variables, while keeping other two variables at their respective center point levels. Among these, interaction between temperature and inoculum size had significantly affected the CDA production (Fig. 16). Other interactions that had fairly affected the production level are the interactions between inoculum age and inoculum size (Fig. 17) and temperature and inoculum age (Fig. 18). The remaining interactions between the variables appeared significant. The least important is the interaction between pH and inoculum size (Fig. 19). Parallel nature of the contour plots for the interaction of pH with temperature and inoculum age indicated that enzyme production was not affected by these interactions, however it was observed to be increased at higher pH values (Fig. 20, 21). Among the independent variables, all were found to play important role in the CDA production as their *F*-values are high and the corresponding *p*-values were small (Table 25). The optimum coded values for these parameters were calculated from the model equation 2 and were found to be 1.929, -0.416, -1.277 and 1.303 for pH, temperature, inoculum age and inoculum size respectively. The corresponding uncoded optimum values were pH, 7.9; temperature, 28.0 ^{0}C; inoculum age, 90.0 hrs and inoculum size, 11.0 % (v/v). The maximum CDA production predicted by the model equation was 648.24 Ul^{-1} under the optimized conditions.

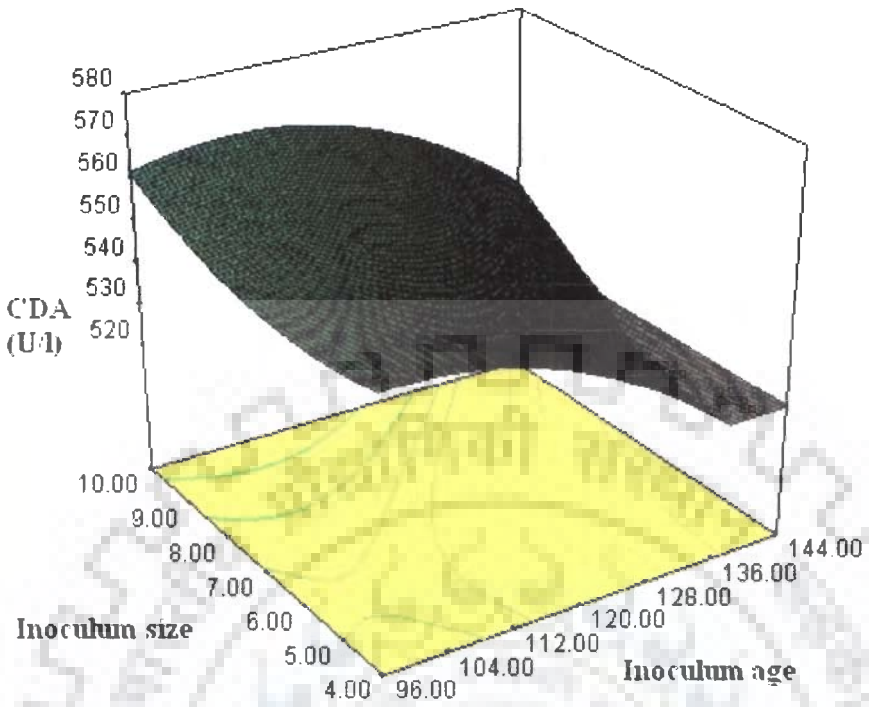


(a)

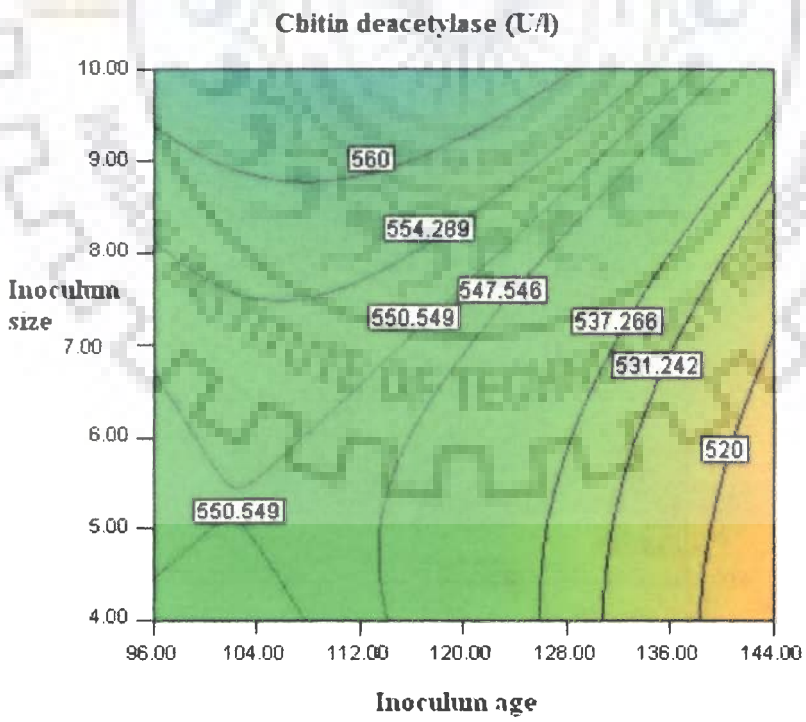


(b)

Fig. 16 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of temperature and inoculum size

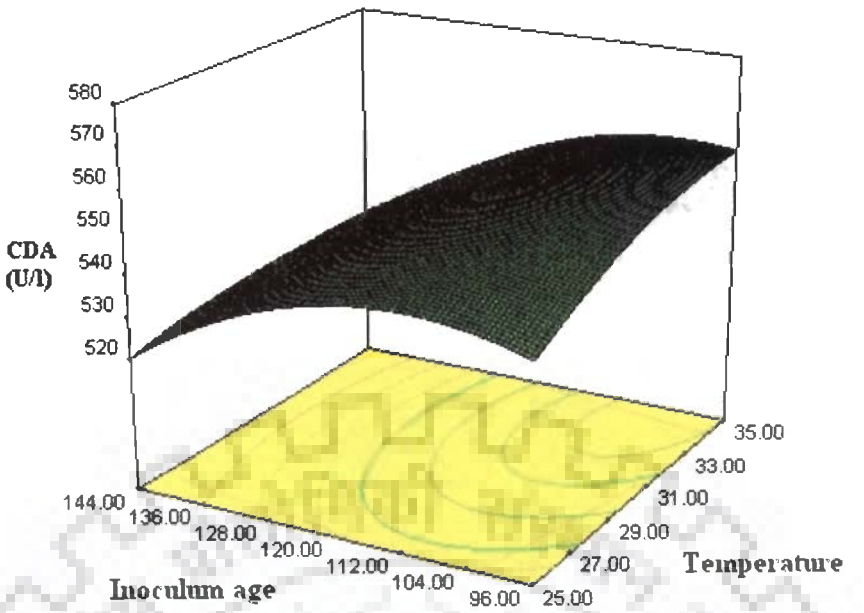


(a)

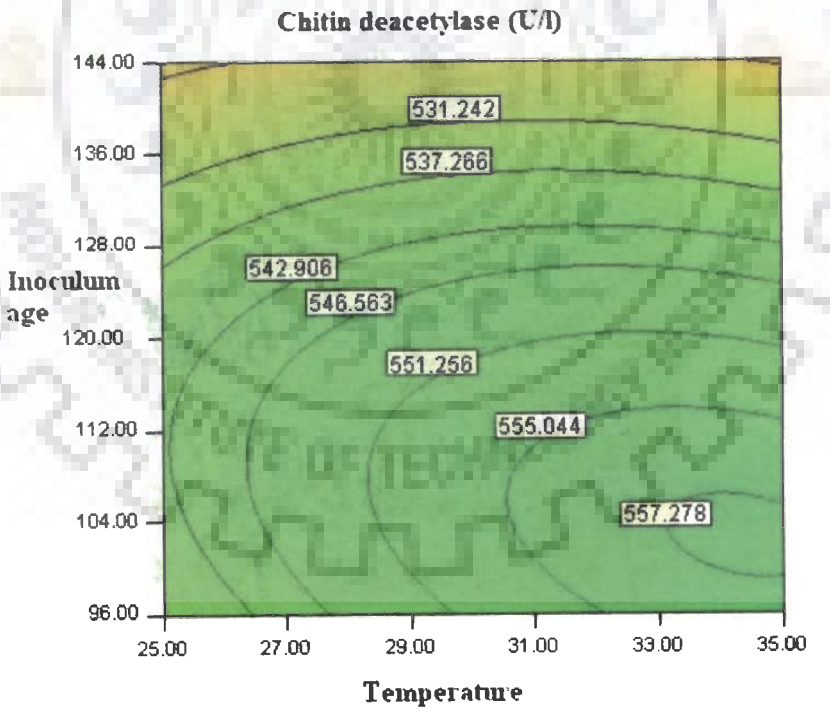


(b)

Fig. 17 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of inoculum age and inoculum size

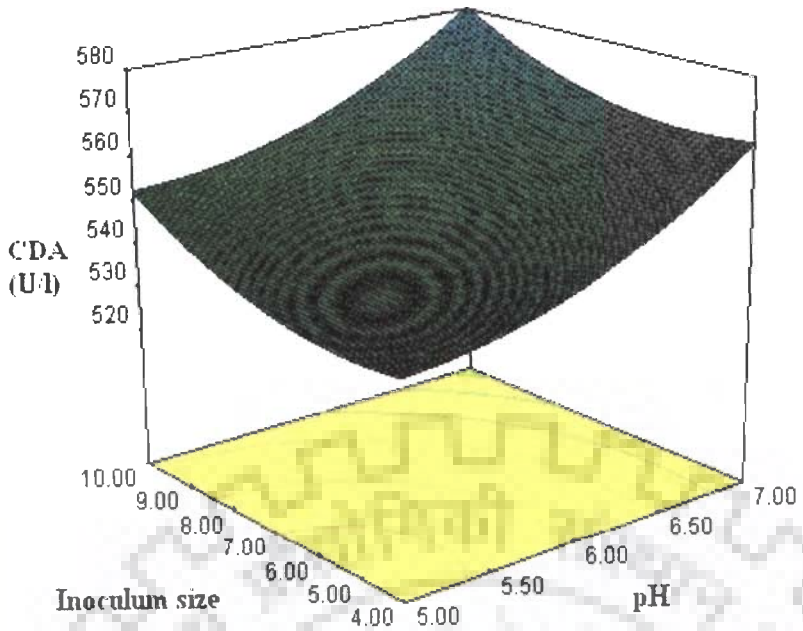


(a)

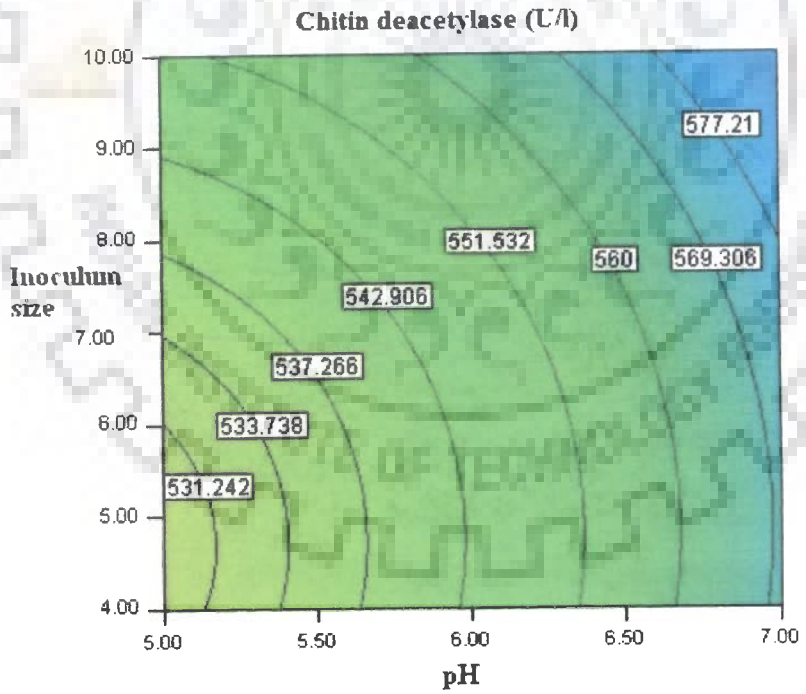


(b)

Fig. 18 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of temperature and inoculum age

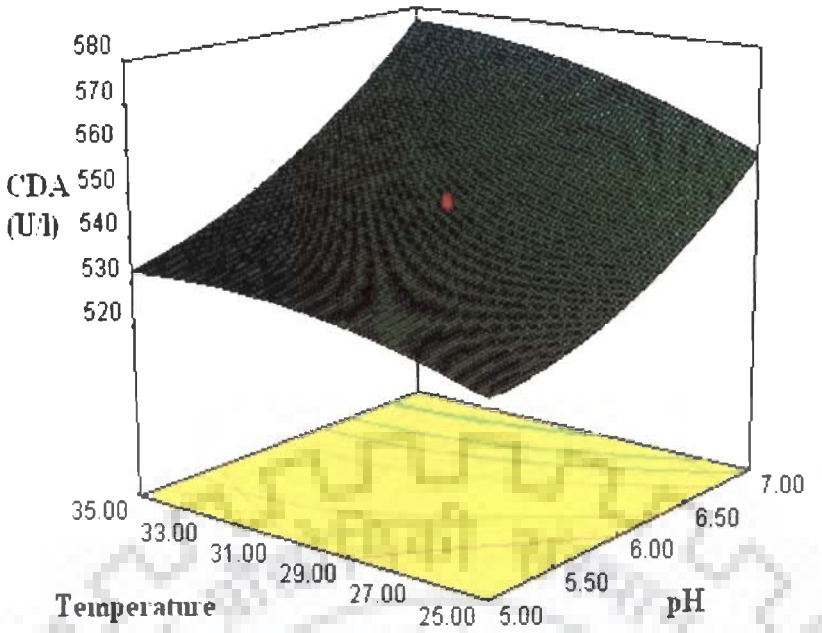


(a)

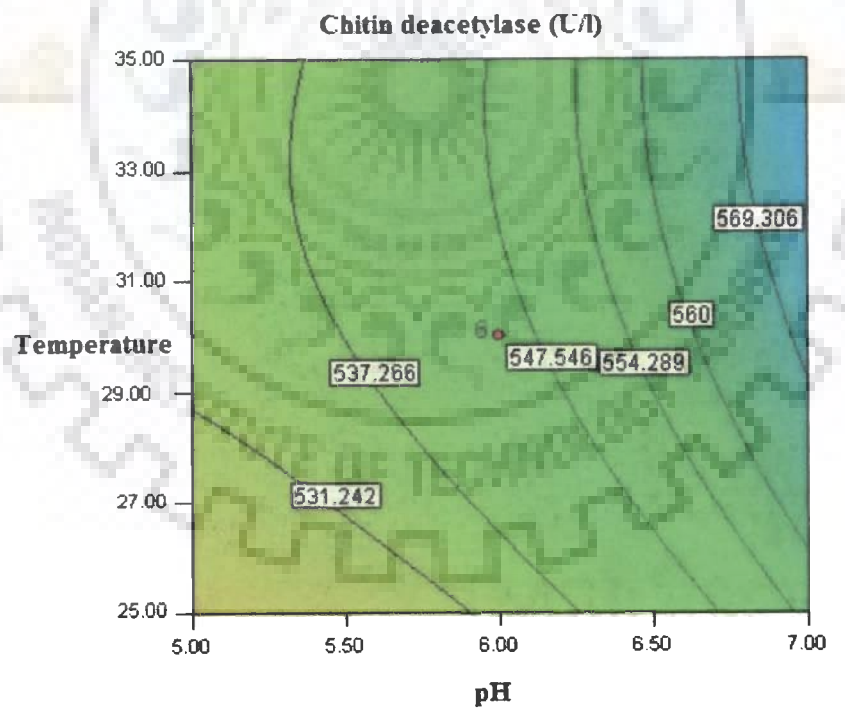


(b)

Fig. 19 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of pH and inoculum size

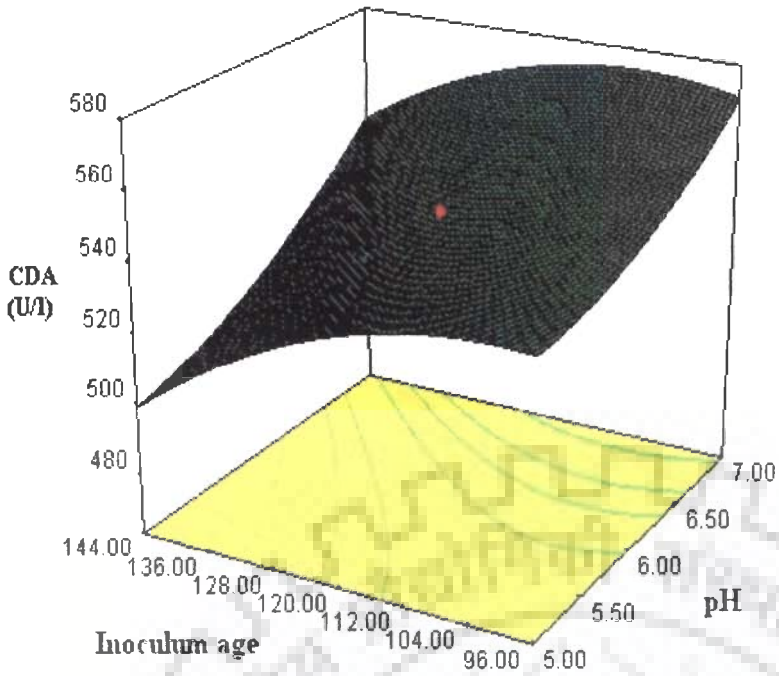


(a)

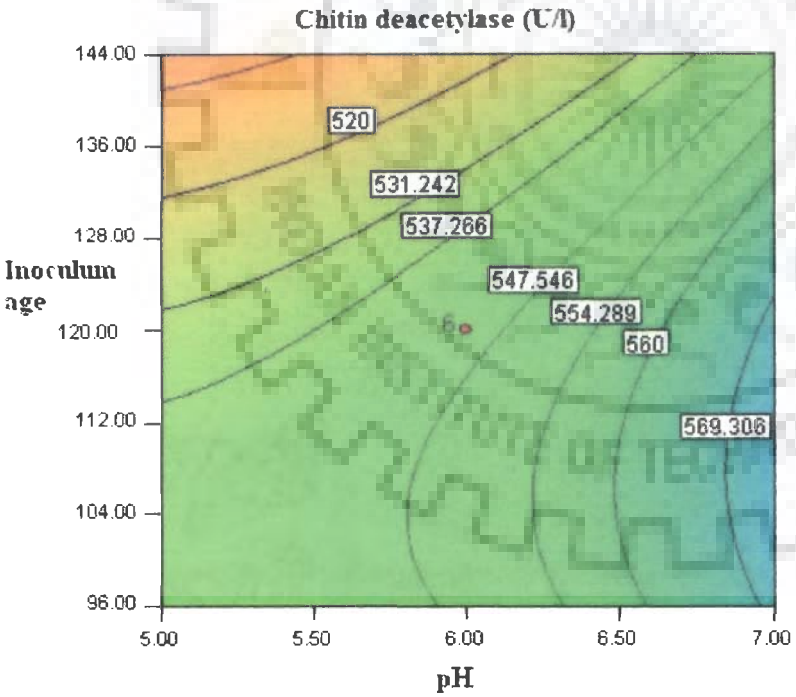


(b)

Fig. 20 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of pH and temperature



(a)



(b)

21 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of pH and inoculum age

4.3.3.3. *Validation of the experimental model*

To validate the model equation, experiments were carried out in triplicate for CDA production under the conditions as predicted by the model and was observed to be 623.57 ± 8.2 Ul^{-1} (Fig. 22). The theoretical prediction for the model equation was (648.24 Ul^{-1}). The closeness between theoretically predicted values and experimental result at optimum condition validates the model experimentally.

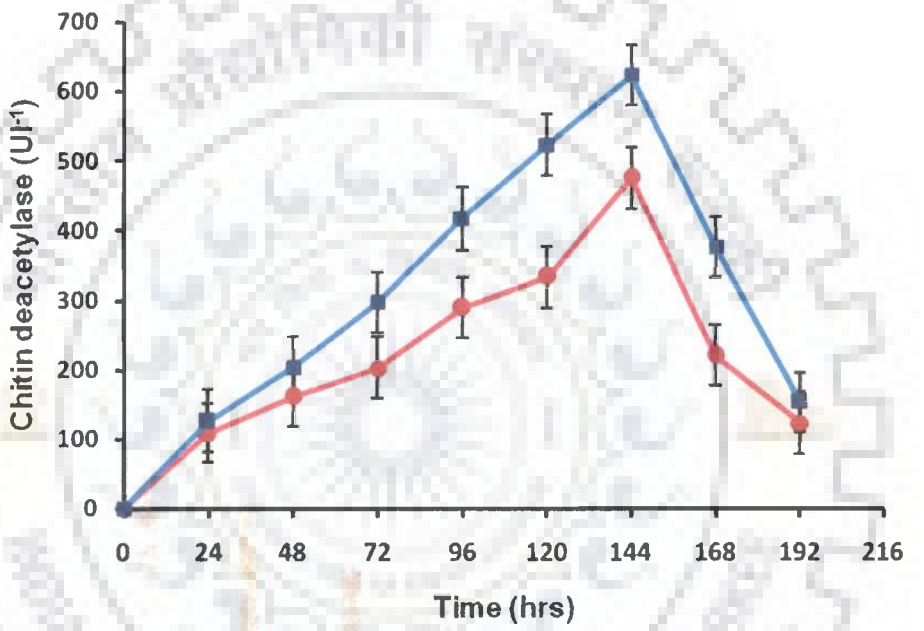


Fig. 22 Chitin deacetylase production at varying time intervals by mutant *P. oxalicum* SAE_M- 51 under submerged fermentation

(■ , derived conditions; ● , basal conditions)

4.4 EVALUATION OF DIFFERENT FERMENTATION CONDITIONS FOR CHITIN DEACETYLASE PRODUCTION

Enzyme production ability of *P. oxalicum* SAE_M-51 strain was analyzed in different fermentation systems i. e. surface, submerged and solid-state fermentation systems (Table 27). Increasing levels of enzyme production was obtained at varying time intervals for upto 144 hrs. However, maximum levels of CDA was obtained during solid-state fermentation (718.98 ± 12.90 UI^{-1}) (Fig. 23). Productivity when determined under these conditions has enumerated that maximum productivity was obtained under SSF process (4.9929 ± 8.42 $UI^{-1}h^{-1}$) (Table 27). Since SSF had resulted into maximum levels of production, hence, further studies were carried out using SSF system to derive critical parameters for achieving further enhanced levels of enzyme production.

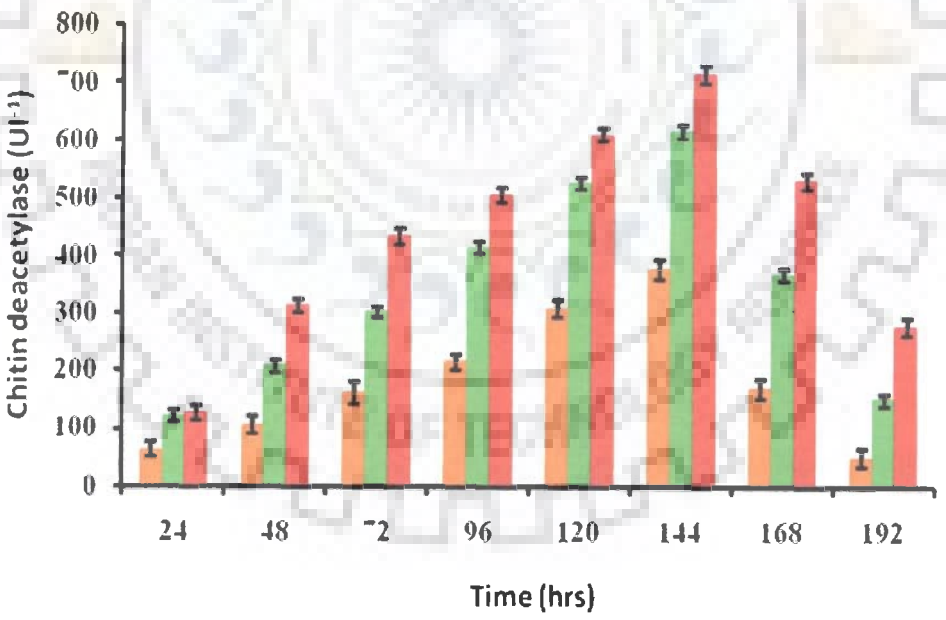


Fig. 23 Enzyme production by *P. oxalicum* SAE_M-51 strain under various fermentation systems (, surface; , submerged; , solid-state system)

Table. 27 Chitin deacetylase production by mutant *P. oxalicum* SAE_M-51 under various fermentation conditions

Fermentation conditions	Chitin deacetylase	
	Activity (Ul^{-1})	Productivity ($Ul^{-1}h^{-1}$)
Surface fermentation	378.43 ± 17.20	2.6279 ± 7.13
Submerged fermentation	618.43 ± 11.20	4.2946 ± 7.38
Solid-state fermentation	718.98 ± 12.90	4.9929 ± 8.42

4.5 ANALYSIS OF SOLID-STATE FERMENTATION CONDITIONS FOR ENZYME PRODUCTION

Solid-state fermentation had resulted into comparatively higher levels of the CDA production as compared to other two fermentation systems. Therefore, in order to economize the process and to attain further higher levels of production, substrate and other major factors affecting the levels of production during solid-state fermentation were evaluated.

