Production, Purification and Characterisation of Chitin Degrading Enzymes from Microbial Cultures isolated from Coastal Environment Samples

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In

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By

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Declaration

I hereby declare that the work presented in this thesis entitled "Production, purification and characterisation of chitin degrading enzymes from microbial cultures isolated from coastal environment samples" is a bonafied record of the research work carried out by my student Mr. Karthik Narayanan (AcSIR Enrollment No. 10BB11A39008), under my guidance and supervision, at the CSIR- National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, Kerala, India. I also declare that all suggestions made by the audience during the Pre-synopsis presentation and those recommended by the Doctoral Advisory Committee have been incorporated in the thesis. The work incorporated in this thesis or any part of it has not been submitted for the award of any other degree, diploma, associateship or any other title or recognition.

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Karthik Narayanan

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Karthik Narayanan

Dedicated to

My Parents and Teachers

Contents

List of abbreviations

%	Percent
µ	micron
µg	microgram
µm	micrometre
µM	micromolar
µL	microlitre
°C	Degree Celsius
AMCase	Acidic mammalian chitinase
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BBD	Box-Behnken Design
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
BSA	Bovine Serum Albumin
CC	Colloidal Chitin
CCD	Central Composite Design
CDA	Chitin Deacetylase
cm	centimetre
CSIR	Council for Scientific and Industrial Research
CSL	Corn Steep Liquor
CTAB	Cetyl trimethylammonium bromide
CYS	Chitin-Yeast extract-Salts (medium)
Da	Dalton
DEAE	Diethylaminoethyl
DNA	Deoxy Ribonucleic Acid
DNS	3, 5- Dinitrosalicylic acid
DOE	Design of Experiments
EDTA	Ethylenediaminetetraacetic Acid
Fig.	Figure
g	gram
g L^{-1}	gram per litre
gds	Gram dry substrate

GH	Glycosyl Hydrolase
h	hour
HPLC	High Performance Liquid Chromatography
IEF	Iso-electric focussing
IPG	Immobilised pH gradient
ITS	Internal Transcribed Repeat
IUBMB	International Union for Biochemistry and Molecular Biology
kDa	kilodalton
K_m	Michaelis constant
L	litre
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
min	minute
mL	millilitre
mM	millimolar
NAG	N-acetylglucosamine
NG	N-glucosamine
NIIST	National Institute for Interdisciplinary Science and Technology
nm	nanometre
OA	Orthogonal Array
PAGE	Polyacrylamide Gel Electrophoresis
PBD	Plackett-Burman Design
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pI	Isoelectric point
pNP	para-Nitrophenol
RNA	Ribo Nucleic Acid
rpm	Rotations per minute
RSM	Response Surface Methodology
rRNA	Ribosomal RNA

SDS	Sodium Dodecyl Sulphate
sec	second
SEM	Scanning Electron Microscopy
SmF	Submerged Fermentation
sp.	Species
SSF	Solid State Fermentation
U	Units
U gds^{-1}	Units per dry gram substrate
U mL^{-1}	Units per millilitre
UV	Ultra-violet
V_e	Elution volume
V_o	Void volume
v/v	Volume per volume
V_{max}	Maximum velocity
vvm	Volume per volume per minute
w/v	Weight per volume
WB	Wheat Bran
YE	Yeast Extract

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Chapter 1

Introduction and Review of Literature

1. Introduction

1.1. Chitin

Chitin, a poly-β-1,4-*N*-acetylglucosamine (NAG) (Figure 1.1) is the next most abundant polysaccharide found in nature after cellulose and is biocompatible, biodegradable and bioabsorbable (Khoushab & Yamabhai, 2010). Henri Braconnot, a French professor of natural history, discovered chitin in 1811 after the discovery of a "material particularly resistant to usual chemicals" by A. Hachett, an English scientist in 1799 (Jeuniaux, 1966). The presence of nitrogen in chitin was demonstrated by Lassaigne in 1843 (Jeuniaux, 1966).

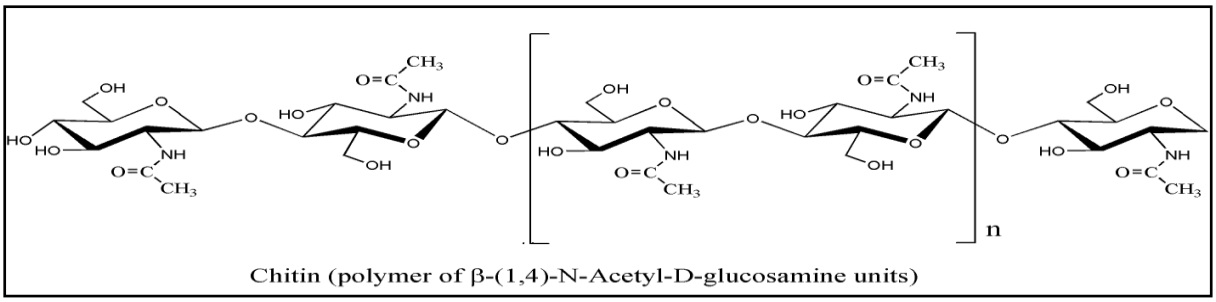

Fig. 1.1. Chemical structure of chitin.