4.5.1 Analysis of the substrates for enzyme production under solid-state fermentation

A variety of agro-horticultural residues that are easily and abundantly available either at considerably lower price or with no cost, were chosen as substrate for solid-state processes (Fig. 24). All the solid supports had significantly encouraged the growth and enzyme production by mutant *P. oxalicum* SAE_M-51 strain but among all, mustard oil cake led to maximum levels of CDA (877.85 ± 10.95 Ul^{-1}) production (Fig. 25). Almost similar CDA levels were detected with black gram husk, corn residues and wheat bran, whereas cotton hull, rice bran, groundnut shell, red gram husk, tea waste and sugarcane bagasse had resulted in comparatively lower CDA activity. This showed that all the substrates could serve as source of carbon and energy for growth of the fungus but luxuriant growth of the *P. oxalicum* SAE_M-51 strain was observed on mustard oil cake which indicated this to be most suitable matrix for colonization and fermentation and also it's a cost effective matrix for fermentation. Hence, mustard oil cake was selected for further studies.

Fig. 24 Different solid supports used for solid-state fermentation

(a) Corn residue; (b) Mustard oil cake; (c) Cotton hull; (d) Wheat bran; (e) Red gram husk; (f) Rice bran; (g) Tea waste; (h) Black gram husk; (i) Groundnut shell; (j) Sugarcane bagasse

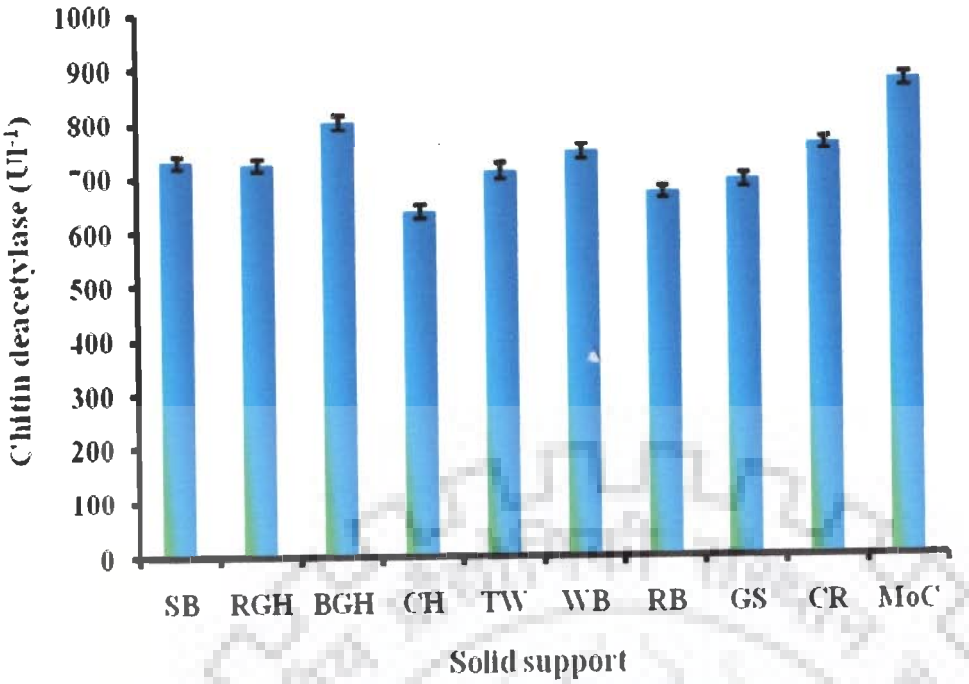
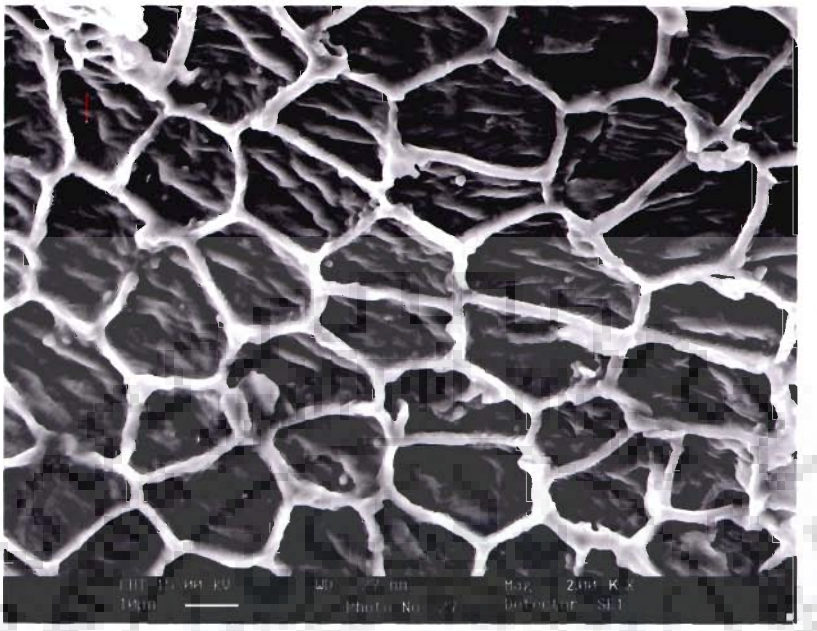


Fig. 25 Evaluation of different solid supports for enzyme production by mutant *P. oxalicum* SAE_M-51 strain

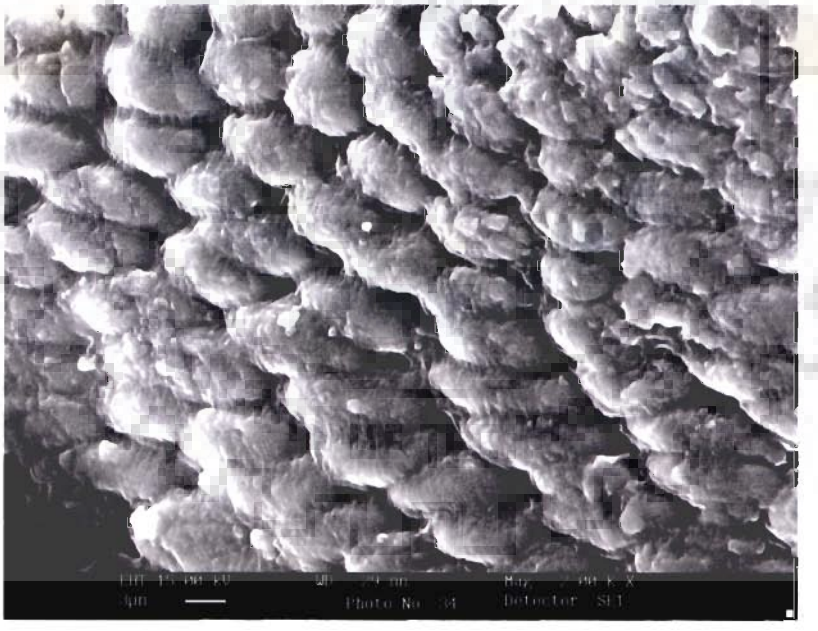
(SB, Sugarcane bagasse; RGH, Red gram husk; BGH, Black gram husk; CH, Cotton hull; TW, Tea waste; WB, Wheat bran; RB, Rice bran; GS, Groundnut shell; CR, Corn residue; MoC, Mustard oil cake)

4.5.2 Mustard oil cake in solid state fermentation

As observed through scanning electron microscopy, morphological features of mustard oil cake seemed to be supportive for fungal entrapment and anchorage. Uninoculated mustard oil cake appeared porous and hence emerged as a better substrate for fungus to adhere and penetrate (Fig. 26 a). Mustard oil cake following inoculation had profound colonization by *P. oxalicum* SAE_M-51 (Fig. 26 b). Thus, it seems to be a suitable matrix supporting the metabolic activity of fungus.



(a)



(b)

Fig. 26 Scanning electron micrograph of uninoculated mustard oil cake (a), and mustard oil cake entrapped with *P. oxalicum* SAE_M-51 (b) under solid-state fermentation

4.5.3 Optimization of solid-state fermentation conditions for CDA production

4.5.3.1 *Determination of optimum conditions*

Optimum values of the three major variables viz. amount of substrate, moisture content and inoculum size for improved CDA production were determined by RSM using 2^3 full factorial CCD. The results of the experiments in terms of actual and predicted values of dependent variable i.e. CDA activity are shown in Table 28. CDA secretion varied markedly in the range between 639.37 and 1817.49 UI^{-1}. The multiple regression analyses when applied to the experimental data, the following equation (3) was found to explain CDA production as a function of the variables studied.

$$Y = 1131.70 + 147.86 X_1 - 26.03 X_2 + 144.96 X_3 - 108.28 X_1^2 + 155.30 X_2^2 + 81.47 X_3^2 - 200.36 X_1X_2 - 68.21 X_1X_3 - 70.74 X_2X_3 \quad (3)$$

The adequacy of the model was checked using ANOVA which was tested using Fisher's statistical analysis and the results are shown in Tables 29 and 30, respectively. The coefficient of determination (R^2) was 0.9723 for CDA production, which explained 97.23% variability in the model. The R^2 value should be between 0 and 1. The closer the R^2 value is to 1.0, the better therefore would be the correlation between experimental and predicted values. The values of correlation coefficients (adjusted R^2 and predicted R^2) for CDA production were 0.9473 and 0.7743, respectively. The observed values of R^2 explain that the fitted model could explain 94.73 % of the total variation and therefore, vouches for adequacy of the model. An adequate precision of 24.84 had indicated an adequate signal and suggested that the model can be used to navigate the design space.. The model *F*-value of 38.98 and values of (Prob > *F*, <0.0001) indicated that the model is significant. Values of 'Prob > *F*' less than 0.0500 indicated that the model terms are significant. Apart from linear term of moisture content, all other linear, squared and interaction terms are found to be significant for CDA production. The 'Lack of Fit *F*-value'

of 327.71 implied that lack of fit is significant for CDA production. There is only a 0.01 % chance that a 'Lack of Fit *F*-value' this large occur due to noise. Also, the CV value indicates the degree of precision with which the treatments are compared, and the low value of CV showed the reliability of experiment. In this study, a relatively lower value of the C.V. (5.63 %) suggested a good precision and reliability of the experiment.

The contour and three-dimensional response surface curves were plotted to delineate the effect of interaction between independent variables on CDA production and also for derivation of their optimal concentration for achieving higher levels of CDA production. Interaction between substrate amount and moisture content has significantly affected the enzyme production (Fig. 27). CDA production was also considerably influenced by the interaction between substrate amount and inoculum size (Fig. 28). Bi-folding nature of the interaction plots between moisture content and inoculum size stated quadratic effect of moisture content on CDA production (Fig. 29). The coded values for these parameters calculated from the model equation 2 were found to be -0.547, -0.319 and 0.526 for substrate amount, moisture content and inoculum size respectively. The corresponding uncoded derived values would be 4.906 g, 73.62 % and 8.578 % for substrate amount, moisture content and for the inoculum size, respectively. Under derived conditions, maximum CDA production was 1162.03 ± 7.2 Ul^{-1} following 144 hrs of incubation.

Table 28 Central composite design matrix for optimization of CDA production under solid-state fermentation

Run	X_1	X_2	X_3	Chitin deacetylase (UI^{-1})	
				Observed	Predicted
1	-1	-1	-1	639.37 ± 4.3	654.07
2	+1	-1	-1	1399.02 ± 5.1	1486.94
3	-1	+1	-1	1041.10 ± 3.7	1144.21
4	+1	+1	-1	1187.12 ± 3.9	1175.65
5	-1	-1	+1	1142.90 ± 4.5	1221.91
6	+1	-1	+1	1817.49 ± 3.7	1781.92
7	-1	+1	+1	1449.46 ± 4.8	1429.08
8	+1	+1	+1	1134.83 ± 4.4	1187.67
9	- α	0	0	649.10 ± 4.1	576.75
10	+ α	0	0	1097.27 ± 5.2	1074.11
11	0	- α	0	1669.01 ± 3.9	1614.72
12	0	+ α	0	1568.40 ± 4.2	1527.17
13	0	0	- α	1201.27 ± 3.8	1118.32
14	0	0	+ α	1618.48 ± 4.3	1605.92
15	0	0	0	1134.79 ± 4.7	1131.70
16	0	0	0	1130.36 ± 4.5	1131.70
17	0	0	0	1135.32 ± 4.9	1131.70
18	0	0	0	1124.98 ± 3.2	1131.70
19	0	0	0	1125.80 ± 3.8	1131.70
20	0	0	0	1122.56 ± 3.6	1131.70

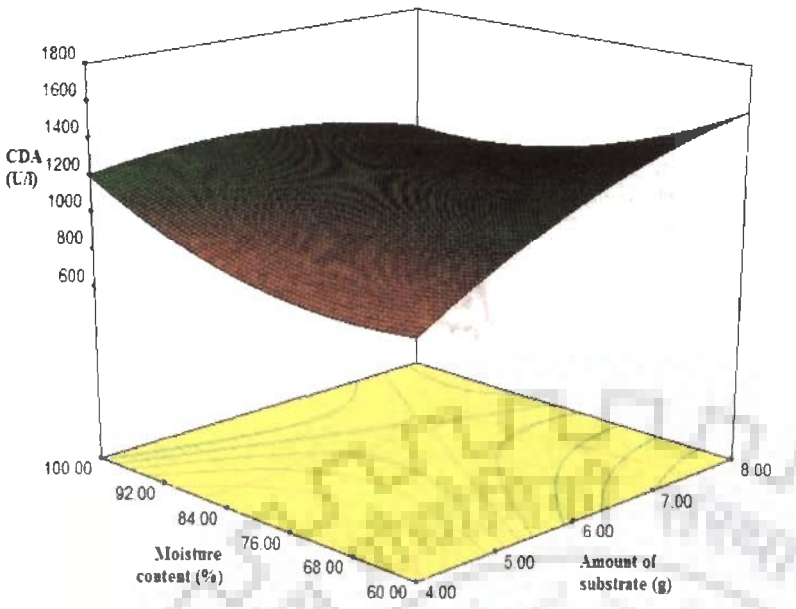
Table 29 Result of multiple regression analyses of CCD for optimization of CDA production under solid-state fermentation

Variables	Coefficient	*F*- value	*P*-value
Intercept	1131.70	-	-
X_1	147.86	63.44	<0.0001
X_2	-26.03	1.97	0.1912
X_3	144.96	60.97	<0.0001
X_1X_2	-200.36	68.23	<0.0001
X_1X_3	-68.21	7.91	0.0184
X_2X_3	-70.74	8.51	0.0154
X_1^2	-108.28	35.90	0.0001
X_2^2	155.30	73.84	<0.0001
X_3^2	81.47	20.32	0.0011

Table 30 ANOVA for quadratic model for optimization of CDA production under solid- state fermentation

Source	SS	DF	MS	*F*-value	*P*-value (Prob>*F*)
Model	1.651E+06	9	1.834E+05	38.98	<0.0001
Residual	47066.72	10	4706.67	-	-
Lack of fit	46923.54	5	9384.71	327.71	<0.0001
Pure error	143.18	5	28.64	-	-
Total	1.698E+06	19	-	-	-

R^2: 0.9723; Adj R^2: 0.9473; C.V.: 5.63 %; Adeq. precision: 24.843



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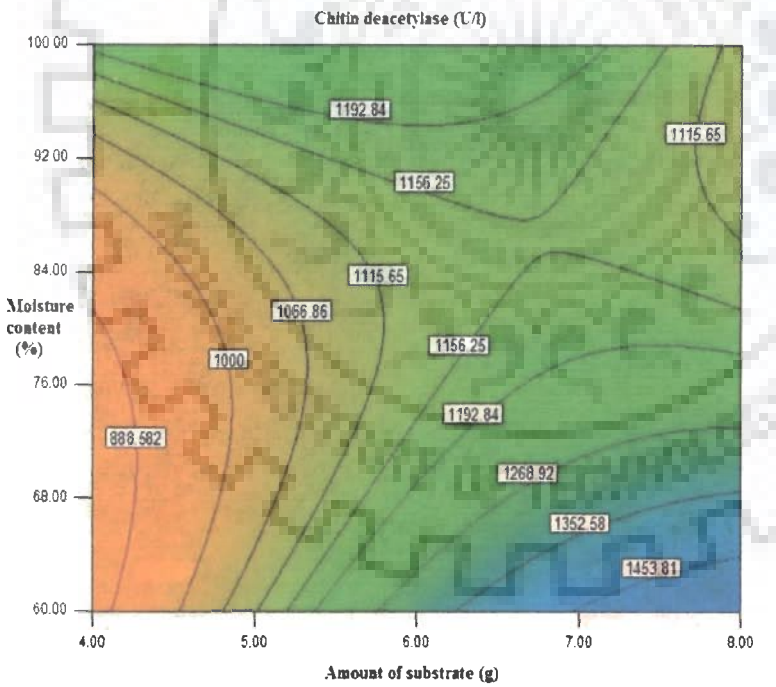
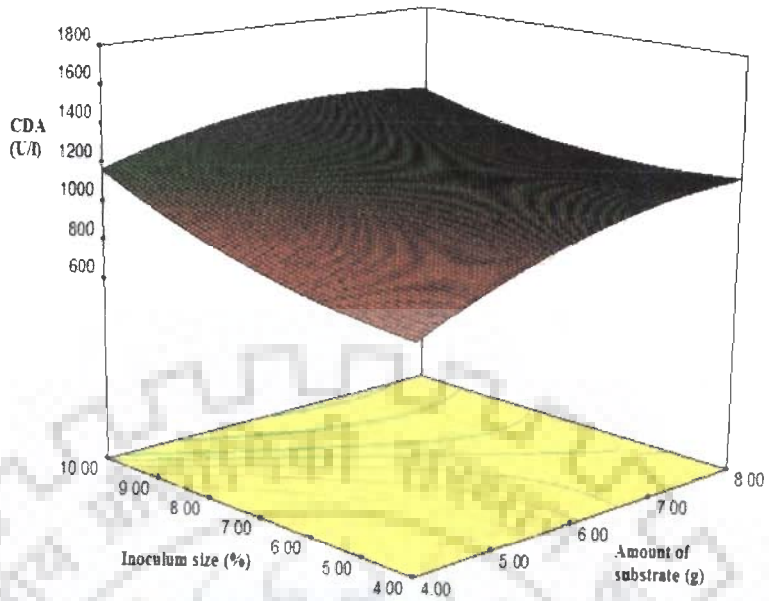


(b)

27 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of amount of substrate and moisture content



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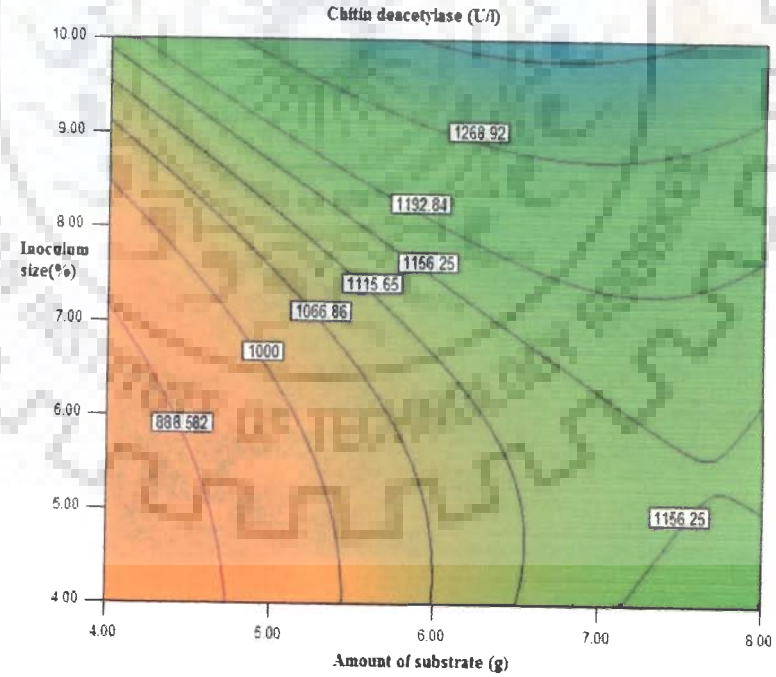
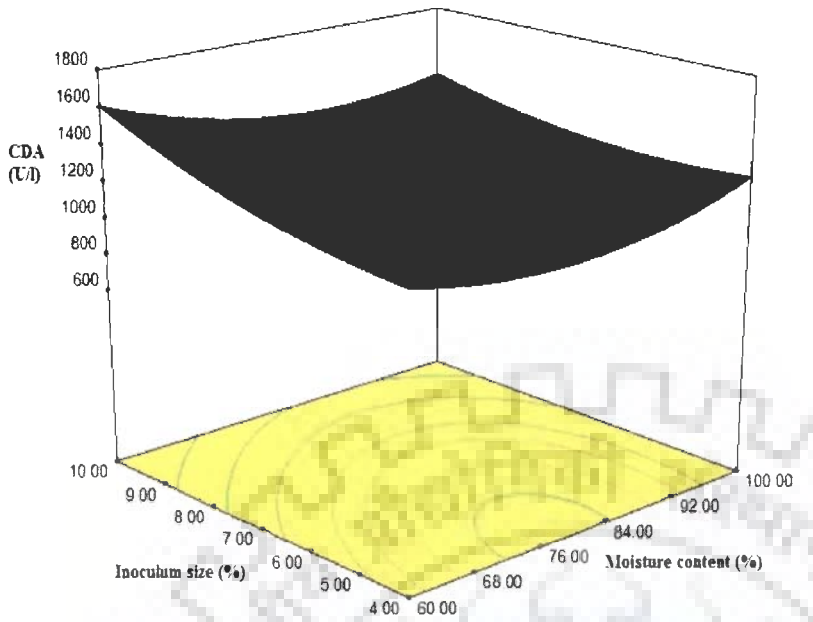
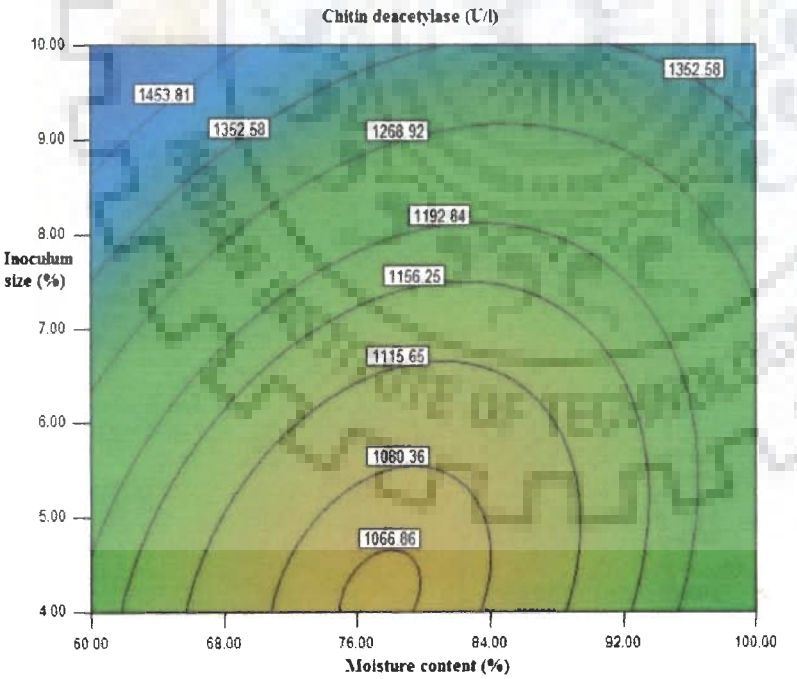


(b)

Fig. 28 Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of amount of substrate and inoculum size



(a)



(b)

29 **Response surface plot (a) and corresponding contour plot (b) of CDA activity as a function of moisture content and inoculum size**

4.5.3.2 *Validation of the model*

Validation of the model and regression equation was performed by conducting the experiments under the derived conditions, as obtained by the model. The predicted response for CDA production (1137.85 Ul^{-1}) (Fig. 30) was in strong agreement with the experimental value (1162.03 ± 7.2 Ul^{-1}), authenticating the validity of the model. A 1.3 fold increase in the production level was obtained when the fermentation was performed under derived conditions as compared to the basal conditions used (875.43 ± 10.4 Ul^{-1}).

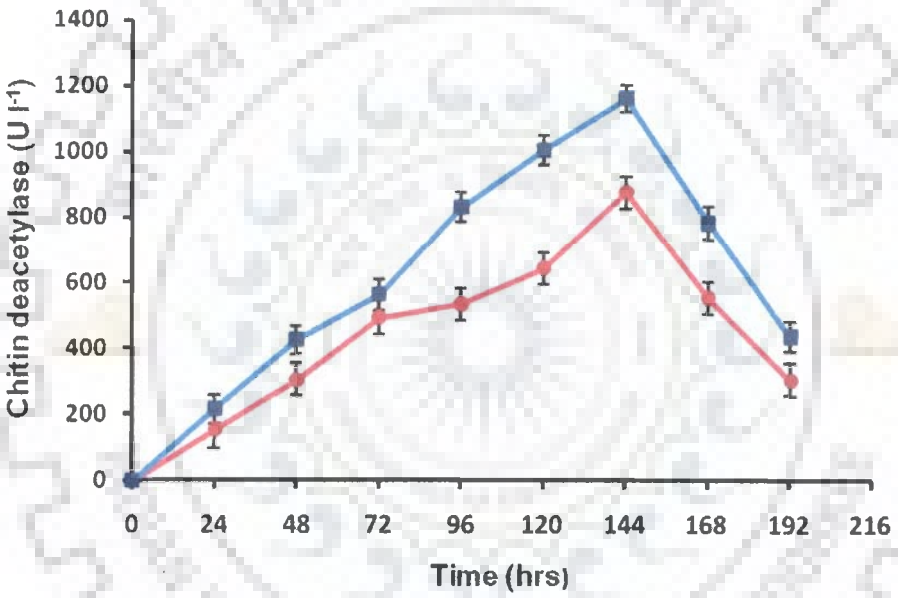


Fig. 30 Chitin deacetylase production at varying time intervals by mutant *P. oxalicum* SAE_M- 51 under solid-state fermentation

(■ , derived conditions; ● , basal conditions)

4.6 PURIFICATION OF CHITIN DEACETYLASE FROM *P. OXALICUM* SAE_M-51

CDA from *P. oxalicum* SAE_M-51 was purified from the culture supernatant to homogeneity using ultrafiltration (Amicon ultra-centrifugal filter unit, 10 kDa molecular weight cut-off, Millipore), cation and anion exchange chromatography. CM-Sepharose chromatography had led into a 9.34 fold increase in the purity with a recovery of 31.86 % of the initial activity (Table 31). In the further step i.e. following DEAE-chromatography the peak activity was obtained at 75 mM NaCl and the enzyme was purified to 88.25 fold with 11.06 % recovery (Table 31). The specific activity had increased during the purification steps from 0.63 to 55.38 Umg^{-1} protein. The purified CDA appeared as a single band with a molecular mass of 53 kDa (Fig. 31) and the pI of purified CDA was observed to be 5.2.

Table 31 Purification of *P. oxalicum* SAE_M-51 CDA from culture supernatant

Purification step	Total activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Yield (%)	Purification fold
Culture supernatant	227.79	363.12	0.63	100	1
Ultrafiltration	125.70	84.80	1.48	54.87	2.37
CM- Sepharose	72.57	12.38	5.86	31.86	9.34
DEAE- Sepharose	21.6	0.39	55.38	11.06	88.25

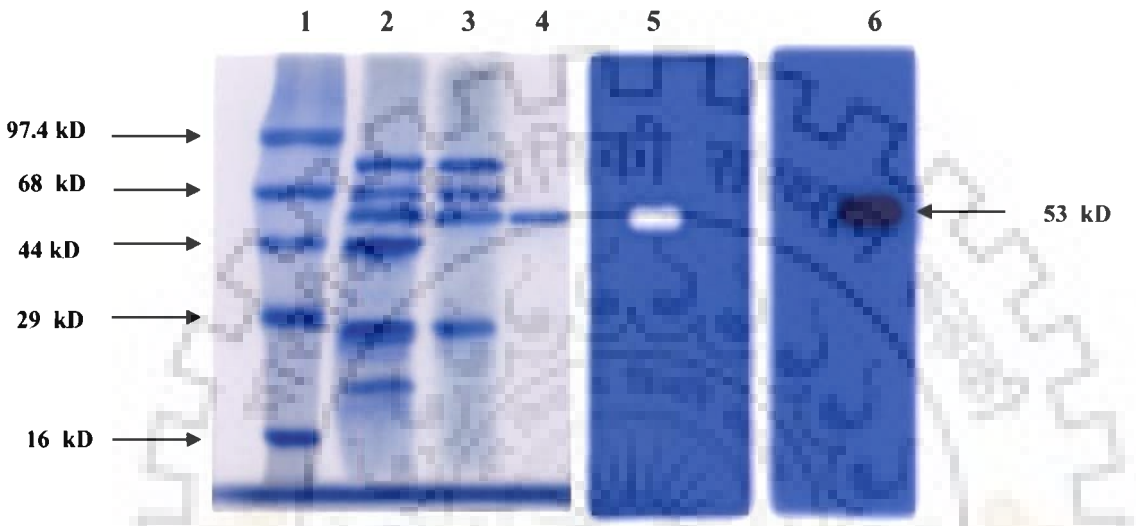


Fig. 31 SDS-polyacrylamide gel and zymogram analysis of purified CDA from *P. oxalicum* SAE_M-51. Lane 1: protein molecular weight markers; lane 2: crude extract; lane 3: fraction obtained after CM-sepharose purification; lane 4: purified CDA following DEAE- sepharose exchange; lanes 5, 6: zymography of purified CDA after incubation of overlay gels containing 0.1 % glycol chitin and following staining with Calcofluor white M2R or by treating with nitrous acid before staining

4.7 CHARACTERIZATION OF PURIFIED CDA OF *P. oxalicum* SAE_M-51

4.7.1 Effect of pH on CDA activity and stability

Purified CDA exhibited maximum activity at pH 9.0, however, notable levels of activity was also detected at pH 6.0 (Fig. 32 a). Enzyme stability analysis had shown that the enzyme retained 100 % of its activity at pH 9.0 after 24 hr of incubation. Further, it retained about 25-55 % of its activity when incubated with buffers of comparatively lower pH of 5.0-7.0 (Fig. 32 b).

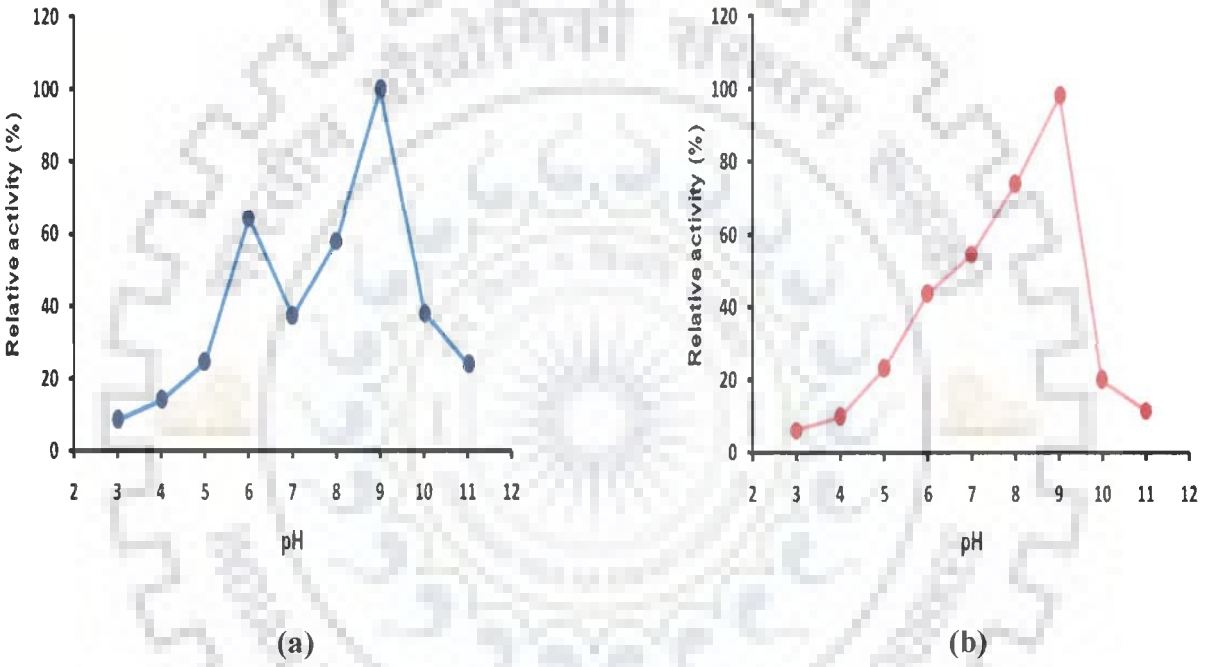


Fig. 32 Effect of pH on the activity (a) and stability (b) of CDA from *P. oxalicum* SAE_M-51

4.7.2 Effect of temperature on CDA activity and stability

4.7.2.1 Kinetics of catalysis

The purified CDA had exhibited optimal activity at 50 ^{0}C. Enzyme activity was reduced when the same was assayed at either lower or higher temperature (Fig. 33). Thermostability of the enzyme was evaluated by studying the time dependent thermal inactivation of enzyme at various temperatures (50-80 ^{0}C). It was observed that the enzyme retained its activity at a wide range of temperature (50-70 ^{0}C) (Fig. 34). Half- life of the enzyme at its optimum temperature was 693.10 min, declining to 53.31 min at 70 ^{0}C (Table 32).

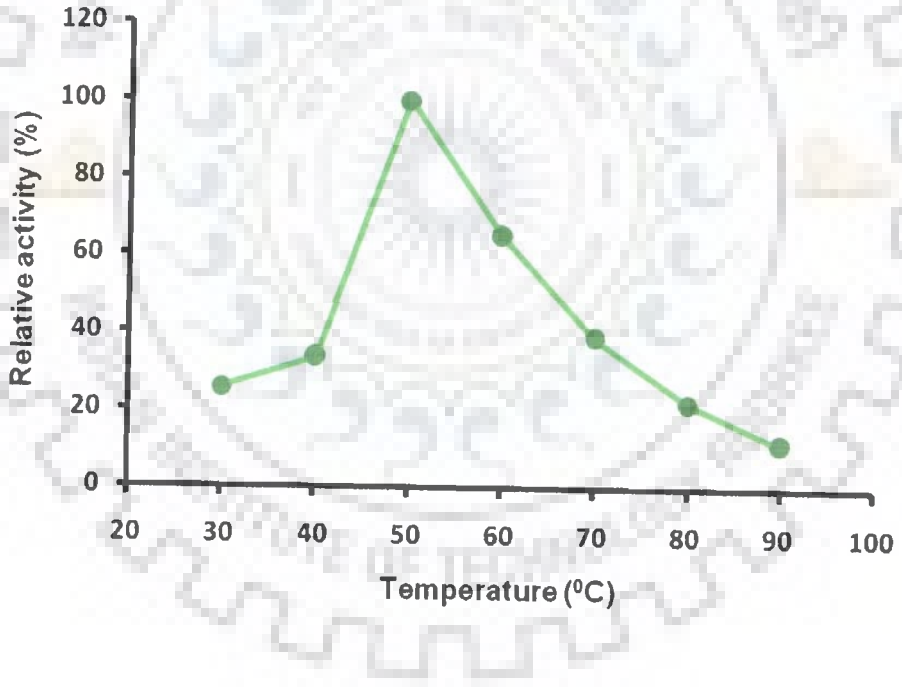


Fig. 33 Effect of temperature on CDA activity from *P. oxalicum* SAE_M-51

4.7.2.2 *Thermodynamics of CDA denaturation*

Stability of the purified enzyme against thermal denaturation can be articulated by studying its thermodynamic parameters viz. half-life of the enzyme, minimum amount of energy needed to denature the enzyme ($E_{a,d}$), the enthalpy of denaturation ($\triangle H_d^*$), the change in the entropy ($\triangle S_d^*$) and the change in the Gibbs free energy ($\triangle G_d^*$) during the thermal denaaturation. Thermal denaturation of enzymes is a two step process, proceeds via formation of an intermediate transition state (U) which could be refolded into its native conformation upon cooling. Complete inactivation of enzymes requires an input of minimum amount of energy i.e. activation energy of denaturation ($E_{a,d}$), which can be determined by Arrhenius plot (ln K_d vs 1/T) . The first-order rate constants (k_d) for thermal denaturation of *P. oxalicum* CDA were determined from the slopes of first-order plots (ln relative activity (%) vs time). The values of k_d at 323, 333, 343, and 353 K were 0.001, 0.004, 0.013 and 0.038 min^{-1}, respectively (Fig. 34). Activation energy for irreversible inactivation '$E_{a,d}$' of the CDA was 114.72 kJ mol^{-1} as calculated by Arrhenius plot (Fig. 35). The thermal inactivation of the enzyme is accompanied by disruption of non-covalent linkages including hydrophobic interactions and opening up of the enzyme structure with concomitant increase in the enthalpy ($\triangle H_d^*$) and entropy ($\triangle S_d^*$) of activation for denaturation. Free energy, enthalpy and entropy of activation for enzyme denaturation at different temperatures are shown in Table 32. It is evident from the Table 31 that Gibbs free energy ($\triangle G_d^*$) of activation for enzyme denaturation is temperature dependent and decreased with a concomitant increase in temperature thereby denoting the decreasing stability of enzyme at higher temperatures. No significant change was observed in the enthalpy ($\triangle H_d^*$) of activation for thermal denaturation of CDA with increasing temperature. Entropy ($\triangle S_d^*$) of activation for complete thermal unfolding of enzyme appeared to be a temperature independent parameter and its positive values implied that the thermal denaturation of the enzyme at higher temperature resulted in an associated increase in disorderliness of the system.

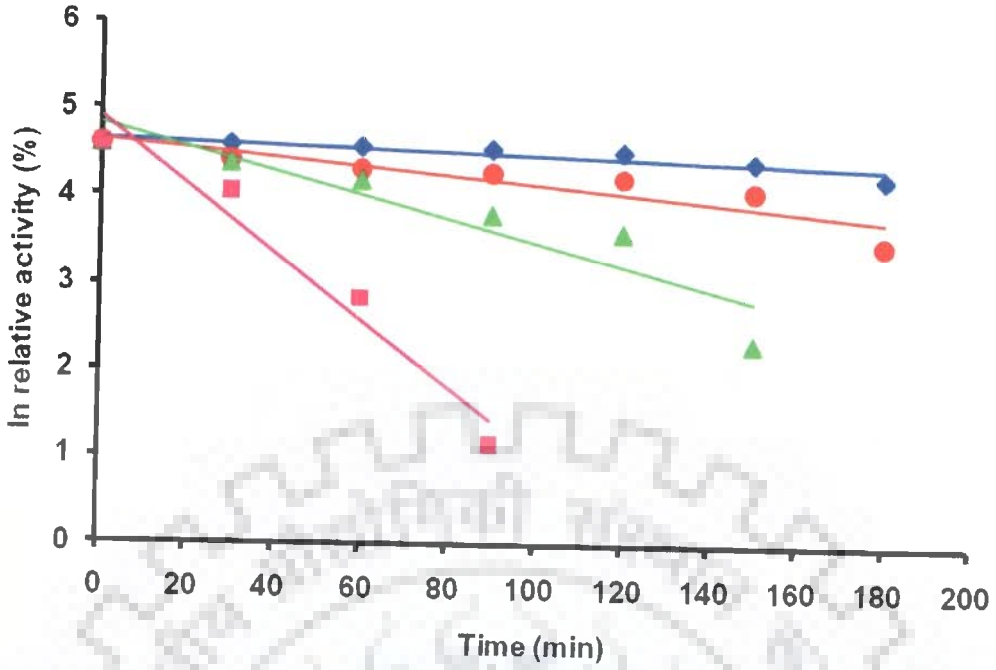


Fig. 34 Pseudo first-order plot for thermal denaturation of CDA from *P. oxalicum* SAE_M-51 CDA (◆ , 50 ^{0}C; ● ,60 ^{0}C; ▲ ,70 ^{0}C; ■ ,80 ^{0}C)

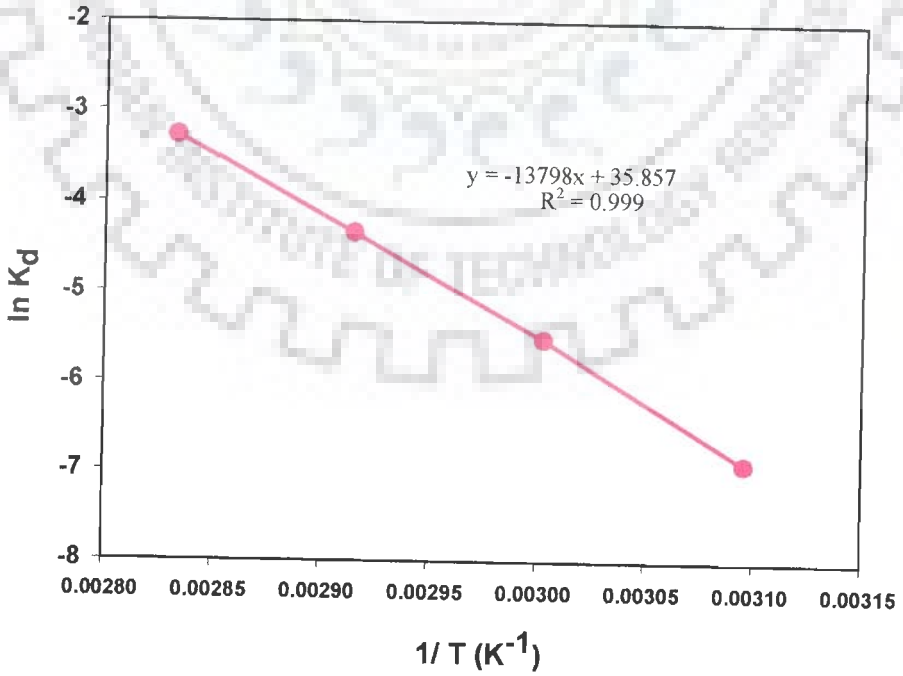


Fig. 35 Arrhenius plot for thermal denaturation of CDA from *P. oxalicum* SAE_M-51

Table . 32 Analysis of parameters during thermal denaturation of CDA from *P. oxalicum* SAE_M-51

T (K)	$t_{1/2}$ (min)	$\triangle G_d^*$ (kJ mol^{-1})	$\triangle H_d^*$ (kJ mol^{-1})	$\triangle S_d^*$ (J mol^{-1} K^{-1})
323	693.10	97.86	112.04	43.93
333	173.27	97.15	111.95	44.44
343	53.31	96.78	111.87	43.99
353	23.10	96.55	111.79	43.20

4.7.3Kinetic parameters

Kinetic parameters viz. K_m, V_{max}, k_{cat} and k_{cat}/K_m and substrate specificity of CDA from *P. oxalicum* SAE_M-51 were determined through Linweaver-Burk plot using various concentrations (0.5 - 5 mg/ml) of five different substrates (glycol chitin, dimeric, trimeric and pentameric forms of GlcNAc and *N*-acetyl glucosamine) (Fig. 36 a - d). The enzyme was not able to deacetylate *N*-acetyl glucosamine, but showed marginal activity towards $(GlcNAc)_2$ and $(GlcNAc)_3$. It was potentially active and had deacetylated chitin oligomers having a degree polymerization to more than four. Enzyme exhibited maximum affinity towards $(GlcNAc)_5$ as evident due to it's lower K_m (3.07 mg ml^{-1}) and higher V_{max} (6.76 µmol min^{-1}) values (Table 33). Values of Michaelis-Menten constant and specificity constant signifies marginal catalytic activity of enzyme towards chitobiose and chitotriose. A simultaneous increase was observed in the enzyme affinity towards oligomers as to their degree of polymerization. The catalytic constant and specificity constant of the enzyme had also followed the similar pattern and had increased along with the degree of polymerization.

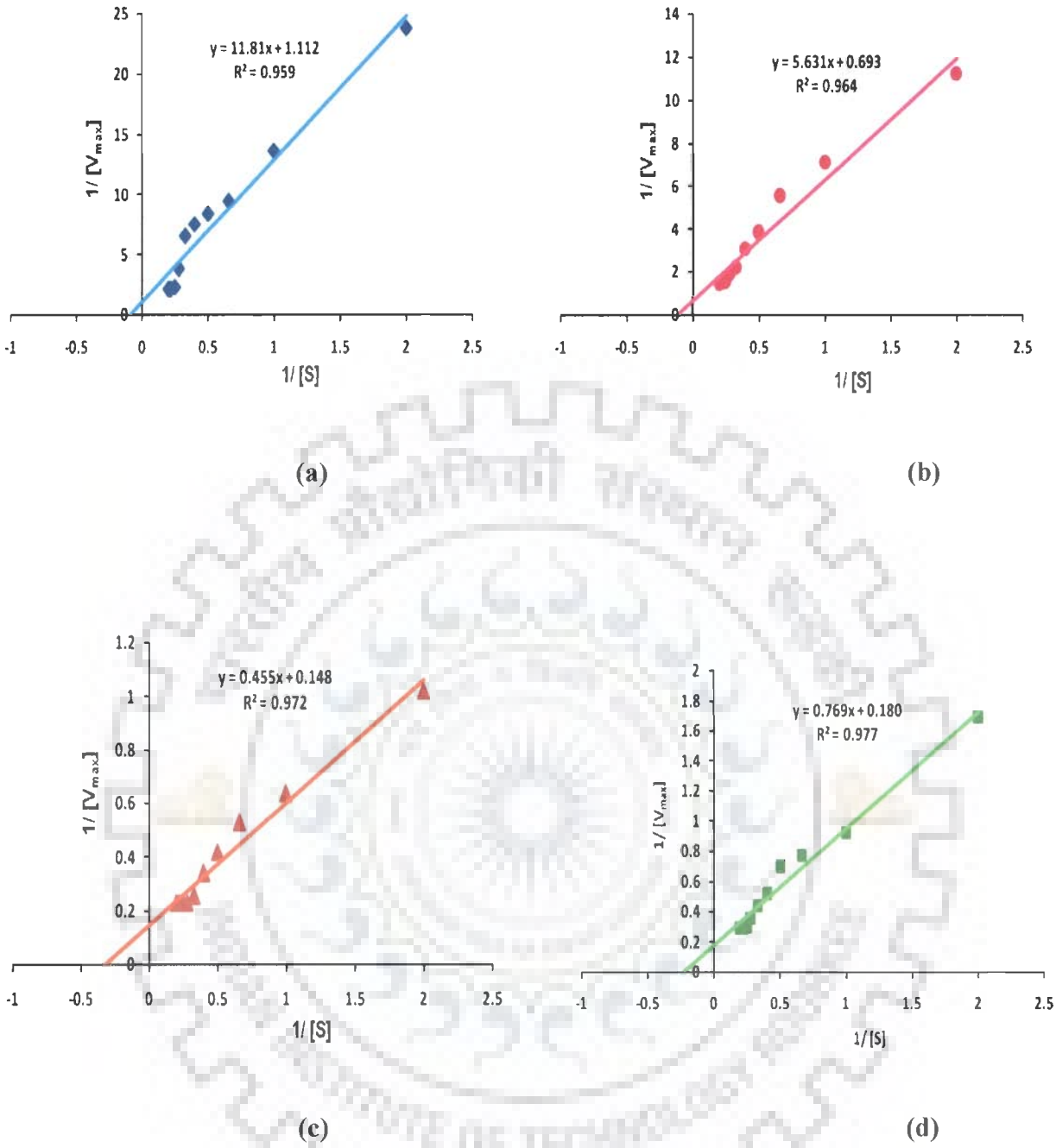


Fig. 36 Double-reciprocal plot for determination of K_m and V_{max} for CDA from *P. oxalicum* SAE$_M$-51 against (a) chitobiose and chitotriose; (b) glycol chitin and chitopentaose (◆, chitobiose; ●, chitotriose; ▲, chitopentaose; ■, glycol chitin)

Table 33 Substrate specificity and kinetic parameters of CDA from *P. oxalicum* SAE_M-51

Substrate	Relative activity (%)	Kinetic parameters			
		K_m (mg ml^{-1})	V_{max} (μmol min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mg ml^{-1} s^{-1})
Glycol chitin	100	4.27	5.55	231.25	54.16
$(GlcNAc)_2$	16.03	10.62	0.89	37.08	3.49
$(GlcNAc)_3$	25.94	8.13	1.44	60.00	7.38
$(GlcNAc)_5$	121.80	3.07	6.76	281.66	91.75

4.7.4 Effect of metal ions

CDA activity was assayed in the presence of various metal ions and EDTA. Among the different metal ions, Cu^{+2}, Co^{+2}, Fe^{+2} and Cd^{+2} led to an increase in the CDA activity (Table 34) whereas enzyme was inhibited by Mo^{+2}, Zn^{+2}, Pb^{+2} and EDTA when added at 1mM concentrations. Inhibition of CDA activity by EDTA had suggested the enzyme to be a metallo-protein.