Chitin is the main component of crustacean and arthropod exoskeletons and connective tissues, fungal cell walls and is also found to a certain extent in other marine organisms. The best characterized sources of chitin are shellfish (including shrimp, crab, lobster, and krill), oyster, and squid; and it is harvested in quantities of about 29.9, 1.4, and 0.7 million tons per year respectively (Synowiecki & Al-khateeb, 2003). The chitin contents in crustaceans usually range from 2-12% of the whole body mass. Up to 75% of waste generated from the processing of shellfish consists of chitin. An approximate composition of shell-fish waste is given in Table 1.1. The chitin content from processing wastes on the basis of dry weight is 13-26% for crabs, 14-42% for shrimps and 34-49% in case of krill (Synowiecki & Al-khateeb, 2003; Dahiya et al., 2006). In the natural state, chitin is complexed with proteins, lipids, pigments and minerals such as calcium carbonate (Synowiecki & Al-khateeb, 2003).

The percent content of chitin and calcium carbonate in the exoskeletons of different marine crustaceans and molluscs has been given in Table 1.2. Hence, demineralisation and deproteinisation of the chitin containing biomass is necessary to obtain chitin in the pure form. Traditionally, this is carried out using concentrated acids or alkalis, which poses corrosion, disposal and safety related environmental problems. The alternative to this is processing of the chitin wastes using chitin degrading enzymes. The production of chitinolytic enzymes from various microbial sources and use of these enzymes for various applications, including the production of N-acetylglucosamine (NAG) and its oligomers has been reported by several authors (Synowiecki & Al-khateeb, 2003; Dahiya et al., 2006; Matsumoto, 2006; Chen et al., 2010; Hamid et al., 2013; Jung and Park, 2014; Stoykov et al., 2015).

Table 1.1. Proximate composition of shellfish waste (Gopakumar, 2002).

Parameter	Prawn waste (%)	Squilla (%)	Crab shell (%)
Moisture	75-80	60-70	60-65
Ash	30-35	33-37	45-50
Protein	35-40	40-45	30-35
Chitin	15-20	12-16	13-15
Fat	3-5	2-3	1-1.5

Table 1.2. Chitin and $CaCO_3$ content in the exoskeletons of different marine crustaceans and molluscs (Kim, 2010).

Type	Chitin (%)	$CaCO_3$ (%)	Location
Phylum Crustacea			
Krill	20-30	20-25	Cuticle/Exoskeleton
Crab	15-30	40-50	Cuticle/Exoskeleton
King Crab	~35	40-50	Cuticle/Exoskeleton
Blue Crab	~14	40-50	Cuticle/Exoskeleton
Shrimp	17-40	20-30	Cuticle/Exoskeleton
Prawn	~40	20-30	Cuticle/Exoskeleton
Lobster	60-75	20-30	Cuticle/Exoskeleton
Goose Barnacle	~60	20-30	Shell
Phylum Mollusca			
Mussels and Clams	~3	85-90	Shell
Oyster	~6	85-90	Shell
Squid	20-40	negligible	Pen

1.2. Chitosan

Chitosan is a random heteropolymer of NAG and its deacetylated counterpart, N-glucosamine (NG). It is present in limited amounts in the exoskeletons of many crustaceans and insects and also in the fungal cell wall. It has gained immense importance due to its physicochemical properties such as biodegradability, biocompatibility, solubility and non-toxicity (Kurita, 2006). Chitosan is utilised in various areas, including biomedicine (burn and wound dressings), food and feed additives (antioxidant and hypocholesterolimic agent), cosmetics (ingredient in hair and skin care products), pharmaceuticals (encapsulating agent for drug delivery), agriculture (antifungal agent and bio-pesticide) and water treatment (removal/ recovery of metal ions and dyes from wastewaters) (Tsigos et al., 2000; Dutta et al., 2004).

At present, chitosan is mainly produced by deacetylation of chitin using harsh thermochemical process, which involves the use of a large amount of concentrated alkali (40-50% NaOH or KOH) and high temperatures (100°C or higher), which in turn causes environmental concern. This process is non-eco-friendly, tedious to control and results in reduced product quality with generation of a heterogeneous range of deacetylated products (Chang et al, 1997; Muzzarelli, 2012). Figure 1.2 shows a pictorial representation of the chemical process for the production of chitin and chitosan.

Most physicochemical properties and biological activities such as crystallinity, hydrophilicity, degradation and cell response of chitosan are essentially dependent on three factors. These are degree of polymerisation (DP) which defines the molecular mass of the polymers, degree of acetylation (DA) which defines its charge density and pattern of acetylation (PA), which defines the distribution of NAG and NG moieties in the chitosan chain. When the degree of deacetylation of chitin reaches about 50% (depending on the origin of the polymer), it becomes soluble in aqueous acidic media and is called chitosan. The

solubilisation occurs by protonation of the $-NH_2$ function on the C-2 position of the D-glucosamine repeat unit, whereby the polysaccharide is converted to a polyelectrolyte in acidic media (Bose et al., 2014).

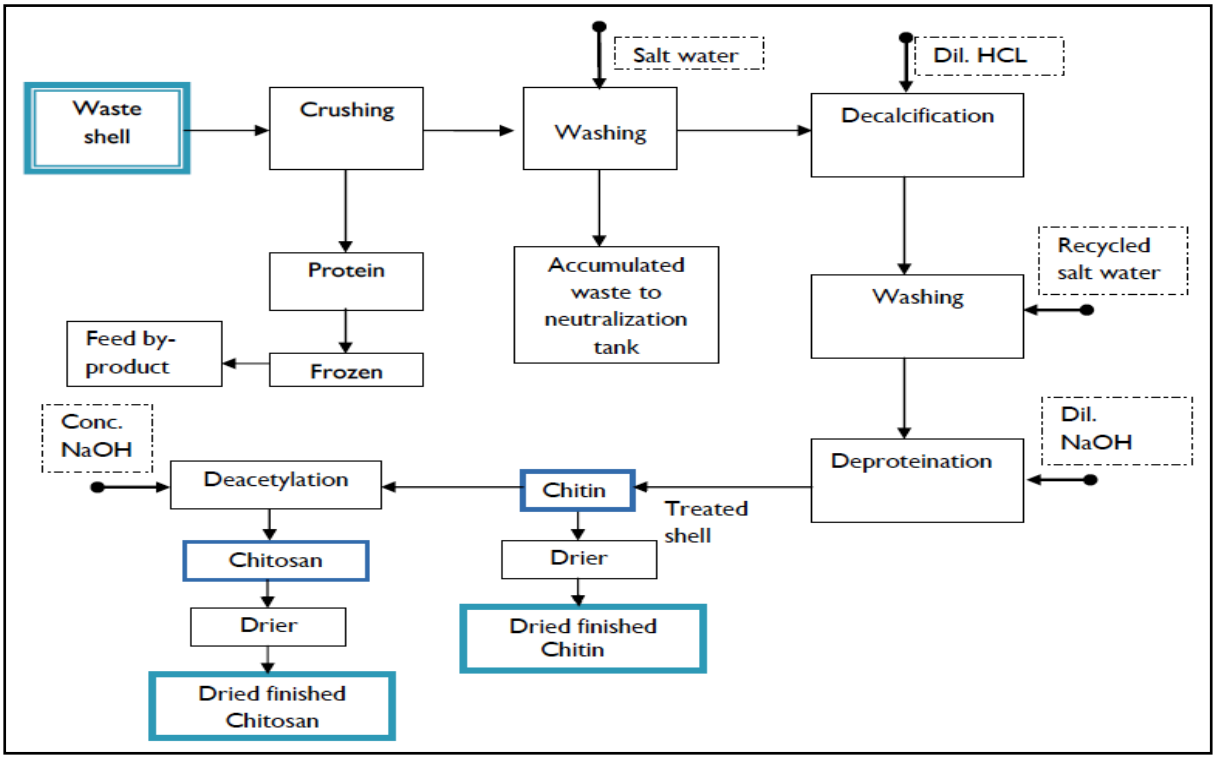

Fig. 1.2. Chemical process for the production of chitin and chitosan (KITCO).

According to the deacetylation grade and presence of other minerals, chitosan is categorized in three types: low purity, medium purity and high purity or medical grade by Global Industry Analysts, 2012 (https://2014.igem.org/Team:ITESM-Guadalajara). Low purity chitosan is used as flocculent for water treatment during industrial processes, as pesticide for agriculture, as pulp for paper and as a component for industrial processes in photography products and textiles. Medium purity chitosan is used for the same purposes as low purity chitosan, but it also has additional applications in protein precipitation, encapsulating agent (for food and some pharmaceuticals) and aqueous thickener. The high purity chitosan is ideal for wound healing and hemostasis, bio-surgery and ophthalmology, scaffold and cell therapy, and drug delivery and vaccines (https://2014.igem.org/Team:ITESM-Guadalajara). Table 1.3 shows various applications of chitin, chitosan and their derivatives.

Table 1.3. Applications of chitin, chitosan and their derivatives (Arbia et al., 2013).

Application area	Specific use
Wastewater treatment	➢ removal/recovery of metal ions from wastewaters, copper, chromium, cadmium, lead, nickel, mercury, iron, silver, zinc, cobalt and arsenic ➢ removal and binding of dyes ➢ removal and binding of heavy metals ➢ sludge treatment and dehydration agent ➢ biological denitrification
Food	➢ food and nutrition ➢ bioconversion for the production of value-added food products ➢ preservation of food ➢ filmogenic properties – food wrapping ➢ filtration and clarification of fruit juices ➢ hypolipidemic and hypocholesterolimic agent (slimming agent) ➢ antioxidant ➢ phenolic compound adsorption ➢ chitosan hydrogels for cell immobilization (lactic acid production) and for pigment ➢ encapsulation (astaxanthin) used in aquaculture to give typical salmon colour ➢ iron extract (to help in preventing bad odours in cooked meat)
Biomedicine	➢ burn and wound dressings for humans and animals ➢ antitumour activity ➢ drug delivery, gene delivery ➢ artificial skin, pharmacy ➢ immunostimulating properties in mammals and plants ➢ antiviral and anti-*Candida albicans* activities ➢ enhancing specific immunity (adjuvant properties) and stimulation of cytokine production ➢ ocular drug delivery vehicles in ophthalmology ➢ as nerve conduit for nerve regeneration due to its ability to facilitate nerve cell attachment ➢ therapeutic agents in the treatment of tumours (chitin and chitosan conjugates of 5-fluorouracil) ➢ encapsulation applications due to chitosan ability to form gels in the presence of certain divalent cations such as calcium, barium and strontium ➢ nutraceutical value as a potent antioxidant and matrix metalloproteinase inhibitor *via* alleviations of radical-induced oxidative damage (water-soluble carboxymethyl derivatives of chitin and chitosan) ➢ self-hardening paste for guided tissue regeneration in the treatment of periodontal bone defects (hydroxyapatite-chitin-chitosan composite bone-filling material) ➢ spermicide
Agriculture	➢ plant elicitor ➢ stimulation of chitinase and glucanase production (increased response to pathogen attack) ➢ stimulation of chitinase activity in compost (change of bacterial and fungal genetic diversity) ➢ antimicrobial (antifungal) agent and bio-pesticide ➢ enhancing plant vitality and ability to degrade walls of fungi upon entry ➢ fertilizer and biocontrol agent ➢ enhancing biocontrol efficiency by addition to plant growth-promoting rhizobacteria
Textile and paper	➢ textile fibres (sizing agent) ➢ paper manufacture (additive)
Biotechnology	➢ chitin affinity chromatography to selectively adsorb chitinase from a fermentation broth ➢ affinity matrix (chitosan) for the separation of wheat germ agglutinin ➢ enzyme and whole cell immobilizer ➢ *N*-acetyl chitobiose production from chitin using commercial hydrolytic enzymes ➢ chitinase and chitosanase production from *L. paracasei*, *Pseudomonas* and *Streptomyces* species ➢ microorganism immobilization for bioremediation of seawater polluted with crude oil ➢ support for biosensors ➢ bioseparation
Cosmetics	➢ ingredients for hair and skin care (moisturizer)

1.3. Chitinase

Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin and are found in a variety of organisms, including micro-organisms.