Table 34 Effect of different metal ions on purified CDA activity

Metal ions (1mM)	Relative activity (%)
None	100
K^{+}	103
$NH4^{+}$	108
Ca^{+2}	110
Cd^{+2}	112
Co^{+2}	118
Cu^{+2}	120
Mn^{+2}	105
Mg^{+2}	111
Zn^{+2}	78
Fe^{+2}	113
Ni^{+2}	102
Li^{+2}	93
Bi^{+2}	88
Ba^{+2}	106
Mo^{+2}	44
Pb^{+2}	81
EDTA	91

4.7.5 Effect of acetate on CDA activity

Sensitivity of enzyme towards end product inhibition was evaluated by assaying the enzyme activity in presence of acetate ranging from 0 to 100 mM concentration. Almost > 85 % of the enzyme activity was retained in presence of 0-70 mM sodium acetate, it declined thereafter with an increase in acetate concentration (Fig. 37). Sodium acetate at 80 – 100 mM led

into 30- 80 % loss in activity; however 50 % of the activity was retained at 90 mM concentration.

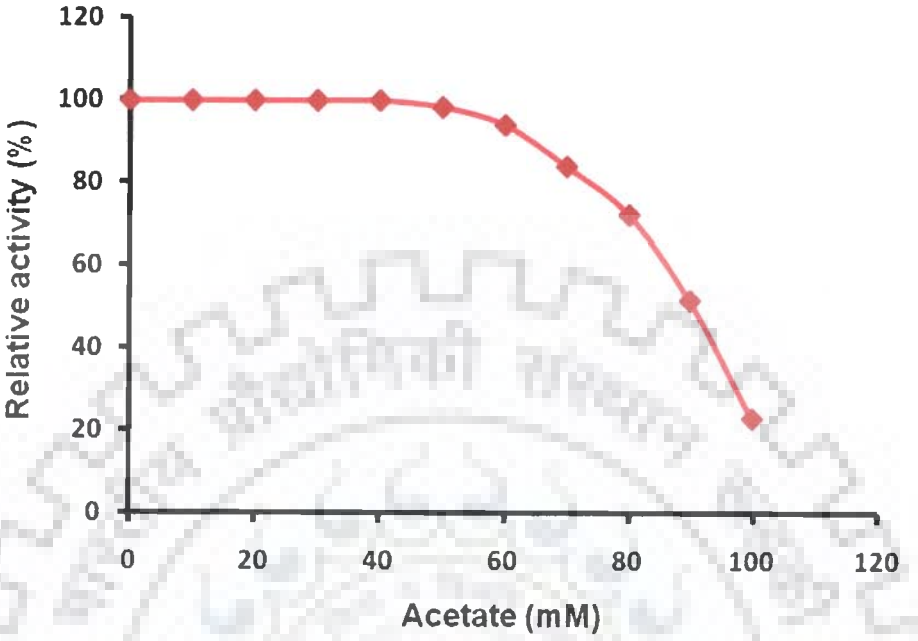


Fig. 37 Effect of acetate ions on CDA activity from *P. oxalicum* SAE_M-51

4.7.6 Circular dichroism spectroscopy

To get an insight into the secondary structural elements and conformational stability of the CDA from *P. oxalicum* SAE_M-51, far-UV CD spectra were recorded at different pH (Fig. 38). Analysis of the far-UV CD spectra of CDA had revealed that the elements of secondary structure are sensitive to the pH. The results suggest that CDA is α, β protein, rather than predominantly β-protein. CDA contains 56.26 % α-helix and 15.63 % β-structure at its optimal pH (Table 35). The ellipticity values at 222 and 217 nm, characteristic for α-helix and β- sheet conformation, respectively, varied with increasing pH (6, 7, 9, 10, 12) and significantly differ from the values observed at lower pH (2 and 4).

Table 35 Secondary structural characteristics of chitin deacetylase at different pH values

pH	α-helix (%)	β-sheet (%)	Random coil (%)
2.0	8.42	17.80	73.78
4.0	14.18	19.24	66.58
6.0	30.56	26.36	43.08
7.0	42.41	28.31	29.28
9.0	56.26	15.63	28.11
10.0	47.13	11.52	58.65
12.0	39.37	5.69	54.94

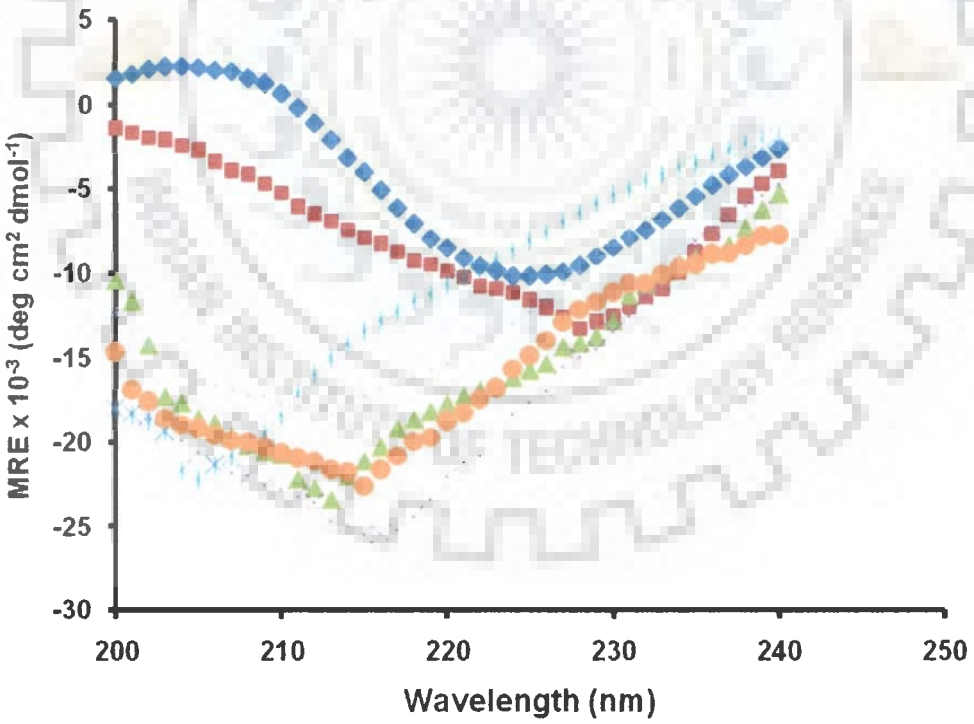


Fig. 38 Far UV-CD spectra of chitin deacetylase from *P. oxalicum* SAE_M-51 as a function of pH (◆ , 2; ■ , 4; ▲ , 6; × , 7; * , 9; ● , 10; + , 12)

4.7.7 Determination of internal peptide sequence

The internal amino acid sequencing of CDA was performed by MALDI-ToF/ToF analysis to find out the pattern of homology the enzyme has with having deacetylase activity. Enzyme from *P. oxalicum* SAE_M-51 possesses resemblance to members of carbohydrate esterase family 4 that include CDAs, rhizobial NodB chitooligosaccharide deacetylase, peptidoglycan *N*-acetylglucosamine deacetylases, acetyl xylan esterases and xylanases A, C, D, E. CDA from *P. oxalicum* SAE_M-51 exhibited significant levels of identity with other CDAs of fungal and yeast origin (*Emericella nidulans*, 54 %, *Saccharomyces cerevisiae*, 50 %; *Rhizopus stolonifer*, 45 %). Some of the peptides also had identity with bacterial deacetylases (*Streptosporangium roseum*, 54 %; *Pectobacterium cartovorum*, 37 %). Peptide 7 showed low level of identity with chitin oligosaccharide deacetylase and acetyl xylan esterase (*Thermobispora bispora*, 32 %; *Vibrio parahaemolyticus*, 26 %) (Table 36).

Table 36 Sequence homology analysis for internal peptides of CDA from *P. oxalicum* SAE_M-51

Accession no	Organism		Sequence		Identity %
Peptide 1	*Penicillium oxalicum*	379	TLQEQEEDVAA	389	-
ZP_07055193.1	*Bacillus cereus*	557	TLQEQEVVNDN	567	54
AAX11701.1	*Rhizopus stolonifer*	87	TSAEVKAAVAA	97	45
AAN 65362.1	*Gongronella butleri*	167	LQEQNLRASM	176	36
Peptide 2	*Penicillium oxalicum*	244	ALTKAAMGGSLE	255	-
YP_002282034.1	*Rhizobium leguminosarum*	223	ALTKAAMAG	231	66
AAT68493.1	*Glomerella lindemuthiana*	09	AAATAALAGSTN	20	42
AAK84438.1	*Blumeria graminis*	102	ALTFDDGPYTSHI	114	33
BAI52768.1	*Magnaporthe grisea*	09	AWLAAAALVSAH	20	33
Peptide 3	*Penicillium oxalicum*	329	APLLESLSVKFASPP	343	-
ZP_03502150.1	*Rhizobium etli*	89	APLIERLGIKFENSPP	106	47
ACB54958.1	*Helicoverpa armigera*	34	APKKDSLEVELCKD	47	33
XP_002793041.1	*Paracoccidioides brasiliensis*	225	HQDLESLSAEQRR	237	33
XP_003175982.1	*Arthroderma gypseum*	307	GRPLLEIGHDVHEQ	320	26
Peptide 4	*Penicillium oxalicum*	298	GVGCIDTELLDR	309	-
ZP_06114571.1	*Clostridium hathewayi*	103	IDTELLDR	110	66
EDN59221.1	*Saccharomyces cerevisiae*	126	GPSASTTKLLDR	137	50
XP_750931.1	*Aspergillus fumigatus*	142	RYTADLLDLLDR	153	33
Peptide 5	*Penicillium oxalicum*	277	STYELLELLTL	287	-
ACF22100.1	*Emericella nidulans*	69	YTEE LLEDLA	78	54
AAW50596.1	*Volvariella volvacea*	196	YTQT LLNLTE	205	45
XP_001394100.1	*Aspergillus niger*	142	YTQDLLDLLD	151	36
Peptide 6	*Penicillium oxalicum*	213	FDVSELWWDRESR	225	-
YP_003343379.1	*Streptosporangium roseum*	207	DVDT LWRDRDS	217	54
EEH48938.1	*Paracoccidioides brasiliensis*	370	FSIPGR WWSNT SR	382	38
XP_002911435.1	*Coprinopsis cinerea okayama*	174	NDASRCWWTCGG	185	31
Peptide 7	*Penicillium oxalicum*	248	AAMGGSLESPIYPRLGGIQ	266	-
ZP_03833676.1	*Pectobacterium cartovorum*	06	AATMYGIESNYPRDLIGYA	24	37
YP_003652570.1	*Thermobispora bispora*	168	AARSHPAVDPERIIVAGIQ	186	32
YP_003937480.1	*Clostridium sticklandii*	51	FDDGGSEENVKSVLETLDK	69	26
BAF65669.1	*Vibrio parahaemolyticus*	236	IAMPANSLTEAEPFLGYVD	254	26

4.8 EVALUATION OF DEACETYLATION OF CHITIN BY PURIFIED CDA FROM *P. OXALICUM* SAE_M-51

Crystallinity and insolubility of the chitin are the two major barriers for its bio-catalytic deacetylation to chitosan. Therefore, in order to obtain chitosan species with higher degree of deacetylation (DDA), a two-stage process, consisting of chemical and enzymatic treatments was adopted. Crab shell chitin was first subjected to different chemical treatments to open up the polymer structure which in turn increases the substrate accessibility for the enzyme.

4.8.1 Effect of chemical treatment on crab shell chitin

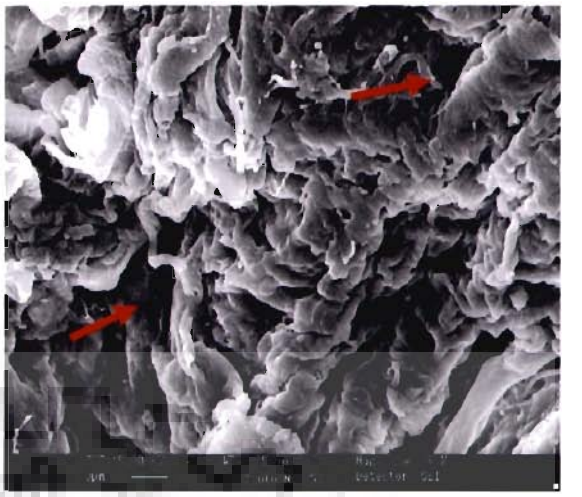
Crab shell chitin was subjected to pretreatment with acids, alkali and calcium chloride/methanol solvent system to deform its regular crystalline microfibrillar structure so that both exterior as well as interior acetyl groups will become accessible to the enzyme. Various morphological and physical variations in the parent polymer were observed following chemical treatment.

4.8.1.1 Scanning electron microscopic analysis of structural properties

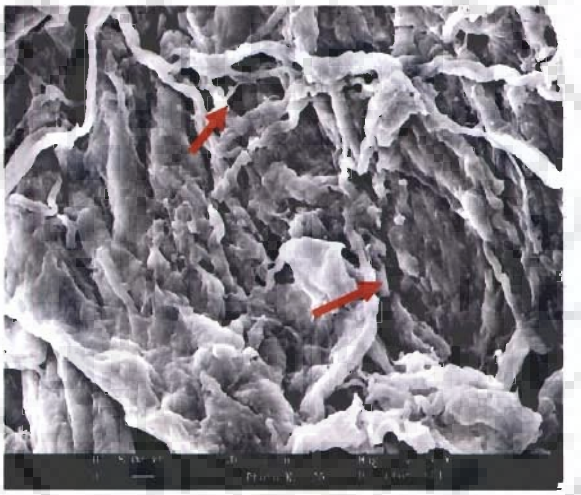
Morphological features of chitin before and following chemical pretreatment were analyzed using scanning electron microscopy. Commercial chitin from crab shell has a compact structure with some distinctly arranged crystalline microfibrils visible at its surface (Fig. 39 a). Both superfine (Fig. 39 b) and amorphous chitin (Fig. 39 c) appeared as the most promising substrates for their further bio-catalytic deacetylation. The substrates loose their characteristic crystalline microfibrillar arrangement and have a porous structure which would allow the enzyme to act on acetyl groups. Marginal porosity of the material makes it a poor substrate for enzymatic deacetylation. Phosphoric acid treated (Fig. 39 d) and colloidal chitin (Fig. 39 e) had almost similar

stature. The characteristic microfibrillar structure is visible in both the substrates but the level of complexity is somewhat low as some major and minor grooves are clearly observed between fibrils in the micrographs due to the deformation of the crystalline structure of the chitin.

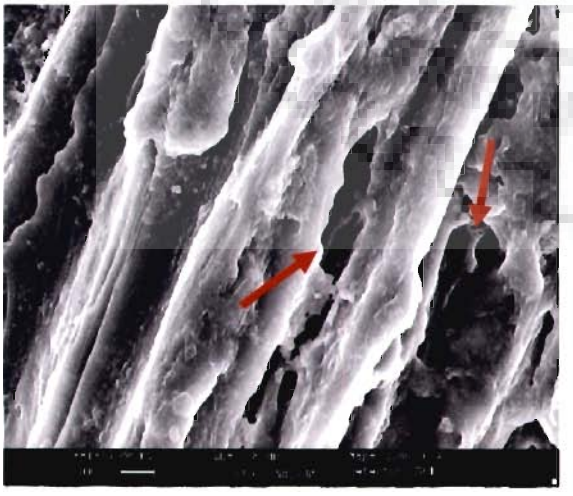
(a)



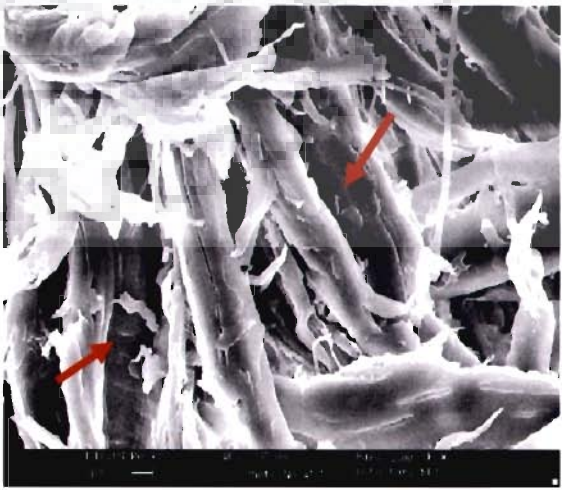
(b)



(c)



(d)



(e)

Fig. 39 Scanning electron microscopic analysis of crab shell chitin (a), superfine chitin (b), amorphous chitin (c), phosphoric acid treated (d) and colloidal chitin (e)

4.8.1.2 X-ray diffraction analysis

Chitin in its native state has a characteristic higher degree of crystallinity, which lowers its susceptibility towards enzymatic deacetylation. Chemical pretreatment had resulted into significant loss of chitin crystalline structure with generation of new amorphous chitin variants. Crystallinity of chitin before and following treatment was determined using wide angle X-ray diffraction analysis in terms of crystalline index (CrI_{020}, CrI_{110}) (Table 37). Fig. 40 (a-e) showed diffraction patterns of treated and untreated chitin. X-ray diffractograms of all the samples exhibited characteristic crystalline peaks of chitin at $2\theta \sim 9\text{-}10^{o}$ and $19\text{-}20^{o}$. Some minor peaks were also observed at 27.31, 25.91, 32.41, 34.31 and 26.21^{0} in X-ray diffractograms of the crab shell chitin, PA-CT, SF-CT, Am-CT and colloidal chitin, respectively. Crystalline index of the samples was determined using peak intensities at $9\text{-}10^{o}$ and $19\text{-}20^{o}$ which were observed to decrease following chemical treatment.

High crystalline index of crab shell chitin showed its higher degree of crystallinity and more ordered structure. CrI_{020} values calculated using peak intensity at $2\theta \sim 9\text{-}10^{o}$ dictated Am-CT as the most promising substrate for enzymatic deacetylation. However, CrI_{110} values based on intensity of peaks at $2\theta \sim 19\text{-}20^{o}$ showed SF-CT as the most accessible substrate for enzyme action. Crystallinity of PA-CT and colloidal chitin was also observed to be significantly reduced (□ 70 %). The order of crystallinity observed after chemical treatment was crab shell chitin > PA-CT > Co-CT > SF-CT > Am-CT.

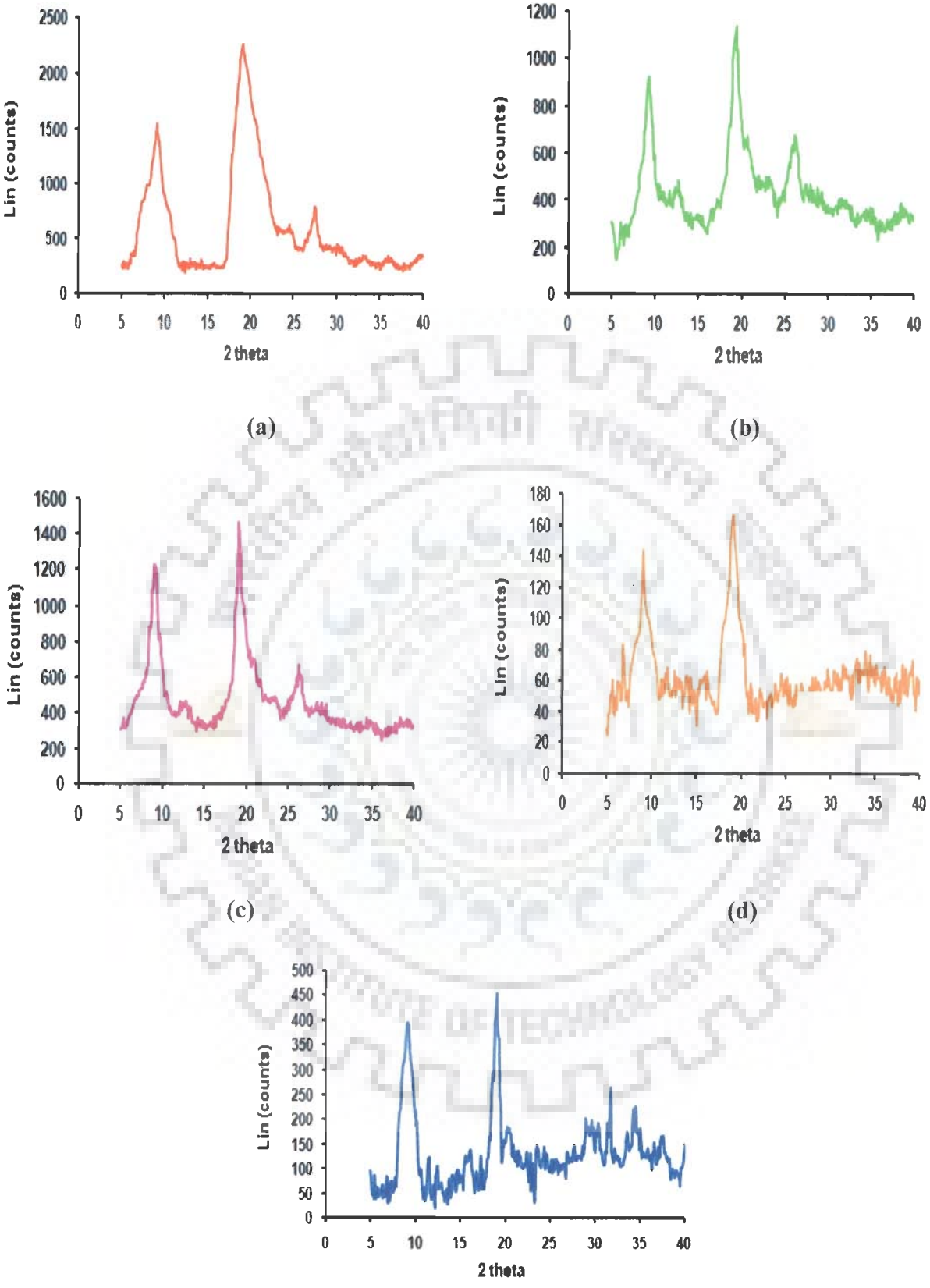


Fig. 40 X-ray diffraction analysis of crab shell chitin (a), phosphoric acid treated chitin (b), colloidal chitin (c), superfine chitin (d) and amorphous chitin (e)

Table 37 X- ray diffraction analysis and crystalline index of crab shell chitin before and following chemical treatment

Chitin samples	2θ (^{0}C)	*d*- spacing (^{0}A)	CrI_{020} (%)	CrI_{110} (%)
Crab shell chitin	9.21, 19.30, 27.31	9.58, 5.64, 3.09, 2.94	84.01	89.08
PA-CT	9.01, 11.70, 19.21, 25.91	9.58, 4.61, 4.27, 3.39	71.86	74.85
SF-CT	9.10, 19.11, 32.41	9.29, 4.58, 3.23	66.66	71.08
Am-CT	9.11, 19.07, 31.71, 34.31	9.87, 7.68, 5.12, 3.91	64.94	69.97
Colloidal chitin	9.20, 12.11, 19.00, 26.21	9.50, 6.94, 4.57, 3.37	68.64	73.72

(PA-CT: Phosphoric acid treated chitin; SF-CT: Superfine chitin; Am-CT: Amorphous chitin)

4.8.1.3 Thermal analysis

Thermal properties of chitin samples following chemical treatment have been studied using TGA and DSC profile from 25 to 600 ^{0}C. The TGA thermograms of various chitin substrates are shown in Fig. 41. Thermograms of all the chitinous substrates have two main decomposition stages. The first stage decomposition started at around 70 ^{0}C with weight loss of about 5.9, 6.2 , 8.8, 9.4, 8.3 % for crab shell chitin, PA-CT, SF-CT, Am-CT and colloidal chitin, respectively. This stage is due to the loss of water content in the polysaccharide backbone. The second stage started at 270, 260, 250, 240, 220 ^{0}C for crab shell chitin, PA-CT, colloidal chitin, SF-CT and Am-CT with corresponding weight loss of 66.7, 71.7, 71.9, 73.6, 74.4 %. This was due to the decomposition of the polysaccharide backbones, including acetylated and deacetylated subunits of chitin. The TGA results had indicated that crab shell chitin has highest thermal stability and following chemical treatment decrease in stability was observed along with the loss of its crystallinity.

The thermal properties of treated and untreated chitin samples were also studied using DSC from 30 to 350 ^{0}C. DSC curves of samples had shown a wide endothermic peak centered between 50 – 200 ^{0}C (Fig. 42). The onset temperatures were in the range of 52 – 188 ^{0}C. Table 38 showed amount of thermal decomposition enthalpy (Δ H) of chemically treated and untreated chitins. The amount of peak enthalpy correlates with the crystallinity of the structure. Higher enthalpy value for crab shell chitin supports its crystalline nature. Chemical treatment would result in the decrystallization of the compact structure and hence a corresponding decrease in enthalpies was observed. Lowest enthalpy values of SF-CT indicated that its amorphous structure and susceptibility towards thermal decomposition. Peak enthalpy values were observed to increase in the order i.e. Am-CT < SF-CT < colloidal chitin < PA-CT < crab shell chitin. Thermograms of chitin moieties before and following treatment revealed differences in endothermic peak area and positioning, indicating that the chemical treatment

brings changes in the molecular structure of the polysaccharides and all the molecules exhibit varying levels of thermal stability.

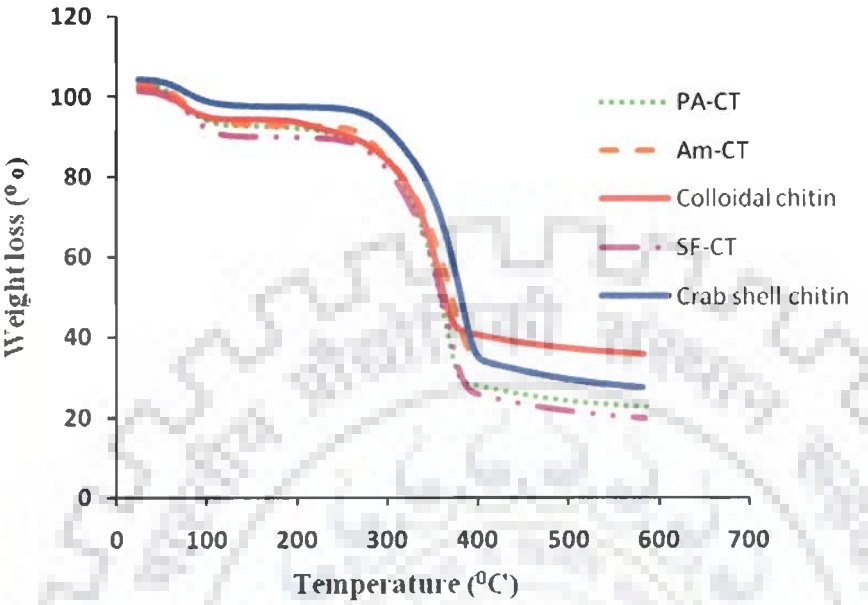


Fig. 41 TGA thermograms of crab shell chitin before and following chemical treatment

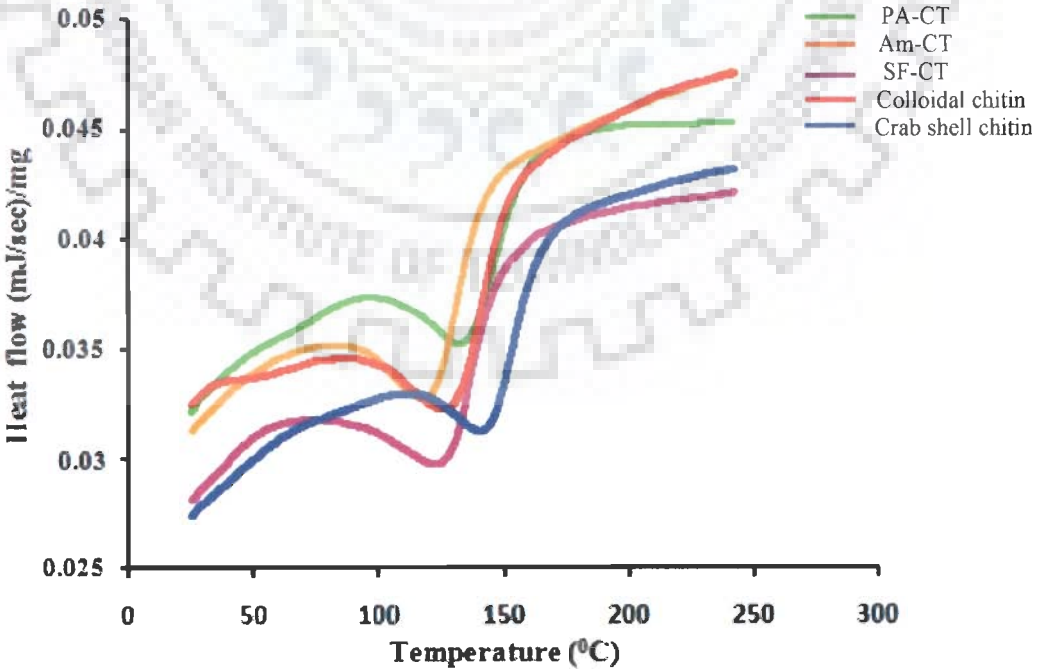


Fig. 42 DSC thermograms of crab shell chitin before and following chemical treatment

Table 38 Differential scanning calorimetric analysis of crab shell chitin before and following chemical treatment

Substrate	Endotherm			
	T_o (^{0}C)	T_p (^{0}C)	T_c (^{0}C)	ΔH (mJ mg^{-1})
Crab shell chitin	124 ± 1.13	148 ± 1.72	188 ± 2.32	172.00 ± 0.82
PA-CT	102 ± 1.28	136 ± 1.51	179 ± 2.71	122.31 ± 0.56
Am-CT	84 ± 1.41	121 ± 1.83	168 ± 1.95	95.10 ± 0.71
SF-CT	52 ± 1.21	116 ± 1.73	152 ± 1.60	77.20 ± 0.78
Colloidal chitin	100 ± 1.16	128 ± 1.52	161 ± 1.78	104.54 ± 0.91

(T_o, onset temperature; T_p, peak temperature; T_c, completion temperature; ΔH (mJ mg^{-1}), peak enthalpy
Each value is expressed at mean ± SE (n=3)

4.8.1.4 Degree of deacetylation

Chemical treatment had resulted into opening up of the polymer backbone along with possibly lower extent of deacetylation of commercial crab shell chitin. DDA of chitin before and after treatment was determined using elemental analysis and FTIR. FTIR spectra of samples showed characteristic peaks of amide I (1655 cm^{-1}) and amide II (1560 cm^{-1}) with subtle differences in absorption intensities due to chemical treatment (Fig. 43 a-e). The characteristic amide I peak appeared at 1658, 1656, 1654 and 1652 cm^{-1} in FTIR spectra of PA-CT, SF-CT, Am-CT and colloidal chitin, respectively. Furthermore, the vibrational mode of amide II was observed at 1558, 1557, 1559 and 1568 cm^{-1}. The peak appeared at 3450 cm^{-1} corresponds to -OH groups. The absorption intensities of these peaks were used to determine the DDA. Chitin from crab shell was observed to have about 28% of deacetylation. Extent of chitin deacetylation was significantly increased following various chemical treatments, due to the loss in ordered crystalline structure of chitin with opening up of polymer backbone and progressive removal of both external and internal acetyl groups. DDA of PA-CT and colloidal chitin was observed to be 32 and 33%, respectively. Am-CT generated via alkali treatment had marginally higher (34.44 %) level of deacetylation. Maximum deacetylation (37.14 %) was observed with superfine chitin obtained following calcium chloride/formic acid treatment. DDA analyzed by FTIR and elemental analysis are almost similar (Table 39).

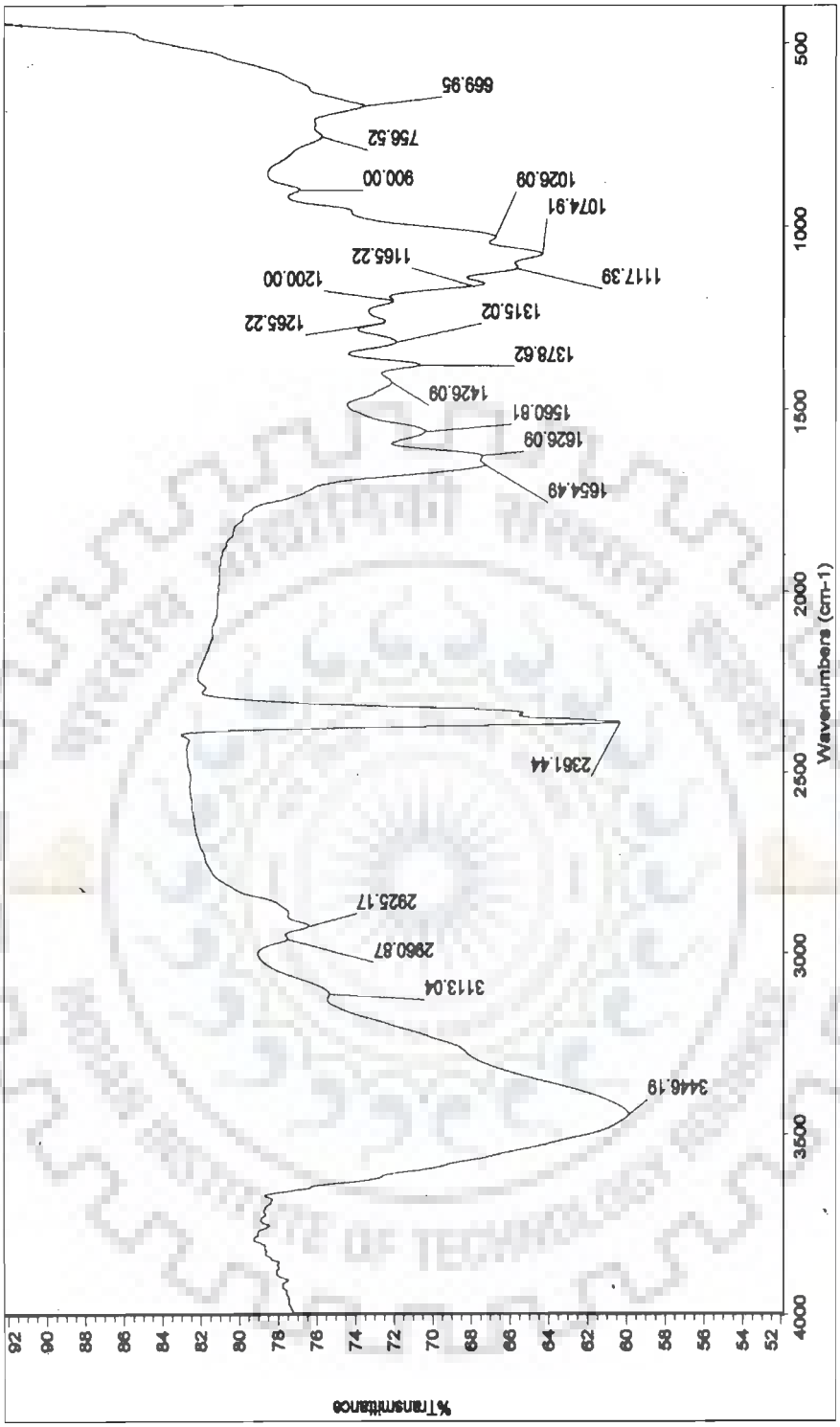


Fig. 43a FTIR specta of crab shell chitin

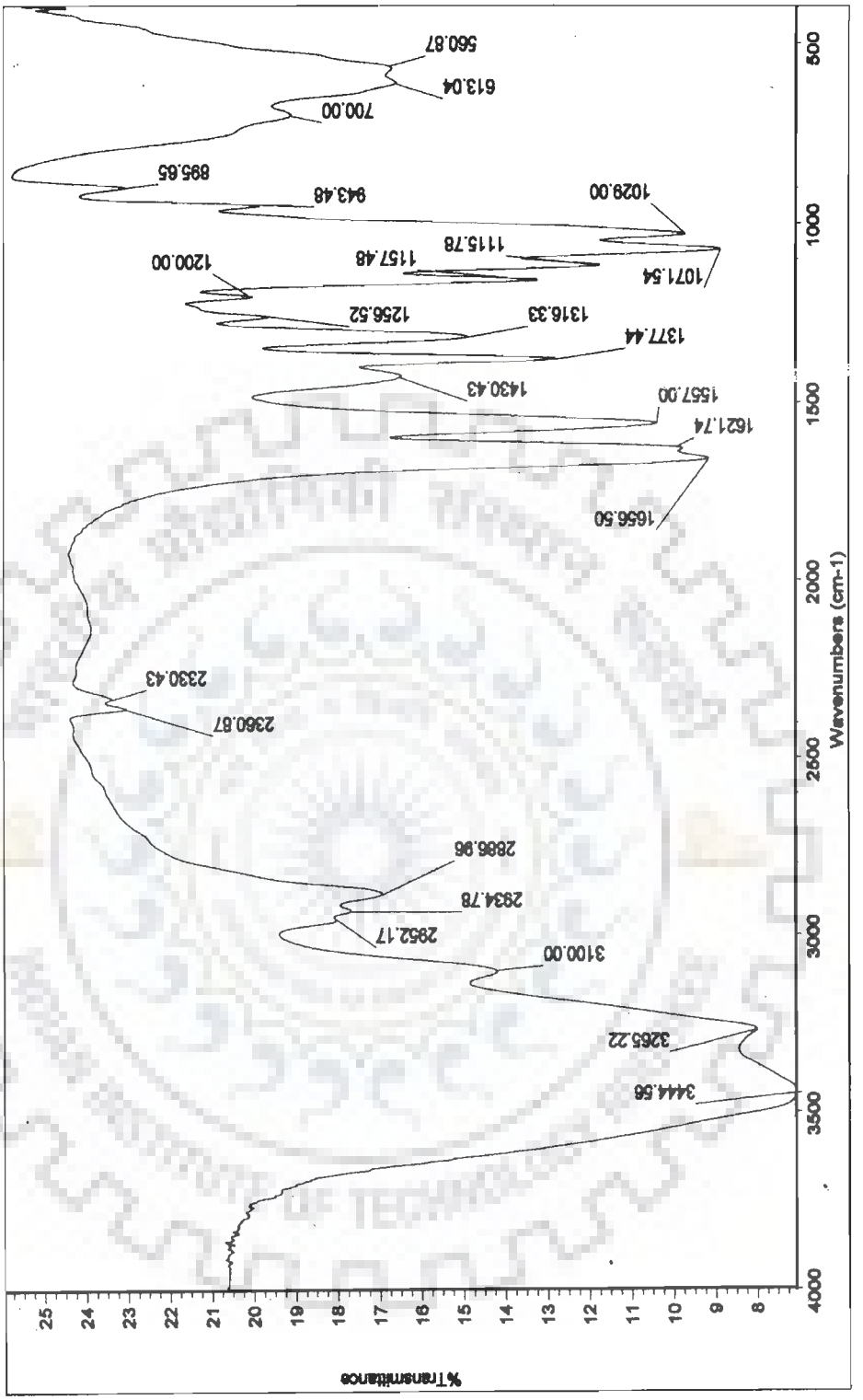


Fig. 43b FTIR spectra of superfine chitin

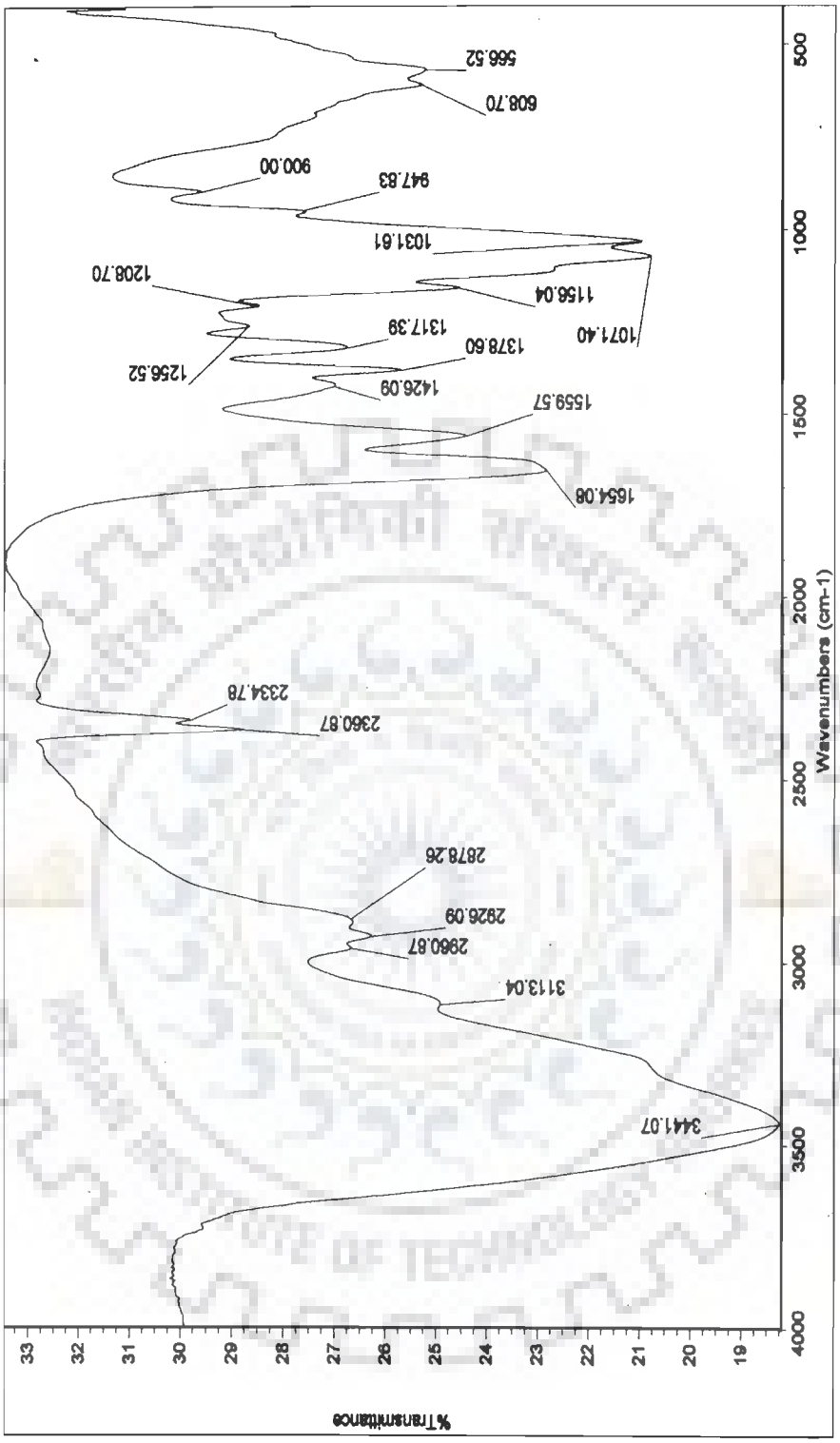


Fig. 43c FTIR spectra of amorphous chitin

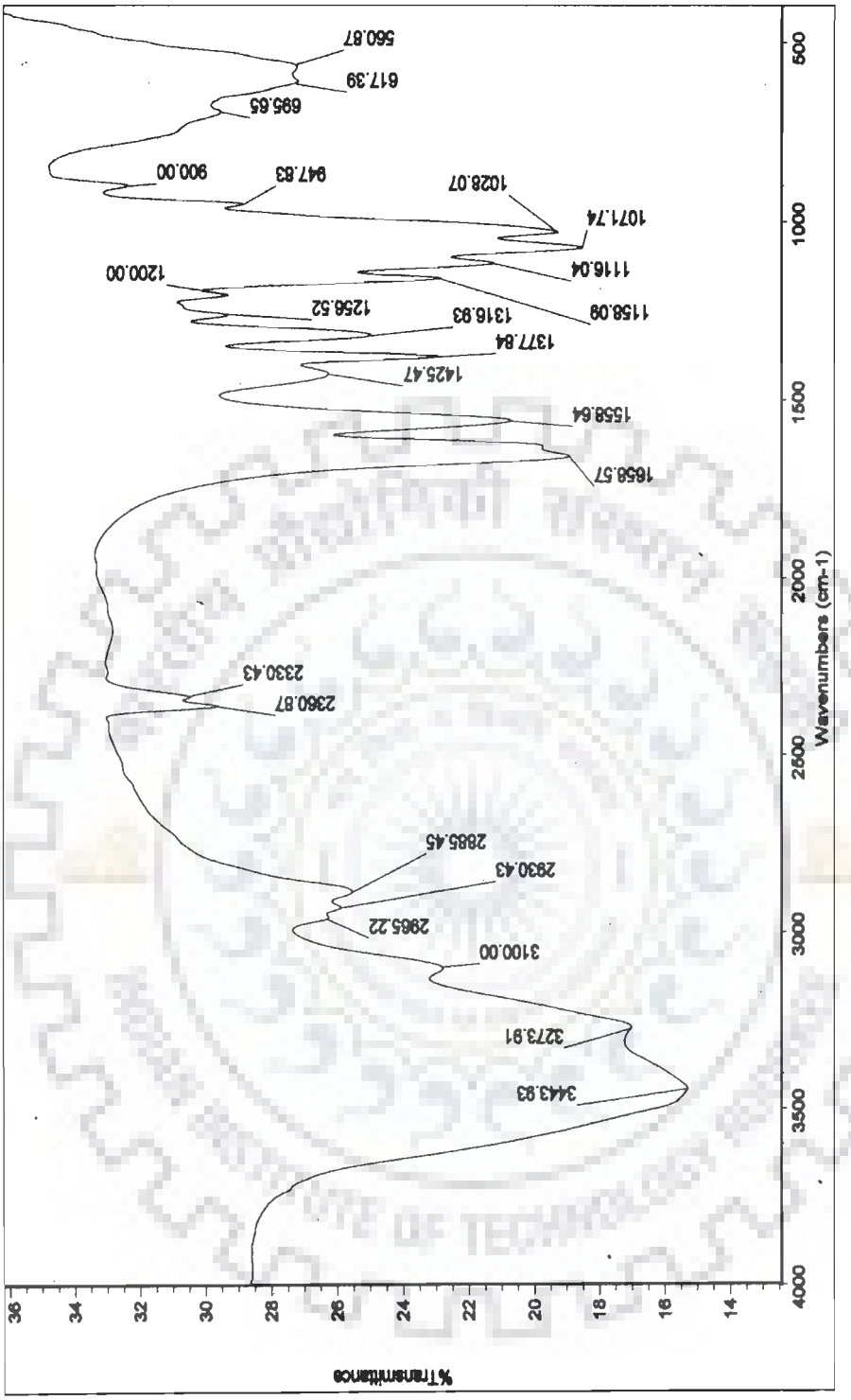


Fig. 43d FTIR spectra of phosphoric acid treated chitin

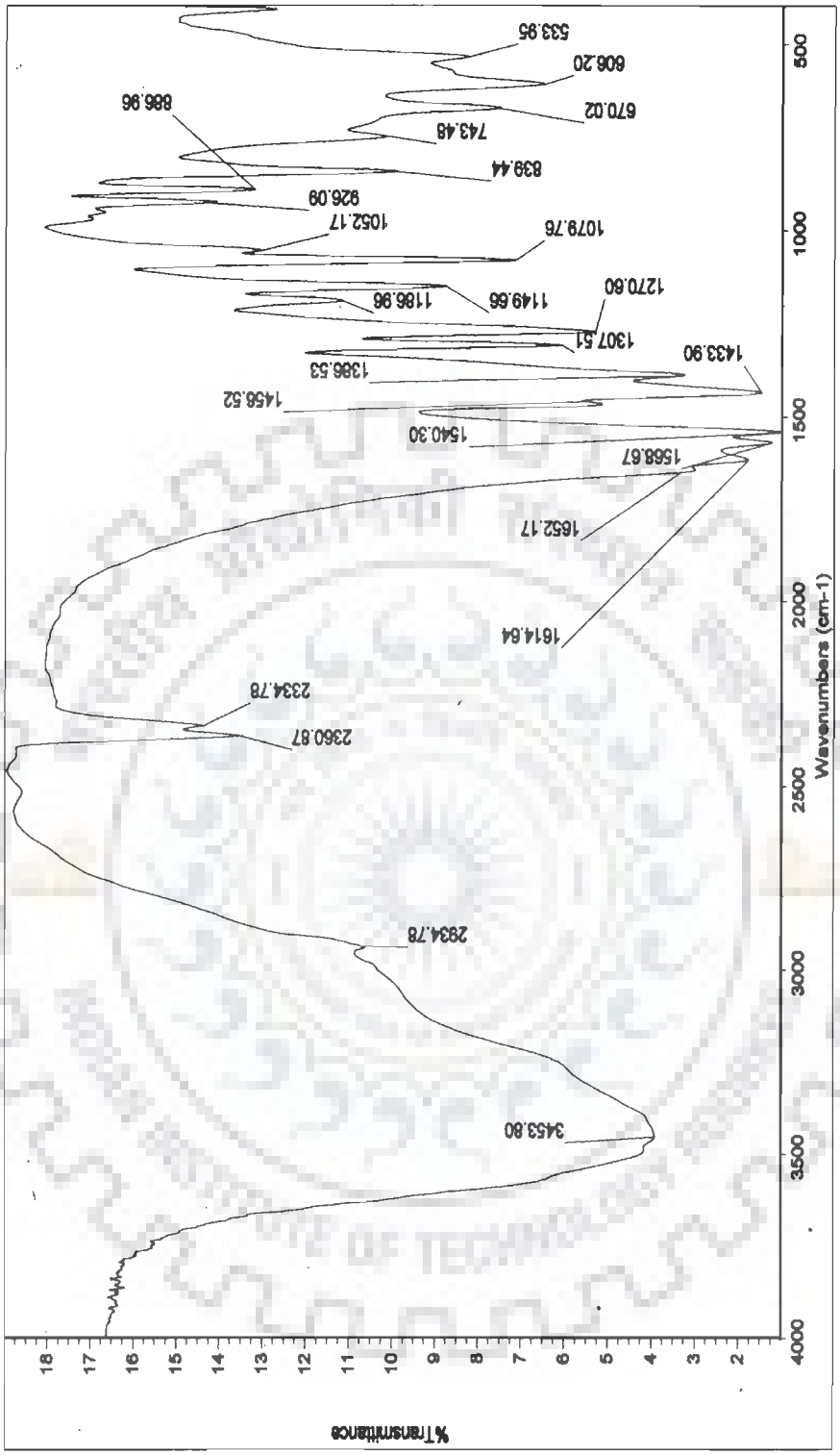


Fig. 43e FTIR spectra of colloidal chitin

Table 39 Degree of deacetylation of crab shell chitin before and following chemical treatment determined using elemental analysis and FTIR