1.3.1. Classification of chitinases

The classification of chitinases is based on the mode of action as proposed by International Union for Biochemistry and Molecular Biology (IUBMB, 1992) and CAZy (http://www.cazy.org/**).** They are classified as endochitinases (E.C. 3.2.1.14), which randomly hydrolyse the chitin polymer to generate soluble low molecular weight polymers; exochitinases, which consists of two categories, chitobiosidase (E.C. 3.2.1.30; now included with E.C. 3.2.1.52), which catalyze the progressive release of di-acetylchitobiose starting at the non-reducing end of the chitin; and, β-N-acetylglucoaminidases (E.C. 3.2.1.52), which successively remove NAG units from the non-reducing end of the products generated by endochitinases and chitobiases (Chen et al., 2010; Matsumoto, 2006). The different modes of action of chitinase enzyme on the chitin polymer has been depicted in Figure 1.3.

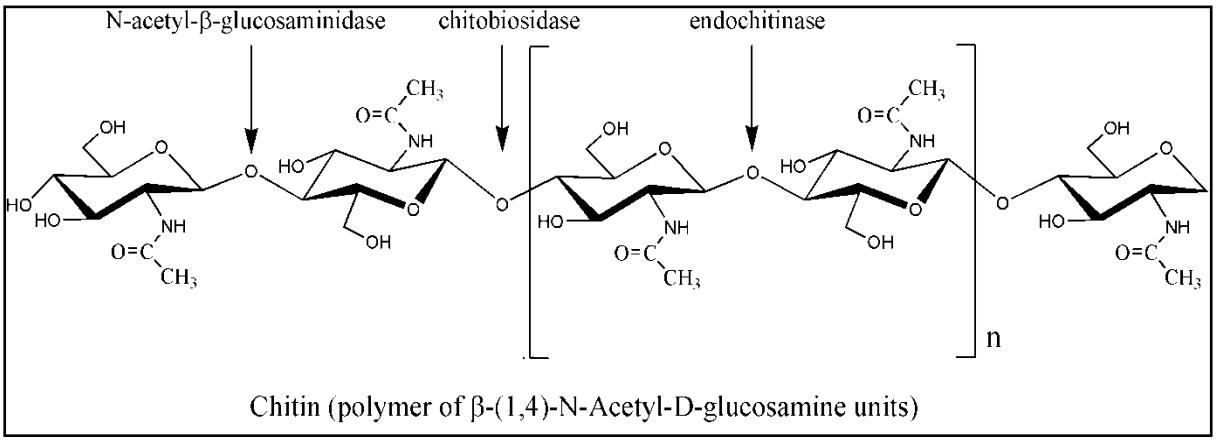

Fig. 1.3. Classification of chitinases according to site of action.

Based on amino acid sequence similarity, chitinases can be grouped into glycosyl hydrolase families (GH) 18, 19 and 20, which are structurally unrelated (Henrissat, 1997).

The catalytic mechanism of chitinases family 18 involves substrate-assisted catalysis, which retains the anomeric configuration of the product. Family 18 chitinases are mainly inhibited by allosaminidin. The catalytic mechanism of family 19 chitinases is a general acid-base mechanism that inverts the anomeric configuration of the hydrolyzed NAG residue. Amidines and amidrazones are the common inhibitors of family 19 chitinases (Kasprzewska, 2003; Matsumoto, 2006). Bacterial and fungal chitinases belong to both families GH18 and 19. Family 19 chitinases have mostly been identified in the plants. Family 20 includes the β-N-acetylhexosaminidases from bacteria such as *Vibrio harveyi*, *Streptomyces*, and humans (Dahiya et al., 2006). Microbial chitinases are divided into three major subgroups, namely, A, B and C, based on the amino acid sequences of their GH 18 modules. These subgroups differ in the architectures of their substrate-binding cleft, and thus, differ in their catalytic activities (exo vs. endo) and also contain different carbohydrate-binding modules (CBM) (Dahiya et al., 2006; Hartl et al., 2012). The presence of CBMs in enzymes enables them to bind more tightly to insoluble substrates, which enhance their processivity. The properties of chitinase subgroups A–C are summarized in Table 1.4.

Table 1.4. Properties of GH family 18 subgroups (Hartl *et. al.*, 2012).

Sub group	**Molecular Mass (kDa)**	**Substrate binding cleft**	**Mode of cleavage**	**Chitinase Class**	**CBM**	**Location of CBM**
A	40-60	Deep and narrow	Exo	Fungal/Bacterial	-	-
B	30-50	Shallow and open	Endo	Fungal/Plant	Varies among species	C-terminal
C	120-200	Deep and narrow	Exo (predicted)	Fungal/Bacterial	CBM 18 and 50	N-terminal

1.4. Sources of chitinase

Chitinases are present in a wide range of organisms, including viruses (Dahiya et al., 2006), bacteria (Bhattacharya et al., 2007), fungi (Matsumoto, 2006), insects (Merzendorfer & Zimoch, 2003), higher plants (Kasprzewska, 2003) and mammals (Boot et al., 1995). Most organisms (bacteria, plants and insects) have large families of chitinases with distinct functions, including digestion, defence against pathogens, cuticle turnover, and cell differentiation.