Sample	Degree of deacetylation (%)	
	Elemental analysis	FTIR
Crab shell chitin	29.80 ± 1.7	28.74 ± 0.2
PA-CT	31.27 ± 1.4	32.02 ± 0.5
SF-CT	38.08 ± 2.1	37.14 ± 0.1
Am-CT	34.42 ± 1.6	34.18 ± 0.6
Colloidal chitin	32.61 ± 1.8	33.44 ± 0.3

4.8.2 Enzymatic deacetylation

Deacetylation potential of purified *P. oxalicum* SAE_M-51 CDA on untreated crab shell chitin and chemically pretreated chitin substrates was evaluated by incubating appropriate amount of substrate with enzyme for 24 h and DDA was detected. Samples were withdrawn at various intervals and analyzed for amount of acetic acid released and DDA. Kinetics of enzyme reaction revealed that relatively higher extent of deacetylation was observed during initial 10 hrs and that decreased thereafter (Fig. 44). DDA increased slowly in the later phase even though prolonged reaction time and presence of higher amount of substrate. Untreated chitin i.e. chitin from crab shell had the minimum level of deacetylation of all the substrates used, due to its higher crystallinity and negligible porosity. SF-CT appeared as the most promising substrate for enzymatic deacetylation (79.52 %). Lower crystalline index and porous morphology of the substrate makes both the interior and exterior acetyl groups accessible for the enzyme. Colloidal chitin was the another promising substrate that was susceptible for enzymatic deacetylation (72.31 %) in spite of its regular crystalline microfibrillar structure, as observed by scanning electron micrographs. Morphological examination and crystallinity of amorphous chitin depicted it as the second potential substrate for enzymatic action. But the deacetylation levels achieved with the later was comparatively lower than that of the colloidal chitin. Higher degree of crystallinity and relatively lower extent of exposed surface area affected into phosphoric acid treated chitin as the less preferred substrate for enzymatic deacetylation (65.00 %).

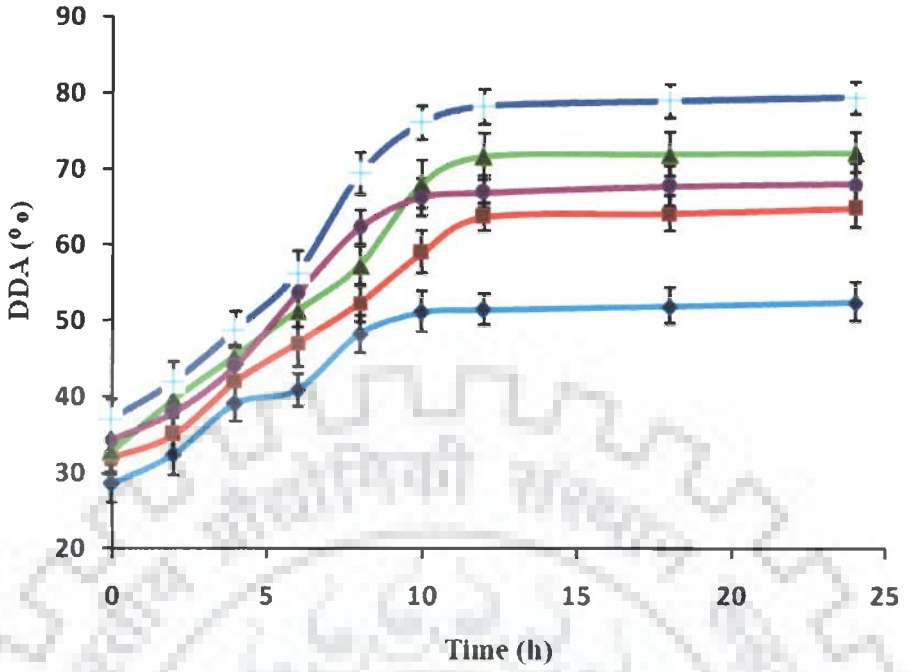


Fig. 44 Analysis of degree of deacetylation of untreated and treated chitin (◆, crab shell chitin; ■, PA-CT; +, SF-CT; ●, Am-CT; ▲, colloidal chitin)

4.8. 3 Derivation of reaction conditions for enzymatic deacetylation

A significant level of deacetylation was achieved with superfine chitin using purified CDA from *P. oxalicum* SAE_M-51. In order to get chitosan with considerably higher level of DDA, 2^2 full factorial CCD was applied to determine the optimum levels of enzyme dose and substrate amount in terms of acetic acid released and DDA. Acetate released was analyzed using analytical kit procured from Boehringer Mannheim GmbH (Germany) and DDA was determined using potentiometric titration. Each factor in the design was studied at five levels ($-\alpha$, −1, 0, +1, $+\alpha$) and a set of 11 experiments were carried out. All variables were taken at a central coded value considered as zero. All experiments were conducted in triplicates and the average value was taken as the response (Y).

The CCD design and the corresponding experimental data are shown in Table 40. Results of regression analyses and ANOVA for acetate released (mM) and DDA (%) are

presented in Tables 41 - 44. By applying multiple regression analysis on the experimental data, the following second order polynomial equations (equation 4 and 5) had enumerated the amount of acetic acid released and DDA as a function of the variables analyzed.

$$Y = 18.26 - 0.13\,X_1 - 0.10\,X_2 - 3.67\,X_1^2 + 1.48\,X_2^2 - 2.42\,X_1X_2 \qquad (4)$$

$$Y = 89.31 + 1.52\,X_1 + 0.17\,X_2 - 5.60\,X_1^2 + 1.95\,X_2^2 - 3.98\,X_1X_2 \qquad (5)$$

The Fisher's *F*-test with a very low probability value (<0.0001) indicated that both the models were highly significant. The goodness of the fit of the model was examined by coefficient of determination (R^2) value. It was calculated to be 0.9716 for acetic acid released and 0.9722 for DDA. This implied that the sample variation of 97.16 and 97.22 % was attributed to the variables and only 2.84 and 2.78 % of the total variance could not be explained by the model. The adjusted R^2 values of 0.9513 for acetate released and 0.9511 for DDA were also satisfactory to denote the validity of the model. The 'adequate precision' values of and 20.13 for acetic acid released and 21.24 for DDA, respectively, indicated that the model can be used to navigate the design space. A lower value of coefficient of variation (CV = 4.60, 1.39 % for acetate released and DDA, respectively) showed that the experiments conducted were reliable and precise.

The 2D contour and 3D response surface curves were plotted to understand the interaction of the variables and also to determine their optimum levels to attain maximum response. The strong interaction between amount of substrate and enzyme dose for both the responses is evident from the response surface contour plots between them (Fig. 45, 46) and also from their low corresponding probability of failure value. The model predicted maximum acetate released (18.26 mM) with 3.02 g substrate treated with 59.72 U of enzyme. However, maximum deacetylation (89.07 %) was attained with 2.96 g substrate treated with 59.10 U of CDA.

Validation of the model and regression equation was performed by taking the values at their optimum levels. The experimental responses (acetate released, 19.54 mM; deacetylation 90.32 %) were in close agreement with the statistically predicted ones, confirming the validity of the model.

Table 40 Central composite design matrix for optimization of conditions for enzymatic deacetylation

Run	X_1	X_2	Acetate released (mM)		DDA (%)	
			Observed	Predicted	Observed	Predicted
1	-1	-1	14.20	13.88	80.12	79.99
2	+1	-1	19.65	18.47	92.83	91.00
3	-1	+1	17.88	18.52	87.78	88.29
4	+1	+1	13.65	13.42	84.56	83.37
5	$-\alpha$	0	11.44	11.10	76.50	75.96
6	$+\alpha$	0	9.86	10.74	78.40	80.26
7	0	$-\alpha$	20.43	21.37	91.87	92.98
8	0	$+\alpha$	21.48	21.08	93.24	93.45
9	0	0	18.20	18.26	89.40	89.31
10	0	0	18.04	18.26	89.04	89.31
11	0	0	17.96	18.26	89.60	89.31
12	0	0	18.43	18.26	89.13	89.31
13	0	0	18.67	18.26	89.37	89.31

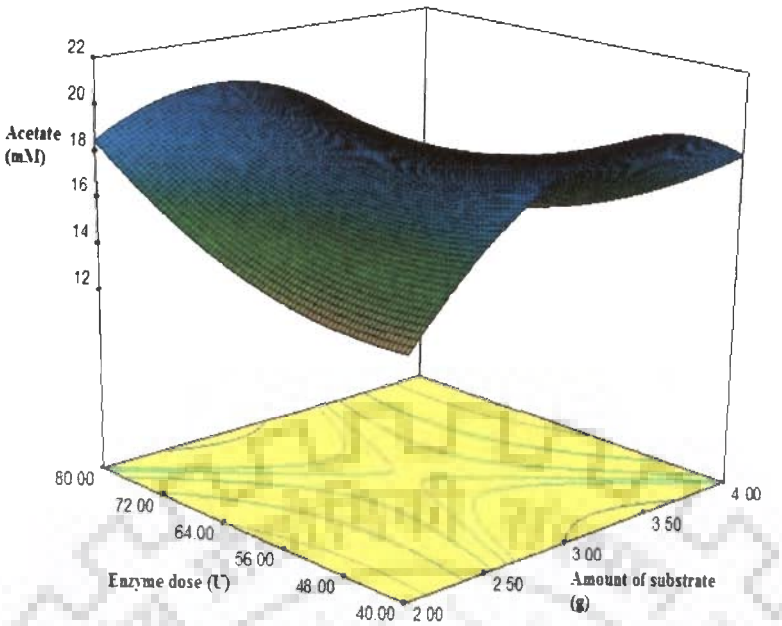
Table 41 Result of multiple regression analysis of CCD for acetate released

Variables	Coefficient	*F*- value	*P*-value
Intercept	18.26	-	-
X_1	-0.13		0.6585
X_2	-0.10		0.7153
X_1X_2	-2.42		0.0004
X_1^2	-3.67		<0.0001
X_2^2	1.48		0.0015

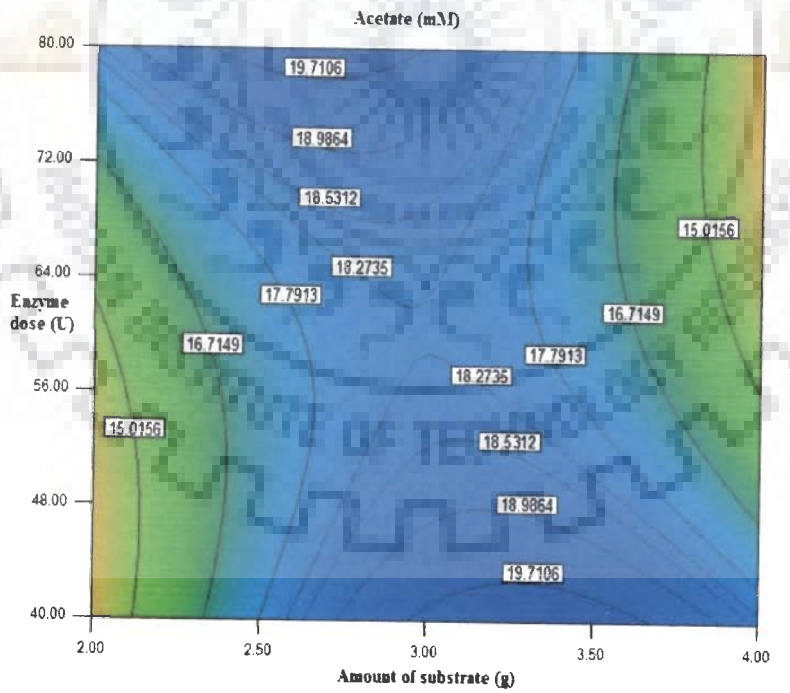
Table 42 ANOVA for quadratic model for acetate released

Source	SS	DF	MS	*F*-value	p-value (Prob>*F*)
Model	144.54	5	28.91	47.85	<0.0001
Residual	4.23	7	0.60	-	-
Lack of fit	3.89	3	1.30	15.30	0.0117
Pure error	0.34	4	0.085	-	-
Total	148.77	12	-	-	-

R^2: 0.9716; Adj R^2: 0.9513; C.V.: 4.60 %; Adeq. precision: 20.135



(a)



(b)

Fig. 45 Response surface plot (a) and corresponding contour plot (b) of acetate released as a function of substrate amount and enzyme dose

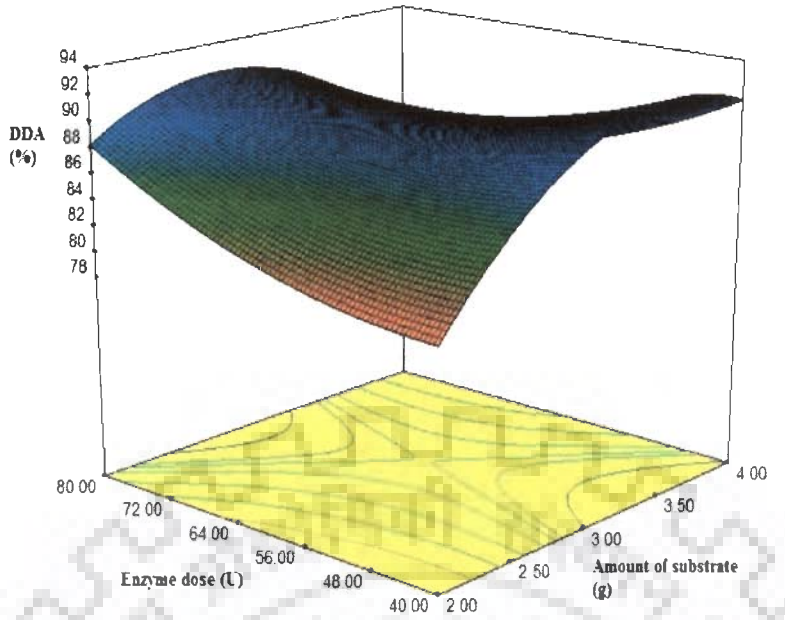
Table 43 Result of multiple regression analysis of Central composite design for DDA (%)

Variables	Coefficient	*F*- value	*P*-value
Intercept	89.31	-	-
X_1	1.52		0.0093
X_2	0.17		0.7101
X_1X_2	-3.98		0.0003
X_1^2	-5.60		<0.0001
X_2^2	1.95		0.0038

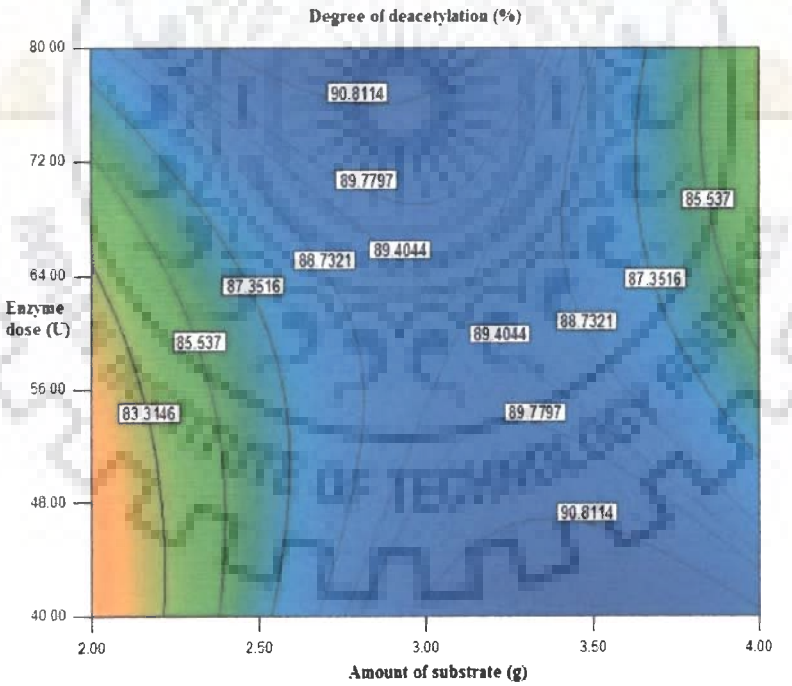
Table 44 ANOVA for quadratic model for DDA (%)

Source	SS	DF	MS	*F*-value	*P*-value (Prob>*F*)
Model	351.25	5	70.25	47.83	<0.0001
Residual	10.28	7	1.47	-	-
Lack of fit	10.08	3	3.36	66.85	0.0007
Pure error	0.20	4	0.050	-	-
Total	361.53	12	-	-	-

R^2: 0.9722; Adj R^2: 0.9511; C.V.: 1.39 %; Adeq. precision: 21.245



(a)



(b)

Fig. 46 Response surface plot (a) and corresponding contour plot (b) of degree of deacetylation as a function of substrate amount and enzyme dose

CHAPTER V

DISCUSSION

Chapter 5

DISCUSSION

5.1 IDENTIFICATION OF CHITIN DEACETYLASE PRODUCING MICROORGANISMS

Microorganisms are considered as "miniature cell factories" and are exploited for the production of enzymes of commercial significance. Enzymes are recognised as potential alternatives to harsh and eco-incompatible chemical technologies. This has led to intensive efforts to explore natural microbial diversity to explore enzymes that could be used for environment friendly "Dream technologies" in the immediate future. Microorganisms surviving in the distinct environmental conditions offer an interesting system to explore and utilize these for industrial applications.

Chitosan is a versatile biopolymer with immense commercial applicability. Its functional characteristics correlate with its structural features. Enzyme aided production of chitosan implying CDA is one of the major areas of research today. CDA assisted enzymatic conversion to chitosan needs intensive screening of novel CDA hyper-producers. One of the limitations for a variety of applications of the enzyme is due to the lack of productive strains and industrially compatible technologies for large scale production of enzymes of economic relevance. A number of research groups have actively been exercising for screening for CDA hyper-producing microbial strains (Nahar et al., 2004; Kim et al., 2008; Zhao et al., 2010b). A major limitation with respect to employing these for bioconversion reactions because the strains explored so far exhibit intracellular production of CDA and further with limited activity and yield. In an attempt to look for strains having higher ability for extracellular production of enzyme, four potential sources namely, sea food processing industry residual materials, insect exoskeleton, decaying snail shell and decomposing fish waste were used to explore for the

potential microbial strains. Eighteen fungal isolates were obtained and their CDA production ability was evaluated by screening on 4-nitroacetanilide containing agar plates. Among the isolated strains, strain SA-1 was selected as it was observed to have maximum levels of extracellular CDA activity (103.78 ± 1.98 UI^{-1}) and also had considerable levels of intracellular activity (27.74 ± 0.45 UI^{-1}). The strain was, therefore, selected for further studies and was identified as *Penicillium oxalicum* ITCC 6965 by the Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi, India.

The development of an industrially viable fermentation process largely depends on the production ability of the microbial strain involved in the process. The strain selected was observed to have significant levels of CDA production. Thus, to explore the possibilities for commercial exploitation of the enzyme, attempts were made for obtaining a further increase in the enzyme production levels. The strain was subjected to genetic manipulation using mutagenesis for achieving an improvement in the levels of production. Further, the production processes were evaluated and attempt was made to explore if low cost agro-industrial residual materials can be supplemented to further economize the process of production of the enzyme.

5.2 MUTAGENESIS OF *P. OXALICUM* SA-1

Strains isolated from natural sources have generally the limited ability for the production of metabolites and can be subjected for genetic manipulation for improvement. The wild type *P. oxalicum* SA-1 was subjected to mixed mutagenesis using ethidium bromide (50 min) and microwave irradiation (10 sec). Several mutants thus obtained were evaluated for their CDA production ability. Mutant *P. oxalicum* SAE_M-51 had exhibited 2.0 fold higher extracellular CDA titres as compared to the wild type strain (Pareek et al., 2010). However, no remarkable increase in the intracellular levels of CDA activity (29.87 ± 0.62 UI^{-1}) was observed. The mutant following repeated subculturing (12 generations) had stable levels of CDA production. Mutagenesis of *Gongronella butleri* through UV irradiation had led into >1.6 fold increased levels of CDA production (Maw et al., 2002b). Induced mutagenesis had been successfully

employed for obtaining increased levels of chitinase (Reid and Ogrydziak, 1981; Lim et al., 1991; Vaidya et al., 2003; Gohel et al., 2004), xylanase (Singh et al., 1995; Bakalova et al., 2002), laccase (Dhawan et al., 2003), tannase (Purohit et al., 2006), cellulase (Chand et al., 2005; Adsul et al., 2007; Fang et al., 2009; Li et al., 2010; Vu et al., 2011) and glucoamylase (Kumar and Satyanarayana, 2009) production. In addition, evaluation of kinetic variables viz. Q_p, Q_s, $Y_{p/x}$, $Y_{p/s}$, q_p, q_s had denoted that mutant strain had ~2.0 fold improved levels for CDA production levels over the parental strain. Similar findings were observed by Lotfy et al. (2007) for citric acid production and Ali and Ashiq (2010) for production of β-D-fructofuranosidase fructohydrolase.

5.3 CRITICAL FACTORS AFFECTING ENZYME PRODUCTION IN SUBMERGED FERMENTATION

The carbon source used in the production medium is one of the major factors affecting enzyme production. Several workers had reported that the production of chitinolytic enzymes mainly chitinase was observed to be induced by chitin in the production medium (Mahadevan and Crawford, 1997; Souza et al. 2005). Chitin, its variant colloidal chitin and glucose were evaluated for their impact on CDA production. Among the substrates analyzed, glucose had resulted into the maximum CDA levels (221.57 ± 1.59 UI^{-1}) followed by colloidal chitin and chitin. However, period required for maximum levels of production was prolonged in the presence of these chitinous substrates. Marginal induction of CDA activity by chitin may be due to its complex structure, higher molecular mass and insoluble nature which may limit its penetration inside the cell. Colloidal chitin, however, since is soluble in water and is comparatively less crystalline than chitin, therefore appear to be a better inducer than chitin.