1.4.1. Plant Chitinases

In plants, chitinases are produced as pathogenesis-related proteins, which are induced by the attack of phytopathogens and confer the plant with self-defence ability against such pathogens. Chitinases can also be induced by the application of chitooligosaccharides and plant growth regulators such as ethylene, which forms the basis of pathogen biocontrol in plants (Kasprzewska, 2003). Chitinases have been reported to inhibit the growth of fungi synergistic with other enzymes, e.g., with β-1,3-glucanasese in different plant varieties such as rice, wheat, potatoes, tobacco, citrus fruits, beans, tomatoes, corn, yam and peas (Matsumoto, 2006). Chitinases have been utilized for biological control of insect pests on transgenic plants either alone or in combination with other insecticidal proteins (Matsumoto, 2006) and have been shown to have a role during embryogenesis, seed germination and growth of seedlings (Kasprzewska, 2003). Chitinases of plants are generally endochitinases with low molecular weight than the chitinases present in insects. Their size usually ranges from 25–40 kDa (Bhattacharya et al., 2007).

1.4.2. Insect chitinases

Chitinases or chitinase-like proteins have been found in all insect species studied belonging to different orders, including dipterans, lepidopterans, coleopterans, hemipterans and hymnopterans and have sizes ranging from 40–85 kDa. The chitinases present in the insects have been described from *Tribolium castaneum, Bombyx mori*, *Manduca sexta*, *Culex pipiens* and *Apis mellifera* (Matsumoto, 2006; Merzendorfer & Zimoch, 2003). The production of enzymes in insects is regulated by the hormones during the transformation of the larvae. These enzymes play important roles as degradative enzymes during ecdysis, where the cuticle is degraded to chitooligosaccharides by the endochtinases, which is further hydrolysed by exochitinases to N-acetyl-glucosamine. This is then salvaged to synthesize a new cuticle. Insect chitinases also have a defensive role against their own parasites (Merzendorfer & Zimoch, 2003).

1.4.3. Mammalian chitinases

All known mammalian chitinases belong to the GH18 family and are further subdivided as true chitinases with chitinolytic activity and chitinase-like proteins, which only have chitin binding ability without any enzymatic activity (Bussink et al., 1995). Chitotriosidase was the first human chitinase to be identified, which was produced by macrophages in Gaucher patients (Boot et al., 2007). Since this enzyme showed antifungal properties, it was proposed that it could be involved in defense against chitin-containing pathogens. After the discovery of chitotriosidase, a second chitinase named acidic mammalian chitinase (AMCase) was shown to be expressed primarily in the gastrointestinal tract and lung of both mouse and human. Based on its expression profile, it was proposed to have a dual function in innate immunity and chitin digestion (Bussink et al., 2007). Recent studies performed on several bat

species suggest that AMCase could actually be involved in chitin digestion in insectivorous mammals (Strobel et al., 2013).

1.4.4. Microbial Chitinases

Chitinases are widely distributed in bacteria such as *Serratia, Chromobacterium, Klebsiella, Bacillus, Pseudomonas, Clostridium, Vibrio, Arthrobacter, Beneckea, Aeromonas,* etc. Among actinomycetes, *Streptomyces* species are considered as good producers of chitinolytic enzymes (Bhattacharya et al., 2007; Narayana & Vijayalakshmi, 2009). Yeasts and fungi with yeast-like growth forms have low numbers of chitinases. Chitinase genes have been identified in *Saccharomyces cerevisiae, Candida albicans, Kluyveromyces lactis* and the dimorphic fungi-yeast *Paracoccidioides brasiliensis* (Li, 2006). They are also found in fungi including *Trichoderma, Oenicillium, Penicillium, Lecanicillium, Neurospora, Mucor, Beauveria, Lycoperdon, Aspergillus, Myrothecium, Conidiobolus, Metharhizium, Stachybotrys* and *Agaricus* (Li, 2006; Matsumoto, 2006; Hartl et al., 2012).

Chitin is generally insoluble in aqueous media and is of high molecular weight and complexity and it has a heterogeneous composition. Therefore, uptake of chitin as such and its degradation within the cell by microorganisms is not possible. Hence, they secrete enzymes with different specificity to transform or hydrolyse chitin. These microorganisms produce chitinases in higher amounts than animals and plants, generally as inducible extracelullar that are of the two types, endochitinases and exochitinases (Matsumoto, 2006). In bacteria, chitinases are also produced as defence against fungi or to colonise other chitin containing organisms, including insects and nematodes. Also, as chitin is a component of the cell walls of fungi and exoskeletal elements of some higher organisms, chitinases are generally found in these organisms modify their own chitin.

1.5. Production of chitinase

Industrial enzymes have been widely produced employing either submerged fermentation (SmF) or solid-state fermentation (SSF) (Pandey and Singhania, 2008; Pandey et al., 2010). A brief comparision of both the production systems is elucidated in Table 1.5.

Table 1.5. Comparision of SmF and SSF processes.