Enzyme production by microorganisms is strongly influenced by various factors, such as nutritional sources and cultivation conditions. Designing of an efficient CDA production medium is a prerequisite to achieve high product yield which would not only improve the yield and quality of CDA but also render the process more economical. The conventional approach of

optimizing one-factor-at-a-time is laborious, time consuming and cannot provide the information on mutual interactions of the variables on the desired outcome. On the other hand, statistical experimental designs provide a systematic and articulated plan to analyze multiple critical factors regulating production process (Bas and Boyaci 2007). Statistical analysis allows visualization of the interactions among several experimental variables, leading to the prediction of data in areas not directly covered by experimentation. The statistical programs create several classes of RSM designs, which provide efficient strategic experimental tools for determination of the optimal conditions of a multivariable system. Among these CCD is widely accepted as it works well in providing derived information on variable's effects and overall experimental error in a minimum number of required experiments. The CCD technique is fairly flexible and allows sequential run of large number of experiments (Montgomery 2000, 2004).

The Plackett–Burman factorial designs allow for the screening of the main factors from a large number of process variables. These designs are thus quite useful in preliminary studies in which the principle objective is to select variables that can be fixed or eliminated in further optimization process. Effect of eleven variables (including three dummy variables) on CDA production by *P. oxalicum* SAE_M-51 was evaluated using Plackett-Burman design (PBD) in twelve experiments. From the regression analyses, it is clear that among the eight nutritional variables studied, four variables (yeast-extract, peptone, $(NH_4)_2SO_4$ and $MgSO_4$) had profoundly affected the CDA production levels, based on their individual probability of failure values (p>F). PBD is a potential tool for screening critical medium components, has successfully been exploited by many workers for optimization studies (Xu et al., 2006; Khambhaty et al., 2007; Kumar and Satyanarayana, 2007; Singh and Tripathi, 2008; Fu et al., 2009; Mukherjee and Rai, 2011). Optimum levels of the critical variables identified by PBD were further determined using 2^4 full factorial CCD with five coded levels. Analysis of variance indicated that the quadratic model came out to be significant (*F*-value, 3.29). Optimum level of each variable and the effect of their interactions on CDA production against any two independent variables were determined

using three response surface curves and the corresponding contour plots. Yeast extract had contributed significantly (quadratic effect) to the response and its interaction with magnesium sulfate was strong. Peptone was also found to be another important component in the medium that influenced the CDA production. However, interactions of peptone with $(NH_4)_2SO_4$ and $MgSO_4$ had no significant impact on CDA production as evident by their response surface plots. Parallel nature of the contour plot between $(NH_4)_2SO_4$ and $MgSO_4$ along the two axes suggested that the two parameters were quite independent of each other and CDA production was critically affected by $MgSO_4$ concentration. Optimum concentration of the variables for enhanced CDA production determined by CCD were (gl^{-1}) : 3.165, 4.754, 0.255 and 0.535 for yeast extract, peptone, $(NH_4)_2SO_4$ and $MgSO_4$, respectively. A 2.0 fold increase in the CDA levels (Q_p = 2.8798 $Ul^{-1}h^{-1}$) was attained in submerged process, when the variables were used at their optimum levels. CDA production (414.7 ± 6.32 Ul^{-1}) in the derived medium was in good agreement with the values predicted by the quadratic model (399.08 Ul^{-1}), confirming the validity of the model (Pareek et al., 2011).

Designing of an appropriate fermentation medium is an essential and prerequisite step for the overproduction of enzymes, furthermore, secretion of metabolic products is accompanied to the survival strategies of some microorganisms occupying certain environments (Dey et al., 2001). Medium optimization studies for enhancement in production of a variety of industrially important enzymes and metabolites employing RSM has been has increased in present-day biotechnology, due to its propensity and relevance (Acikel et al., 2010; Bari et al., 2010; Karan et al., 2011; Sanchez-Otero et al., 2011; Su et al., 2011). Enhanced yields of chitinolytic enzymes viz. chitinase and chitosanase due to yeast extract and $MgSO_4$ was also observed by other groups (Vaidya et al., 2003; Nawani and Kapadnis, 2005; Sun et al., 2007). These studies also signify the requirement of nitrogen rich sources for the production of chitinolytic enzymes. Statistical derivation of medium components for chitinase and chitosanase production was successfully employed by previous workers (Vaidya et al., 2003; Nawani and Kapadnis, 2005;

Gohel et al., 2006; Sun et al., 2007; Han et al., 2008; Lopes et al. 2008). Use of derived medium components would result in maximizing the production of microbial deacetylase, thus further concentrating the prospects for biological production of chitosan.

Further, derivation of optimal processing parameters plays a major role for the fermentation owing to the economy and efficacy of the process. CCD was employed in the present investigation to derive the optimal conditions mainly pH, temperature, inoculum age and inoculum size for enhanced CDA production. Each factor was analyzed at five coded levels and a set of 30 experiments were carried out. The optimal conditions that stimulated maximal CDA production were detected to be: pH, 7.9; temperature, 28 ^{0}C; inoculum age, 90 hrs and inoculum size to 11.0 % (v/v). Close relationship was observed between experimental (623.57 ± 8.2 Ul^{-1}, Q_p = 4.3264 $Ul^{-1}h^{-1}$) and predicted (648.24 Ul^{-1}) values which further validates the model for derivation of suitable conditions to attain maximum CDA yields. Derivation of parameters for CDA production through CCD had resulted into 6.0 fold enhancement in CDA production levels under submerged condition as compared to the production levels as obtained by the wild type strain.

Enzyme production by microorganisms is normally associated with the growth of the organism. The effect of inoculum age and size with respect to any fermentation process is well established. Inoculum consisting of cells in the log phase cells induces rapid biomass formation and therefore promotes the production of higher enzyme levels relatively in shorter durations. However, inoculum size more than the required level may affect into increased biomass level and therefore may lead into nutritional imbalance in medium which in turn may attenuate enzyme production, while suboptimal inoculum level leads into inadequate cell growth and mitigation of enzyme production. A concomitant increase in enzyme production was observed with increasing inoculum size up to a defined level, thereafter, CDA level declined with further increase in inoculum size. Maximum CDA production was observed using 11.0 % (v/v) of inoculum level of 90 hrs old culture. Growth of the organism and metabolite production are also

influenced by pH of the production medium and incubation temperature. CDA production increased in a linear fashion with increase in pH. The statistical analysis had indicated relatively fewer interactions between pH and temperature suggested that their interaction was not a critical one for CDA production. These parameters individually had profound effect on the production because of their very low corresponding probability of failure value as observed. Elliptical nature of the contour plot between temperature and inoculum age had suggested prominent interactions between them. Interaction of pH with other parameters viz. inoculum age and size also didn't exert any noticeable effect on CDA production.

Response surface methodology had been successfully implicated for enhancement in pectinolytic activity by *Kluyveromyces wickerhamii* (Moyo et al. 2005), xylanase production by *Aspergillus carneus* (Fang et al. 2007), cellulase production by *Penicillium decumbens* (Liu et al. 2008) and asparaginase production by *Escherichia coli* ATCC 11303 (Kenari et al., 2010) under submerged condition. Statistical optimization for production of various enzymes of chitinolytic enzyme system was also attempted by a number of researchers (Vaidya et al. 2003; Gohel et al. 2005, 2006; Juarez-Jimenez et al. 2008; Lopes et al. 2008). But majority of the studies are related to the optimization of medium components and very few are related to optimization of culture conditions (Nawani and Kapadnis, 2005).

5.4 EVALUATION OF SOLID-STATE FERMENTATION PROCESS FOR ENZYME PRODUCTION

In an attempt to explore if the cost for the process of production can further be minimized, solid-state fermentation, an alternative process was further evaluated. The solid-state process been demonstrated to be productive and cost effective for the production of enzymes and metabolites (Krishna, 2005). It has gained renewed interest in recent years since the process mainly makes use of agro-horticultural residual materials for production of value added products. Further, it delineates a number of merits over submerged process due to its having superior volumetric productivity, yields into concentrated form of end products with improved

product recovery, lesser effluent generation, low capital investment and due to the reduced energy requirement for the process (Lonsane et al., 1985). A large number of agro-residues mainly sugar cane bagasse, wheat bran, wheat straw, corn cobs, rice husk, maize bran and many more which are generated in enormous amounts during agricultural practices are considered as the promising substrates for SSF (Pandey et al., 1999). Utilization of these residual materials for bioprocesses also eases the pollution menace and demarcates these materials that otherwise are a source of concern and no value to potential providers (Pandey et al., 2000). Filamentous fungi (Senthilkumar et al., 2005; Souza et al., 2001) and actinomycetes (Ninawe and Kuhad, 2005) are the organisms of choice for SSF process because of their ability to grow on complex solid substrates for obtaining higher yields of a wide spectra of extracellular enzymes and metabolites (Mitchell and Lonsane, 1992; Ustok et al., 2007; Levin et al., 2008; Hashemi et al., 2010; Bhattacharya et al., 2011; Liu et al., 2011).

For SSF, ten different solid supports viz. sugarcane bagasse, red gram husk, black gram husk, cotton hull, tea waste, wheat bran, rice bran, groundnut shell, corn residues and mustard oil cake impregnated with the fermentation medium as derived in this study, were utilized. Among the solid substrates employed, mustard oil cake had led into maximum production levels (877.85 ± 10.95 Ul^{-1}). Solid support plays a vital role in SSF because it provides not only anchorage to microbial cells but also provide nutrients for fungal growth and subsequent enzyme production. Oil cakes have high nutritional value, especially due to their protein content ranging from 15 to 50 % (Ramachandran et al., 2007). Mustard oil cake being a rich source of crude protein, crude fiber, calcium, phosphorus and other basic nutrients would enable into substantial fungal growth and therefore the enzyme production (Kuo, 1967). Its porosity further helps into absorption of nutrient thus leading into proper growth and production of enzyme. It has previously been utilized by several workers for lactic acid production and mushroom cultivation (Tuli et al., 1985; Bano et al., 1993; Shashirekha et al., 2002).

Success of any fermentation process largely depends on derivation of critical physico-chemical parameters viz. selection of a suitable strain, appropriate substrate, temperature, pH, inoculum age and size (Pandey et al., 2001). To further improve the production level, optimal values of the three variables viz. amount of substrate, moisture content and inoculum size were further deciphered by RSM using 2^3 full-factorial CCD. The conditions thus derived for maximal CDA production were detected to be 4.906 g, 73.62 % and 8.578 % for the amount of the substrate, moisture content and inoculum size respectively. The experimental CDA production (1162.03 ± 7.2 Ul^{-1}, Q_p = 8.0696 $Ul^{-1}h^{-1}$) under derived condition was observed to be in close agreement with the values predicted by the quadratic model (1137.85 Ul^{-1}). The CDA production by *P. oxalicum* SAE_M-51 increased significantly to 1.3 fold, compared to the production levels obtained under un-derived conditions (877.56 ± 8.9 Ul^{-1}).

Production of chitinolytic enzymes mainly chitinase was extensively studied by various groups under solid state fermentation (Suresh and Chandrasekaran, 1999; Gkargkas et al., 2004; Matsumoto et al., 2004; Binod et al., 2005; Patidar et al. 2005; Binod et al., 2007; Marin-Cervantes et al., 2008). CDA production under SSF was studied by Aye et al. (2006) using soybean residues as solid substrate, but the process parameters were not evaluated to possibly achieve an improvement in the production levels. Derivation of the optimal levels of the various parameters i.e. amount of substrate, moisture content, particle size, temperature, pH and inoculum size is essential for attaining high yield of the metabolites. Moisture plays an important role in the production of metabolites and enzymes in SSF. Thus, it is crucial to provide optimized water content, and control the water activity (a_w) of the fermenting substrate as the availability of water in lower or higher concentrations affects microbial activity adversely. High moisture content decreases porosity and exchange of oxygen to the microorganism thus decreasing the enzyme yield during the process. Lower moisture level may affect into reduced solubility of nutrients, inappropriate swelling of substrates and elevated water tension resulting into lower yield of the enzyme (Lonsane et al., 1985; Gawande & Kamat, 1999). Moreover,

water has profound impact on the physico- chemical properties of the solids and this, in turn, affects the overall process productivity. A moisture content of 73.62 % had resulted into maximum production of the CDA. Almost similar level of moisture content was required by *Penicillium aculeatum* NRRL 2129 for chitinase production (Binod et al., 2007) and by *Fusarium oxysporum* F3 for *N*-acetyl-β-d-glucosaminidase production (Gkargkas et al., 2004). However, comparatively higher moisture level (85 %) was required, when *Lecanicillium lecanii* was inoculated using polyurethane foam for chitinase production (Marin-Cervantes et al., 2008). Metabolite production during SSF also depends on inoculum size. Production levels increased with increase in inoculum size up to a certain level, this might be because of increasing biomass. However, after a certain limit the competition for the nutrients may affect into the decreased metabolic activity of the organism, resulting into the decrease in the product yield. Chitinase production by *Enterobacter* sp. was detected to be enhanced to 2.3 fold following statistical optimization using wheat bran as solid support (Dahiya et al., 2005).

5.5 CHARACTERISTIC FEATURES OF PURIFIED CHITIN DEACETYLASE FROM MUTANT *P. OXALICUM* SAE_M-51

CDA with a molecular mass of 53 kDa was purified to homogeneity from culture supernatants of *P. oxalicum* SAE_M-51 using ion exchange chromatography. The enzyme was purified to 88.25 fold with 11.25 % recovery. Molecular mass of the fungal CDAs purified and studied so far vary in the range of 25 – 80 kDa (Alfonso et al., 1995; Nahar et al., 2004; Cai et al., 2006) and the pI of purified CDA was observed to be 5.2. However, the pI of CDA from *C. lindemuthianum* (Tokuyasu et al., 1996) and *M. anisopliae* (Nahar et al., 2004) had been detected to vary from 3.7-4.1. Utilization of purified enzyme preparation free of chitinase and chitosanase is preferable for enzymatic deacetylation in order to get a reasonably higher molecular weight product.

The optimal pH for CDA activity was found to be 9.0, however considerable level of CDA activity was also detected at pH 6.0. The enzyme was quite stable and maintained 100%

residual activity at pH 9.0 for 24 h. A 25-55 % residual activity was observed when enzyme was incubated for 24 h at lower pH (5.0-7.0). Optimal pH of most of the extracellular CDAs observed so far lies in neutral or alkaline range (Tokuyasu et al., 1996; Nahar et al., 2004; Cai et al., 2006). Further, the optimum pH of intracellular CDA from *M. rouxii* and *A. nidulans* was respectively 5.5 (Kafetzopoulos et al., 1993) and 7.0 (Alfonso et al., 1995). In addition, many of the CDAs analysed so far appeared to be relatively stable at higher pH (Zhao et al., 2010).

Optimum temperature for maximum CDA activity was observed to be 50 ^{0}C, however, the enzyme was observed to be quite stable over broad range of temperature (40 - 70 ^{0}C). The purified enzyme exhibited half-life of 693.10 min at its optimum temperature. Optimum temperature of the CDAs known so far is around 50 ^{0}C (Alfonso et al., 1995; Cai et al., 2006). CDA from *C. lindemuthianum* DSM 63144 exhibited remarkable thermostability at its optimum temperature with almost no loss of activity after preincubation at 50 °C for 45 hr (Tsigos and Bouriotis, 1995). Further, the enzyme from the *A. nidulans* was found to be quite stable over a broad range of temperature (30 – 100 ^{0}C) (Alfonso et al., 1995). The CDA from *C. lindemuthianum* UPS9 had the optimum activity at 60 ^{0}C and > 50 % of the activity was retained after heating of the enzyme at 80 ^{0}C for an hour or at 90 ^{0}C for 30min (Shrestha et al., 2004).

Enzymes, in general, are protein molecules having specific primary, secondary, tertiary and quaternary structures. At high temperatures, enzymes loose their native conformations which are essential for their activity. Thermostability refers to the resistance of enzymes towards unfolding upon heating. The effect of temperature on the rate of enzyme denaturation can be expressed by usual thermodynamic values and may provide evidences about the mechanism involved in the reaction. Half life of an enzyme is the time after which it looses 50 % of the initial activity. A longer half life is an indication of the stability of the enzyme at that particular temperature. Purified CDA had a half life of 693.10 min at its optimal temperature, which was observed to decrease thereafter at higher temperatures.

Thermal denaturation of enzymes requires an input of minimum amount of energy, the activation energy of denaturation ($E_{a,d}$), necessary to initiate the denaturation process. The denaturation processds via formation of an intermediate transition state (U*) (Siddiqui et al., 1997a, b). As long as the initial input of energy is less than the $E_{a,d}$, the unstable intermediate can be refolded into the active conformation upon cooling. This activation barrier is crucial to the stability and survival of the biomolecules. Once the $E_{a,d}$ barrier has been overcome, the enzyme is completely denatured and cannot refold to the native form, known as the irreversible thermal denaturation of the enzyme (Javed et al., 2009). The $E_{a,d}$ for complete denaturation of CDA was determined from the Arrhenius plots (ln K_d vs 1/T) and observed to be 114.72 kJmol^{-1}. Considerably higher value of activation energy ($E_{a,d}$) is an indication of the higher thermostability of the enzyme. The $E_{a,d}$ is directly related to another important thermodynamic parameter, the enthalpy of activation for denaturation. It is the total amount of energy required to bring the enzyme from the ground state to the activated intermediate. The high, and positive, values of $E_{a,d}$ and $\triangle H_d^*$ are therefore associated with the higher thermostability of the enzyme (Marangoni, 2003). In the present study, higher and positive values for the enthalpy of activation of denaturation ($\triangle H_d^*$) were observed, conferring the higher thermal stability of the enzyme. However, no significant change was observed in the enthalpy ($\triangle H_d^*$) of activation for thermal denaturation of CDA with increasing temperature.

Gibbs free energy of denaturation ($\triangle G_d^*$) is a more reliable indicator of enzyme stability. A lower or negative value of the change in the Gibbs free energy indicates the lesser stability and susceptibility of proteins towards thermal denaturation (Purich and Allison, 2000; Marangoni, 2003; Damodaran, 2006). The Gibbs free energy of a substance results from the stabilizing forces present in its structure. The values of ($\triangle G_d^*$) were observed to be in the range of 97.86 – 96.55 kJ mol^{-1} at 50 – 80 ^{0}C. $\triangle G_d^*$ is observed to be temperature dependent parameter and decreased with a concomitant increase in temperature thereby denoting the decreasing stability of enzyme at higher temperatures. The thermal inactivation of the enzymes

is accompanied by the disruption of non-covalent linkages including hydrophobic interactions and opening up of the enzyme structure with concomitant increase in the entropy ($\triangle S_d^*$) of activation for denaturation (Petsko and Ringe, 2004). Entropy ($\triangle S_d^*$) of activation for complete thermal unfolding of CDA appeared to be a temperature independent parameter and its positive values implied that the thermal denaturation of the enzyme at higher temperature resulted in an associated increase in disorderliness of the system. Similar studies were also performed for thermal denaturation of adenosine deaminase (Alrokayan, 2002), ribonuclease (Xiong et al., 2005), glucoamylase (Riaz et al., 2007) and endoglucanase (Saqib et al., 2010).

Kinetic parameters viz. K_m, V_{max}, k_{cat} and k_{cat}/K_m and substrate specificity of CDA from *P. oxalicum* SAE_M-51 were determined through Linweaver-Burk plot using various concentrations (0.5 - 5 mg ml^{-1}) of five different substrates (glycol chitin, dimeric, trimeric and pentameric forms of GlcNAc and *N*-acetyl glucosamine) The enzyme had notably deacetylated glycol chitin and chitin oligomers having degree of polymerization more than four, but was less active with chitobiose and chitotriose and inactive with *N*-acetylglucosamine. Enzyme had maximum affinity for chitopentaose and followed Michaelis-Menten kinetics as observed by its K_m and k_{cat} values, that were 3.07 mg ml^{-1} and 281.66 s^{-1}, respectively. Enzyme requires a minimum degree of polymerization of two to exhibit catalytic activity. Values relative to kinetic parameters had increased along with the degree of polymerization of chitin oligomers. CDAs from other microbial sources had also exhibited the similar pattern for glycol chitin and chitin oligomers (Alfonso et al., 1995; Tokuyasu et al., 1996; Wang et al., 2009).

Increase in enzyme activity was observed with some metal ions in the order of Cu^{+2} > Co^{+2} > Fe^{+2} > Cd^{+2} > Mg^{+2} > Ca^{+2} > Ba^{+2} > Mn^{+2}, while Zn^{+2}, Mo^{+2}, Li^{+2}, Pb^{+2}, Bi^{+2} and EDTA had inhibited the CDA activity. Previous studies had reported that CDA activity was highly influenced by divalent cations especially cobalt. Catalytic activity of CDA from *S. cerevisiae* could be fully restored by addition of $CoCl_2$ (1 mM). Catalytic ability of CDA from *C. lindemuthianum*, *Mortierella* sp. DY-52 and *Flammulina velutipes* was observed to be enhanced

by Zn^{+2}, Ca^{+2} and Co^{+2} (1 mM) (Shrestha et al., 2004; Kim et al., 2008; Yamada et al, 2008). However, Zn^{+2}, Mn^{+2} and Mg^{+2} were inhibitory for CDAs from various sources (Martinou et al., 2002).

Acetate being an end product of the deacetylation reaction, can inhibit CDA activity. CDA from *P. oxalicum* SAE_M-51 was observed to be quite stable in the presence of acetate (0-70 mM). The enzyme retained 50 % of the initial activity at 90 mM sodium acetate concentration. Most of the fungal CDAs are not inhibited when acetate is present at lower (1-10 mM) concentrations (Alfonso et al., 1995; Nahar et al., 2004) and inhibition was more pronounced when acetate was present at 40 mM or higher concentrations. A 50 % inhibition in CDA activity from *A. nidulans* and *S. cerevisiae* was observed in presence of 40- 50 mM of acetate (Alfonso et al., 1995; Martinou et al., 2002; Wang et al., 2010). However, an extracellular CDA from a plant pathogenic fungus *C. lindemuthianum* was not inhibited by sodium acetate even at it's higher concentrations (Tokuyasu et al., 1996).

Secondary structure determination and conformational stability of CDA as a function of pH was determined using far-UV CD spectroscopic analysis. Spectral analysis suggested the enzyme to be a protein consisting of both α and β helical structures. The ellipticity values at 222 and 217 nm were characteristic for α-helix and β- sheet conformation respectively and hence the enzyme in native state was observed to be influenced by pH of the reaction buffer. CDA was observed to possess 56.26 % α-helix, 15.63 % β-structure and 28.11 % random structures at its optimal pH.

Elucidation of internal peptide sequence CDA from mutant *P. oxalicum* SAE_M-51 was performed by MALDI-ToF/ToF analysis, the sequences derived were subjected for homology search with the sequence of CDAs reported in the database. The enzyme possesses significant identity with members of the carbohydrate esterase family 4. Maximum degree of identity was revealed with CDAs from fungal species i.e. *Emericella nidulans*, *Rhizopus stolonifer*, *Glomerella lindemuthiana*, *Aspergillus niger*, etc. A considerable level of identity was also

observed with CDAs from bacterial and yeast strains, i.e. *Streptosporangium roseum*, *Pectobacterium cartovorum*, *Saccharomyces cerevisiae*.

5.6 BIOCONVERSION OF CHITIN TO CHITOSAN BY CHITIN DEACETYLASE FROM *P. OXALICUM* SAE_M-51

To date, chitosan is produced from thermo-alkaline deacetylation of chitin from crustacean shells (Prashanth et al., 2002; Guinesi and Cavalheiro, 2006; Yen et al., 2009). The process is incompatible as the conversion to chitosan, using a strong base solution at high temperature causes variability of the product properties viz. broad range of molecular weight and heterogeneous range of deacetylation and increased cost of production as well (Chang et al., 1997). At the same time, the effluent generated contains larger amounts of concentrated alkali and is deleterious for the environment. Degree and distribution of deacetylated subunits of chitosan has been found to influence its physical and chemical properties, biological applications viz. drug delivery (Desai and Park, 2006a, b), film forming ability (Fisk et al., 2008) and further the metal absorption ability (Varma et al., 2004) of the polymer. Therefore, as an alternative, a controlled, non-degradative and a well defined enzymatic process needs to be developed for chitosan production. Enzymatic process utilizes CDA to deacetylate nascent *N*-acetyl glucosamine subunits of chitin for generating novel chitosan polymers via a controllable process in which monitoring of the DDA of the product could be possible as per the requirement for industrial applications.

Till date, CDA production and enzymatic deacetylation has been studied by several groups (Martinou et al., 1995; Tokuyasu et al., 1996; Win and Stevens, 2001; Caufrier et al., 2003; Aye et al., 2006; Beaney et al., 2007). Crystallinity and insolubility of chitin are the two major barriers that hinder the development of a productive enzymatic deacetylation process (Kolodziejska et al., 2000; Beaney et al., 2007). Both the parameters affect substrate accessibility to the enzyme and hinder the enzyme action on interior acetyl groups that therefore negatively affects the production of highly deacetylated chitosan species. Enzymatic

deacetylation is mainly affected by three factors i.e. structural properties of chitin, mode of interaction between enzyme and chitin and mechanism of action of CDA (Martinou et al., 1995). The enzyme has largely been found to be ineffective in deacetylating insoluble chitin substrates. Win et al. (2000) had observed that treatment of natural chitin with CDA had resulted into about 1% increase in its DDA even after prolonged incubation. Therefore, pretreatment of chitinous materials prior to enzyme addition is recommended for a desired conformational modification of the parent polymer, which in turn affects the enzyme substrate interaction as well as the subsequent deacetylation reaction. Chitin can be treated by both physical and chemical means, but physical treatment such as heating, grinding and sonication haven't been reasonably effective. On the other hand, chemical pretreatments had resulted into significant loss of crystallinity, hence, produces more amorphous substrates for enzyme action (Win and Stevens, 2001; Beaney et al., 2007). Hence, a two stage chemical and enzymatic process could be more advantageous as the pretreatment of crystalline chitin substrates would improve the accessibility of enzyme to the acetyl groups. Therefore, the treated chitin would be a suitable substrate for obtaining enhanced yield and rate of deacetylation reaction for producing novel chitosan polymers.

Enzymatic biotransformation of insoluble chitin is heterogeneous in nature as the deacetylation takes place at solid-liquid interface, where the enzyme would reside and catalyze the reaction. An amorphous and soluble structure is preferred due to homogeneity of the reaction as well as for the access of the enzyme to an increased surface area. Chitin from crab shell is subjected to pretreatment with acids, alkali and calcium chloride:methanol:formic acid solvent system, that has resulted into deformation of the regular crystalline arrangement of chitin microfibrils. Thus a range of chitinous substrates viz. PA-CT, colloidal chitin, SF-CT and Am-CT which differ in their physical and chemical properties viz. DDA, crystallinity, solubility, thermal stability and morphology could be generated. The substrates generated via chemical treatment were characterized to assess the extent of deformation or the crystallinity. Among the

pretreatment approaches used, $CaCl_2.2H_2O$, methanol and formic acid solvent system appeared to be most promising since it is inexpensive and had resulted into formation of a fine and amorphous chitin having lower levels of crystallinity and seemingly appropriate for further enzymatic action.

5.6.1 Surface morphology

Chitin before and following chemical treatment was observed to have distinct changes in the surface architecture. Chemical treatment had led the loosening and swelling of chitin microfibrils, which facilitates the penetration of enzyme in subsequent deacetylation processes. Morphological changes such as loss of characteristic crystalline microfibrillar arrangement along with the formation of some major and minor grooves were evident after treatment. Morphological variations of similar nature were observed as has been detected by other groups (Win and Stevens, 2001; Beaney et al., 2007). Maximum defibrillation was observed in SF-CT, hence it appeared to be the most suitable substrate for enzymatic action. Earlier groups had also recommended the same to pretreatment of chitin (Tokura et al., 1998; Win and Stevens, 2001).

5.6.2 Crystallinity

Crystallinity of chitin is a significant criterion that enables the understanding of its susceptibility towards enzymatic deacetylation. Crystallinity of chitin substrates generated following chemical treatment was expressed in terms of crystalline index (CrI_{020}, CrI_{110}). All the chitin substrates, showed strong reflection at $2\theta \sim 9\text{-}10^{\circ}$ and $19\text{-}20^{\circ}$, however, their intensity was observed to decrease following chemical treatment. According to CrI_{020} values, Am-CT appeared as the most potential candidate for enzymatic deacetylation, however, CrI_{110} values dictated SF-CT as the most promising substrate for enzymatic treatment. Crystallinity of the two acid treated substrates viz. colloidal chitin and PA-CT was observed to be almost similar and somewhat higher than the other substrates but the former substrate appeared to be more amorphous than the later one. Substrate accessibility of enzyme was observed to be notably

improved following chemical treatment due to the decrystallization of regular microfibrillar arrangement (Martinou et al., 1997; Win and Stevens, 2001; Beaney et al., 2007).

5.6.3 Thermal analysis

Chitin and chitosan are biopolymers and high thermal energy is needed for dissociation of their structure (Bershtein and Egorov, 1994). TGA and DSC spectra of substrates before and following the chemical treatment were recorded (25 - 600 ^{0}C). TGA thermograms denoted two decomposition stages, first correspond to loss of water content and second due to decomposition of polysaccharide backbone with weight loss ranging from 66-74 %. DSC thermograms (30 - 350 ^{0}C) of chitinous substrates before and following chemical treatment denoted a wide endothermic peak around 140 – 240 ^{0}C with variations in their onset, peak and completion temperatures. The difference in the position and shape of the endothermic peak indicate differences in the water holding capacity and strength of water-polymer interaction (Sakurai et al., 2000; Kittur et al., 2002). However, considerable variations were observed in dissociation enthalpies (77-172 mJ mg^{-1}) of chitin substrates following chemical treatment. Thermal analysis revealed that crab shell chitin had highest thermal stability followed by SF-CT, Am-CT, colloidal chitin and PA-CT. The discrepancy in the peak enthalpy might be due to the diversity of chitin fiber aggregation following chemical treatment (Jang et al., 2004). Lowest value of peak enthalpy was observed for SF-CT and highest for crab shell chitin as evident by their degree of crystallinity. Amount of peak enthalpy correlated with the compactness of supra-molecular chitin structure (Prashanth et al., 2002; Jang et al., 2004; Sajomsang and Gonil, 2010). The higher the peak enthalpy, the denser the crystallinity would be. Higher peak enthalpy values revealed less susceptibility of substrate towards melting and dissociation process (Yen and Mau, 2007 a, b; Yen et al., 2009).

5.6.4 Degree of deacetylation

DDA to a greater extent influences the performance of chitosan for a variety of applications (Mao et al., 2004). Pretreatment of substrates not only affects into opening up of the

structure but also results into progressive removal of some exterior as well as interior acetyl groups. The FTIR spectra of various chitinous substrates though similar to each other as a whole, however, characteristic variations were observed in the absorption intensities of the functional groups of chitin. Substrate pretreatment is associated with the progressive weakening of the band occurring at 1655 cm^{-1} (amide I). Furthermore, the vibrational mode of amide II at 1550 cm^{-1} appeared at 1558, 1557, 1559 and 1568 cm^{-1} for PA-CT, SF-CT, Am-CT and colloidal chitin. Similar variations were also observed in the peak corresponding to 3450 cm^{-1} (-OH group). The band that appeared around 1425 cm^{-1} was assigned to CH_2 bending, due to the rearrangement of hydrogen bonds on the most favorable orientation of –OH groups, probably in the amorphous region of the polysaccharide (Focher et al., 1992a, b). Apart from this, C-H stretching band (2870-2880 cm^{-1}) and C-O-C starching bands (1070 cm^{-1}) had also appeared in the spectra of treated substrates.

Absorption intensities of peaks at 1655, 1550 and 3450 cm^{-1} were used to calculate DDA of chitin before and following chemical treatment. Crab shell chitin was observed to be 72 % acetylated. DDA had significantly increased following chemical treatment. SF-CT has highest DDA (37.14 %) among all the substrates followed by chemical treatment as evident by its low crystallinity and amorphous structure. DDA of PA-CT and colloidal chitin was observed to be almost similar (> 32 %). Am-CT had more deformed structure than PA-CT and colloidal chitin, thus deacetylated to a higher extent (34.44 %).

5.6.5 Bioconversion to chitosan

Functional properties and in turn industrial utility of chitosan depends on its DDA (Aranaz et al., 2009; Zhao et al., 2010). Higher DDA can be achieved through chemical deacetylation but the resulting chitosan has lower molecular weight and thus product quality is considered to be poor. Fungal CDAs reported so far are able to perform heterogeneous enzymatic deacetylation on solid substrate, but only up to a limited level. So, a two stage chemical and enzymatic process was adopted for bioconversion to chitosan. Chemically

pretreated chitinous substrates were subjected to further enzymatic deacetylation in order to get a product with high DDA. The enzyme was allowed to react with substrate for 24 hr and samples withdrawn at proper intervals were analyzed for DDA and amount of acetate released.

Untreated crab shell chitin had the lowest level of deacetylation of all the substrates used due to its high crystallinity, low porosity and closely packed structure. Similar observations were made by Martinou et al. (1995) and Beaney et al. (2007). PA-CT and Am-CT were the substrates with almost similar efficiency. Am-CT although had a more porous structure and low crystallinity, but their deacetylation levels were almost similar. This might be due to the heterogeneous nature of the Am-CT, although some parts of the sample are amorphous, there would possibly be the crystalline regions within the sample causing hindrance to enzymatic action. Colloidal chitin appeared to be next more susceptible substrate following Am-CT for CDA action. The substrate has open structure having major and minor grooves between microfibrils which increases the substrate accessibility to the enzyme. Its lower crystallinity had also conferred this to be more susceptible for enzymatic action. Highest deacetylation was achieved with SF-CT due to its porous structure and lower crystallinity. This might be attributed to calcium chloride and formic acid solvent system. Formic acid causes the substrate causes the weakening of the crystal structure of chitin and make it more amorphous. But its concentration should be less; as at higher concentrations it affects into partial depolymerization of the chitin chain with considerable reduction of molecular weight of the final product. Also the residual calcium chloride present may draw water into the structure due to its high affinity for water and it may get dissolved at reaction temperature leaving a more porous structure than its native one. Efficacy of this system for chitin pretreatment was also observed by various research groups (Win and Stevens, 2001; Aye et al., 2006; Beaney et al., 2007). They had observed that CDA from *C. lindemuthianum* and *R. oryzae* were able to convincingly deacetylate the treated chitin and had generated highly deacetylated chitosan.

Kinetic study of the deacetylation reaction had revealed that, the formation of acetate occurred initially at a rapid rate that reached a plateau after around 10 hrs. Kinetics of acetic acid release has been explained in terms of accessibility of acetyl groups (Stevens et al., 1997). Initially the exterior acetyl groups are removed, afterwards the rate of removal decreases as the interior acetyl groups are not accessible to enzyme and reached to a plateau. A similar deacetylation pattern was observed with the CDA from *C. lindemuthianum* and *A. coerulea* (Win and Stevens, 2001). CDA from both the sources had affected into rapid deacetylation during the initial stages but the deacetylation had declined during the later stages. The plateau reached depended on the enzyme and substrate ratio in the heterogeneous system. Further, optimal levels of these parameters were detected using statistical approaches in order to achieve the product with higher DDA. Maximum release of acetate (19.54 mM) was observed with a reaction mixture having 3.02 g, substrate along with 59.72 U, CDA. However, maximum deacetylation (90.32 %) was attained when the reaction had (2.96 g, substrate; 59.10 U, CDA). The experimental values detected were in close agreement with the values predicted by the quadratic model (acetate released, 18.26 mM; DDA, 89.07 %) under derived conditions. Contour plot had demonstrated that interaction of both the concentration of the substrate and the enzyme dose had profoundly affected the acetic acid released and DDA of the final product. Derivation of suitable levels of both the factors had resulted in a further increase of about 10 % in the DDA and consequently in the amount of acetate released. Therefore, significant enhancement in enzymatic deacetylation can be achieved through derivation of the major reaction parameters for production of chitosan with desired degree of deacetylation.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Chapter 6

SUMMARY AND CONCLUSIONS

Attempt was made to find the feasibility for using an eco-friendly route for producing high quality chitosan from chitin biopolymer. With an aim for developing an industrially compatible technology, microbial sources from sea food industry wastes were explore for production of CDA. Overall, eighteen fungal strains were isolated, among which strain (SA-1) having a notable extracellular CDA activity (103.78 ± 1.98 Ul^{-1}) along with lower level of intracellular activity (27.74 ± 0.45 Ul^{-1}) was selected and identified as *Penicillium oxalicum* SA-1 (ITCC 6965) by Indian Agricultural Research Institute, New Delhi, India. Attempt was further made to achieve an enhancement in the level of CDA through mutagenesis of the strain.

The wild type *P. oxalicum* SA-1 when mutagenized using successive treatments with ethidium bromide and microwave irradiation, had around a 2.0 fold improvement in the levels of CDA (210.71 ± 1.65 Ul^{-1}) as compared to the wild type strain. Evaluation of kinetic parameters viz. Q_s , Q_p, $Y_{p/x}$, $Y_{p/s}$, q_p, q_s has denoted that strain *P. oxalicum* SAE_M-51 is a hyper producer of CDA with notably improved productivity of 1.46 ± 0.82 $Ul^{-1}\,h^{-1}$ as compared to the wild type strain (0.75 ± 0.53 $Ul^{-1}\,h^{-1}$). Mutant and wild type strains of *P. oxalicum* could be differentiated on the basis of growth patterns as well as morphological features of hyphae and spores. The hyphae of mutant *P. oxalicum* SAE_M-51 were elongated, flattened with a smooth surface, whereas those of parental strain *P. oxalicum* SA-1 were irregular, smooth surfaced and coiled. Similarly, spores of mutant were oval and irregular in appearance, whereas the wild type spores were crescent shaped with rough surface.

Inducibility of chitinous substrates and glucose for CDA production was evaluated under submerged condition. Glucose as compared to chitin or colloidal chitin had led into markedly higher levels of enzyme production (221.57 ± 1.59 Ul^{-1}). However, replacement of glucose with

chitinous substrates had prolonged the duration for enzyme production primarily due to their insoluble nature.

In order to derive a process for production, the level of production was initially analyzed in the submerged process. Medium constituents and fermentation conditions for enhanced CDA production were derived using RSM. Derivation of suitable conditions and the medium is a prerequisite to achieve higher yields of CDA, that can be further utilized in bioconversion reactions for production of novel high quality chitosan species. Among the eight nutritional factors studied, yeast-extract, peptone, $(NH_4)_2SO_4$ and $MgSO_4$ were identified as the most critical variables for CDA production by Plackett–Burman design. Further optimization of these variables was performed by four-factor CCD. The statistical software package "Design Expert Version 7.0", Stat-Ease Inc., Minneapolis, USA was used for analyzing the experimental data. The model developed was authenticated as significant and statistical analysis of the results showed that yeast extract had contributed significantly to the response and its interaction with magnesium sulfate was strong. Peptone was also observed to be another important component in the medium that influences the CDA production. However, interactions of peptone and $MgSO_4$ had no considerable impact on CDA production as is evident from the parallel nature of the contours. Optimum concentration of the variables for enhanced CDA production determined by CCD were: yeast extract, 3.165 gl^{-1}; peptone, 4.754 gl^{-1}; $(NH_4)_2SO_4$, 0.255 gl^{-1} and $MgSO_4$, 0.535 gl^{-1}. The experimental value for CDA production (414.7 ± 6.32 Ul^{-1}) using optimized medium was in good agreement with the values predicted by the quadratic model (399.08 Ul^{-1}), proves the validity of the model.

Furthermore, the other major factors mainly pH, temperature, inoculum age and size were analyzed under submerged process using CCD. Enzyme production was observed to be significantly affected by all the variables studied. Among the variables studied, interaction between temperature and inoculum size had the most significant effect on CDA production. Other interactions that are fairly significant are, inoculum age and inoculum size; temperature

and inoculum age. Following CCD analysis, the various factors for achieving the maximal CDA production were derived to be: pH, 7.9; temperature, 28 ^{0}C; inoculum age, 90 hrs and 11.0 % inoculum size. Under these conditions, the maximal level of CDA production obtained was 623.57 ± 8.2 UI^{-1} (Q_p = 4.3264 $UI^{-1}h^{-1}$) which was in good agreement with the values predicted by the quadratic model (648.24 UI^{-1}), confirming the validity of the model. Optimization of enzyme production under submerged condition through statistical analysis had resulted into 6.0 fold enhancement in CDA production levels.

Level of enzyme production was further analyzed under SSF process to find out if the process of production can be made more economical by utilizing low cost and freely available agro-horticultural residual materials as solid support and substrate for fermentation. Among ten different solid supports employed, mustard oil cake appeared to be the most suitable substrate for CDA production (877.85 ±10.95 UI^{-1}). Scanning electron micrographs of the fermented mustard oil cake revealed a notable growth of the mould with abundant conidia. The CCD analysis has denoted that interaction between substrate and moisture content had profoundly affected the enzyme production. The values derived by CCD for achieving maximum CDA production were, substrate amount, 4.906 g; moisture content, 73.62 % and inoculum size, 8.578 % (w/v). This had resulted into a further 1.3 fold improvement in CDA production. The experimental CDA production (1162.03 ± 7.2 UI^{-1}) observed under derived conditions was in close agreement with the values predicted by the model (1137.85 UI^{-1}) and thus validates the model. An overall 10.9 fold enhancement in CDA production was achieved by derivation of major parameters affects the production process as compared to the levels of production as obtained by the wild type strain.

CDA from *P. oxalicum* SAE_M-51 was purified from the crude culture supernatant to homogeneity using ion exchange chromatography to 88.25 fold with a final recovery of 11.06 %. The purified CDA appeared as a single band with a molecular mass of 53 kDa and the pI was observed to be 5.2. The optimal temperature and pH of the purified enzyme were 50 ^{0}C and 9.0

respectively. Enzyme appeared stable at a pH range of 4.0 – 11.0 and at temperature 50 - 80 ^{0}C. Half life of the purified CDA was 693.10 min at its optimal temperature. Evaluation of thermodynamic parameters of purified enzyme revealed various factors for its stability against thermal denaturation. Activation energy ($E_{a,d}$), free energy (ΔG_d^*), enthalpy (ΔH_d^*) and entropy (ΔS_d^*) of activation for enzyme denaturation at optimal temperature were 114.72 kJ mol^{-1}, 97.86 kJ mol^{-1}, 112.04 kJ mol^{-1}, 43.93 J mol^{-1} K^{-1}, respectively. Kinetic parameters viz. K_m, V_{max}, k_{cat} and k_{cat}/K_m and substrate specificity of CDA from *P. oxalicum* SAE$_M$-51 were determined through Linweaver-Burk plot using various concentrations (0.5 - 5 mg ml^{-1}) of five different substrates (glycol chitin, dimeric, trimeric and pentameric forms of GlcNAc and *N*-acetyl glucosamine). Minimum degree of polymerization required for enzyme action was two. The enzyme had notably deacetylated glycol chitin and chitin oligomers having degree of polymerization more than four, however, activity levels were observed to be significantly reduced with chitobiose and chitotriose and there was no activity detected with *N*-acetylglucosamine. CDA exhibited maximum affinity towards chitopentaose and followed Michaelis-Menten kinetics as observed by its K_m (3.07 mg ml^{-1}) and k_{cat} (281.66 s^{-1}) values. Cu^{+2}, Co^{+2}, Fe^{+2}, Cd^{+2}, Mg^{+2}, Ca^{+2}, Ba^{+2}, Mn^{+2} appeared as activators of CDA activity, while Zn^{+2}, Mo^{+2}, Li^{+2}, Pb^{+2}, Bi^{+2} and EDTA had inhibitory action on enzyme. Enzyme was active in the presence of considerably higher concentrations of acetate (0-70 mM). Far-UV CD spectroscopic analysis revealed presence of both α and β- helical structures in the native conformation of enzyme. CDAs from different groups of organisms exhibit considerable diversity and the degree of identity of CDA from *P. oxalicum* SAE$_M$-51 could be observed with various groups of organisms. The amino acid sequence of the CDA from *P. oxalicum* SAE$_M$-51 shared significant identity with CDA sequences from *Cryptococcus neoformans*, *Bacillus cereus*, *Saccharomyces cerevisiae*, *Ajellomyces capsulatus*, *Aspergillus niger.*

Deacetylation potential of purified CDA was evaluated in two stages; chemical and enzymatic process. High crystallinity, low porosity and closely packed structure of untreated

chitin conferred it as a poor candidate for enzyme action. Since, crystallinity of chitin imposes a barrier to substrate accessibility of enzyme, so crystalline chitin was pretreated prior to enzyme addition using various chemicals i.e. acid, alkali and calcium chloride:methanol:formic acid solvent system. Modifications in the morphological and structural properties of chitin following chemical and enzymatic treatments were analyzed using scanning electron microscopy, X-ray diffraction, FTIR, thermo-gravimetric analysis and differential scanning calorimetry. Among all the substrates analyzed, maximum deacetylation (79.52 %) was achieved using SF-CT, owing to its porous structure and low crystallinity. In order to get a product with considerably higher degree of deacetylation, suitable levels of major factors affecting enzymatic action were further analyzed using CCD in terms of acetate released and DDA. Treatment of 2.96 g of SF-CT with 59.1 U of CDA had resulted into production of remarkably higher levels of deacetylated (90.32 %) chitosan species. The factors thus analyzed through CCD had led into a further 10 % enhancement in DDA.

Therefore, chitin deacetylation using extracellular CDA from *P. oxalicum* SAE_M-51 in a two stage chemical and enzymatic process appears to be productive, eco-friendly and a relatively simpler process for production of high quality chitosan with desired degree of deacetylation. The following are the major conclusions derived from the present study,

a. Among the eighteen fungal isolates, *P. oxalicum* SA-1, isolated from the residual materials of the sea food industry, was detected to be a potential strain with higher yields of extracellular CDA.

b. Strain SA-1 was subjected to mutagenesis by successive treatments employing ethidium bromide and microwave irradiation. Mutant *P. oxalicum* SAE_M-51 thus obtained exhibited 2.0 fold higher levels of CDA production. Evaluation of kinetic parameters viz. Q_s , Q_p, $Y_{p/x}$, $Y_{p/s}$, q_p, q_s had denoted that strain *P. oxalicum* SAE_M-51 to be a hyper producer of CDA.

c. Derivation of fermentation conditions under submerged process had led into notable enhancement in CDA production. The optimal conditions for submerged process were derived employing response surface methodology and this had led into about 6.0 fold enhancement in CDA production (Q_p = 4.3264 $UI^{-1}h^{-1}$) by mutant *P. oxalicum* SAE_M-51.

d. To further economize, production of enzyme was also evaluated in solid-state process to explore if the process could be further economical by using low cost and abundantly available agro-residual matrices as solid support and substrate for the production process. Mustard oil cake was found to be the most persuasive solid substrate for enzyme production. Analysis of major factors affecting enzyme production using CCD has led into 10.9 fold enhancement in the levels of enzyme production as compared to the production obtained by the wild type strain *P. oxalicum* SA-1.

e. Purified CDA produced by *P. oxalicum* SAE_M-51 was observed to be a 53±1 kDa protein. The enzyme was detected to be active and stable over a broad range of temperature and pH with higher catalytic potential and lower susceptibility towards end product inhibition. Thermodynamic parameters when analyzed had significantly enabled in understanding the complex process of thermal deactivation of the enzyme. Far-UV CD spectroscopic analysis revealed the presence of 56.26 % α and 15.63 % β helical structures at the optimal pH for enzyme activity. The amino acid sequence of the *P. oxalicum* SAE_M-51 CDA had considerable identity with the CDA of *Emericella nidulans*, *Rhizopus stolonifer*, *Saccharomyces cerevisiae* *Streptosporangium roseum* and *Pectobacterium cartovorum*.

f. A two stage chemical and enzymatic deacetylation process was evaluated for bioconversion of chitin to chitosan. Pre-treatment of crab shell chitin prior to enzymatic treatment had led into formation of various chitinous substrates with lower

crystallinity and with a more porous structure, among which SF-CT was detected to be the most promising substrate for enzymatic deacetylation. Deacetylation of SF-CT with CDA from *P. oxalicum* SAE_M-51 had resulted into the production of chitosan with 79.52 % of deacetylation. The parameters affecting deacetylation were analyzed through CCD and which had led into a further 10 % improvement in deacetylation and thus producing chitosan with 90 % of the deacetylation.

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1. **Pareek N**, Singh RP and Ghosh S (2011). Optimization of medium composition for enhanced chitin deacetylase production by mutant *Penicillium oxalicum* SAE_M-51 using response surface methodology under submerged fermentation. *Proc Biochem.* (doi:10.1016/j.procbio.2011.05.002)
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