Factor	SmF	SSF
Substrates	Substrates and nutrients solubilised in aqueous medium.	Insoluble substrates like cellulose, starch which are moistened with nutrient containing aqueous medium.
Aseptic conditions	Sterilization by autoclaving and relatively easier to maintain aseptic conditions.	Steam based sterilisation, non ability to maintain strict sterile conditions.
Water	High volumes of water consumed since it is the base medium for growth and dissolving nutrients and substrates and hence growth.	Limited consumption of water; low A_w. Only required in limited quantities for extraction of product.
Metabolic Heating	Easy control of temperature due to homogenous mixing.	Low heat transfer capacity and heat pockets generated in the medium.
Aeration	Limited by solubility of oxygen but may be controlled by supplying high levels of air or oxygen as required and efficient mixing.	Relatively easier aeration and high surface exchange between air and substrate, but ultimately depends on substrate bed height.
pH control	Easy pH control due to homogenous mixing.	The natural solid substrates have self buffering capacity.
Mechanical agitation	Good homogenization achieved.	Static conditions are preferably maintained with occasional mixing if required.
Scale up	Relatively easier to scale up and obtain desired levels of product.	Maintaining optimum conditions at scaled up levels becomes more difficult and hence may not be able to obtain desired levels of product.
Inoculation	Spore/mycelial inoculation in case of fungi, batch/continuous process	Spore inoculation in case of fungi, batch process
Contamination	Lower risk of contamination, but contamination generally observed due to fast growing bacteria.	Higher risk of contamination due to lower sterility conditions of the process, usually contamination by fungi.
Energetic consideration	High energy consuming	Low energy consuming
Volume and cost of Equipment	High volumes and sophisticated equipment and controls required and hence high cost of technology involved.	Comparatively low volumes and simpler setup required and hence low costs of equipment and technology involved.
Effluent & pollution	High volumes of polluting effluents released after the bioprocess is completed.	Very limited or no effluents, less pollution.

Chitinases from microorganisms have been produced predominantly by SmF in batch, fed-batch and continuous modes (Das et al., 2016). But attempts have also been carried out to produce chitinase by SSF method. In SSF, different substrate types, including agricultural residues such as wheat bran, rice bran, chitin flakes, waste products obtained from different marine organisms like crabs, shrimps and prawns etc. (Nampoothiri et al., 2004; Binod et al., 2005; Dahiya et al., 2005; Sudhakar & Nagarajan, 2010, 2011a; Suresh & Kumar, 2012; Das et al., 2016) have been used. The production of extracellular chitinase is influenced by various physiochemical parameters such as pH, incubation temperature, aeration and media components, e.g., carbon sources, nitrogen sources and other micronutrients.

Addition of carbon sources other than chitin to the production medium could have a mixed effect. For example, the addition of simple sugars such as glucose, fructose, lactose, maltose, mannose, arabinose, xylose and sucrose in the medium in combination with colloidal chitin had varying effects on bacterial chitinase production, including increased production (Kavitha & Vijayalakshmi, 2011; Gomaa, 2012; Kuddus & Ahmad, 2013), decreased production (Narayana & Vijayalakshmi, 2009; Singh, 2010; Chakrabortty et al., 2012) and remaining unaffected (Al Ahmadi et al., 2008) by different bacterial strains. The addition of starch and fructose resulted in the increased production of chitinase by a fungus *Trichoderma asperellum* (Kumar et al., 2012).

Nitrogen sources play a crucial role in the synthesis of enzymes involved in various primary as well as secondary metabolic pathways. Supplementation of different nitrogen sources in the production medium and their effects on the chitinase production has been elucidated in several reports. Organic nitrogen sources, which enhanced chitinase production included yeast extract (Al Ahmadi et al., 2008; Narayana & Vijayalakshmi, 2009; Kumar et al., 2012; Sharaf, 2005), corn steep liquor (CSL) (Narasimhan & Shivakumar, 2012), peptone (Al Ahmadi et al., 2008), malt extract (Kuddus & Ahmad, 2013; Meena et al., 2014). The

inorganic nitrogen sources, which enhanced chitinase production were urea (Singh, 2010; Hao et al., 2012), ammonium sulphate (Al Ahmadi et al., 2008; Chakrabortty et al., 2012; Meena et al., 2014), potassium nitrate (Narasimhan & Shivakumar, 2012), sodium nitrate (Binod et al., 2005; Sharaf, 2005) and ammonium sulphate (Farag, et al., 2014; Kumar et al., 2012; Wasli et al., 2009).

The initial pH of the medium and the incubation temperature play an important role in the production of chitinase. It has been generally observed that bacterial chitinase production is highest at near neutral pH of 6-8 (Table 1.6). In an exceptional case, an optimum initial pH of 9.0 was reported for a *Micrococcus* sp. (Annamalai et al., 2010). It was generally observed that fungal chitinase production took place at acidic pH of less than 6.0 (Table 1.6). However, in case of SSF, the generally used substrates being agro-residues, which possess very good buffering capacity, there is no need to adjust the medium pH (Nampoothiri et al., 2004). For both SSF and SmF, chitinase production is generally highest in the mesophilic temperature range of 25-35°C for both bacteria and fungi (Table 1.6). An exception includes a *Bacillus* sp. which had an optimum production temperature of 55°C (Chang et al., 2010).

The addition of metal ions to the production medium influenced the production of chitinase by different microbes. Addition of $CaCl_2$ (Gohel et al., 2006a; Narasimhan et al., 2013), $MgCl_2$ (Gohel et al., 2006a; Al Ahmadi et al., 2008) and $CoCl_2$ (Narasimhan et al., 2013) enhanced chitinase production by different microbial cultures.

An alteration in the porosity of the cell membrane can be caused by the surfactants at low concentrations without the total disruption of the cells causing an increased release of intracellular metabolites, including enzymes into the extracellular environment. Though only a few reports were available, addition of non-ionic surfactants enhanced chitinase production, while addition of ionic detergents inhibited chitinase production in *Aeromonas* sp. (Al

Ahmadi et al., 2008), *Bacillus subtilis* (Narasimhan & Shivakumar, 2012) and *Paenibacillus* sp. (Singh, 2010).

The period of incubation determines the time point until which the culture is to be incubated in the nutrient medium to obtain maximum enzyme production. The reasons for decreased enzyme production after prolonged incubation may be due to the depletion of nutrients or production of inhibitory chemicals in the medium, resulting in the inactivation of secretory machinery of the enzymes or the degradation of the enzyme itself. Different bacterial strains produced maximum chitinase after two days (Kuddus & Ahmad, 2013), three days (Sharmistha et al., 2012), four days (Meena et al., 2014) and five days (Gomaa, 2012) of incubation, while fungal strains required longer incubation periods of four days (Nampoothiri et al., 2004; Wasli et al., 2009), six days (Sudhakar & Nagarajan, 2010), seven days (Sharaf, 2005) or eight days (Thadathil et al., 2014) of incubation.

The type of inoculum is another important factor in fungal chitinase production, which influences the production period and level. Usually spore suspension has been used as inoculum for production of chitinases by fungal strains (Sharaf, 2005; Sudhakar & Nagarajan, 2011a; Kumar et al., 2012; Meena et al., 2014; Patil & Jadhav, 2014). However, Matsumoto et al., (2004) found that mycelial inoculum resulted in faster and higher chitinase production as compared to spore inoculum for *Verticillium lecanii*.

Table 1.6. Production of microbial chitinases.

Microorganism	Method of Production	Initial *pH* of medium	Incubation temperature (°C)	Substrate	Yield	Reference
Aeromonas hydrophila (a), Aeromonas punctata (b)	SmF	8.0[a] / 7.0[b]	37	Colloidal chitin	97.35[a] / 89.87[b] U mL^{-1}	Kuddus & Ahmad, 2013
Alcaligenes faecalis	SmF	8.0	37	Shrimp and crab shell powder	258 U mL^{-1}	Annamalai et al., 2011
Aspergillus terreus	SmF	6.0	30	Fish scale waste	4.31 U mL^{-1}	Ghanem et al., 2010
Bacillus cereus	SmF	7.5	37	Shrimp head powder	11.2 U mL^{-1}	Wang et al., 2012
Bacillus licheniformis	SmF	8.0	30	Colloidal chitin/ dried fungal mat	23 U mL^{-1}	Gomaa, 2012
Bacillus subtilis	SmF	7.0	45	Shrimp and crab shell powder	166 U mL^{-1}	Wang et al., 2006
Basidiobolus ranarum	SmF	9.0	25	Colloidal chitin	3.47 U mL^{-1}	Mishra et al., 2012
Beauveria bassiana	SSF	5.0	40	Wheat bran supplemented with silkworm chrysalis	126 U gds^{-1}	Zhang et. al., 2004
Enterobacter sp.	SSF	8.0	30	Wheat bran supplemented with chitin flakes	1475 U gds^{-1}	Dahiya et al., 2006
Fusarium Oxysporum	SSF	6.0	30	Wheat bran supplemented with chitin flakes	23.6 U gds^{-1}	Gkargkas et. al., 2004
Oerskovia xanthineolytica	SSF	N.S.	45	Wheat bran supplemented with colloidal chitin	170 U gds^{-1}	Waghmare and Ghosh, 2010
Paenibacillus sp.	SmF	6.0	46	Chitin powder	2.24 U mL^{-1}	Dai et al., 2011
Paenibacillus sp.	SmF	7.0	30	Crab shell chitin	56.43 U mL^{-1}	Singh, 2010
Penicillium aculeatum	SSF	5.0	30	Wheat bran supplemented with chitin flakes	12.53 U gds^{-1}	Binod et al., 2005
Penicillium ochrochloron	SSF	7.0	30	Wheat bran[a], Rice bran[b], Red gram bran[c]	2463[a], 1228[b], 965[c] U gds^{-1}	Patil and Jadhav, 2014
Rhizopus oryzae	SmF	N.S.	30	Starch	3.56 U mL^{-1}	Chen et. al., 2013
Serratia Marcescens	SSF	6.0	30	Sugarcane bagasse supplemented with colloidal chitin	38.64 U mL^{-1}	Sudhakar & Nagarajan, 2011
Streptomyces rimosus	SmF	7.5	28	Shrimp shell waste/ crab shell powder chitin	3.5 U mL^{-1}	Brzezinska et. al., 2013a
Streptomyces sporovirgulis	SmF	8.5	25-30	Shrimp shell waste/ crab shell powder chitin	7.5 U mL^{-1}	Brzezinska, et al., 2013b
Streptomyces tendae	SmF	7.0	30	Colloidal chitin/ fungal biomass	11.3 U mL^{-1}	Kavitha & Vijayalakshmi, 2011
Streptomyces violaceusniger	SmF	7.0	30	Colloidal chitin	3.5 U mL^{-1}	Nagpure & Gupta, 2013
Trichoderma harzianum	SSF	4.5	30	Wheat bran supplemented with colloidal chitin	3.18 U gds^{-1}	Nampoothiri et al., 2004

N.S. - Not specified

1.6. Application of chitinases

Microbial chitinases have gained interest in different biotechnological applications due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton. This leads to their use as antimicrobial or insecticidal agents. They have been reported to be effectively as a biocontrol agent against many fungal pathogens especially those of plants and insects (Dahiya et al., 2006; Gohel et al., 2006b; Nagpure et al., 2014). The use of chitinase in the control of mosquito populations has also been explored (Dahiya et al., 2006).

Microbial chitinases have also been described useful for the degradation of chitinous waste which is a major product of the seafood industry. This leads to another useful application of chitinase i.e. the bioconversion of chitin to pharmacological active products, namely NAG and chito-oligosaccharides. These are useful as antimicrobials, immunoenhancers and activation of host defence system, drug delivery carriers, antioxidants, in haemostasis and wound healing, blood cholesterol control and food preservation (Synowiecki & Al-khateeb, 2003; Chen et al., 2010; Khoushab & Yamabhai, 2010). Other interesting applications of chitinases include the preparation of protoplasts from filamentous fungi and the production of single cell protein (Dahiya et al., 2006; Nagpure et al., 2014).

1.7. Chitin deacetylase

Bioconversion of chitin to chitosan using the enzyme chitin deacetylase (CDA) (EC 3.5.1.41) is competent alternative to the chemical method as it overcomes the various disadvantages of the chemical process (Trudel and Asselin, 1990). CDA catalyzes the deacetylation of NAG residues of chitin by multiple attack mechanism (Tsigos et al., 2000). Chitosan produced by the enzymatic method offers the possibility of a controlled, non-degradable deacetylation process that results in a more regular pattern of deacetylation (Tsigos et al., 2000). In spite of their industrial importance and increasing demand of chitosan in various commercial fields, the high cost of production of CDA has hindered its industrial application in bioconversion of chitin to chitosan.

1.8. Sources and production of chitin deacetylase

CDA has been reported in several microorganisms like fungi, yeast and bacteria, and is also present in some insect species (Zhao et al., 2010b). Fungal CDAs have been studied more widely than those from bacterial and insects. On the basis of localization of CDA in fungi, it has been divided broadly into two subgroups: intracellular CDA and extracellular CDA. Intracellular CDA is secreted into the periplasm while extracellular CDA is secreted into the external medium. Numerous micro-organisms, predominantly fungi, displaying both intracellular and extracellular CDA production have been identified in the past few years (Table 1.7). The biological roles of CDA include biosynthesis of cell wall and ascospore wall in fungi and yeasts respectively, appresorium formation in plant pathogens and in fungus-insect interactions for self defence against host chitinases, thus facilitating easier colonisation of the host (Ghormade et al., 2010). CDA-assisted enzymatic conversion of chitin to chitosan needs intensive screening of novel CDA hyper-producers. For industrial application, exploration of novel CDA hyper producers with an inherent capability to secrete the CDA, and further optimization of the production process is necessary.

Table 1.7. Production of microbial CDA. (**N.S. - Not specified**)

Microorganism	Method of Production and localisation of Enzyme	Initial pH of medium	Incubation temperature (°C)	Substrate/Medium	Yield	Reference
Absida corymbifera	SmF, extracellular	9.0	28	Powdered chitin, yeast extract, peptone	Glucose 21.5 U Powdered chitin 0.9 U	Zhao et al., 2010a)
Absida coerulea	SmF, intracellular	4.5	30	Glucose, yeast extract, K_2HPO_4, NaCl, $MgSO_4$, $CaCl_2$	0.24 U	Win & Stevens, 2001
Aspergillus nidulans	SmF, extracellular	N.S.	25	glucose, ammonium tartrate, KH_2PO_4, $MgSO_4$, KCI, yeast extract,	N.S.	Alfonso et.al., 1995
Colletotrichum lindemuthianum	SmF, extracellular	N.S.	25	glucose, glutamic acid, K_2HPO_4, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$, thiamine, and nicotinic acid	1245 U	Tsigos and Bouriotis, 1995
Colletotrichum gloeosporioides	SmF, extracellular	6.0	28	glucose, glutamic acid, K_2HPO_4, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$, thiamine, and nicotinic acid	0.018 U mg^{-1}	Pacheco et al., 2013
Metarhizium anisopliae	SmF, extracellular	5.5	28	Glucose, yeast extract, peptone	0.26 U	Nahar et al., 2004
Mucor rouxii	SmF, intracellular	4.5	30	Glucose, yeast extract, peptone	1127 U	Araki & Ito, 1975
Bacillus amyloliquifaciens	SmF, extracellular	6.0	37	Glucose/starch, yeast extract	17.84	Zhou et al., 2010
Cryptococcus neoformans	SmF, extracellular	N.S.	25	Glucose, yeast ex, peptone	N.S.	Baker et. al., 2007
Mortierella sp.	SmF, extracellular	4.5	28	Glucose, yeast extract, peptone	0.6 U mL^{-1}	Kim et al., 2008
Paenibacillus sp.	SmF, extracellular	6.0	46	Chitin powder, yeast extract	0.68 U mL^{-1}	Dai et al., 2011
Penicillium oxalicum	SmF, extracellular	6.0	30	Glucose, yeast ex, peptone, KH_2PO_4, K_2HPO_4, $MgSO_4$, $(NH_4)_2SO_4$, NaCl, $CaCl_2$.	220 U L^{-1}	Pareek et al., 2011a
Penicillium oxalicum	SSF, extra	N.S.	30	Mustard oil cake	1162 U gds^{-1}	Pareek et al., 2014b
Rhizopus japonicus	SmF, extracellular	6.0	30	Glucose, yeast extract, peptone, KH_2PO_4, K_2HPO_4, $MgSO_4$, $(NH_4)_2SO_4$, NaCl, $CaCl_2$.	547 U L^{-1}	Zhang et al., 2014
Rhizopus oryzae	SmF/SSF extracellular	6.5	30	glucose, $NaNO_3$, KCl, KH_2PO_4, $MgSO_4$, yeast extract (SmF); soyabean residue (SSF)	13 U L^{-1} (SmF); 914 U L^{-1} (SSF)	Aye et al., 2006
Rhodococcus erythropolis	SmF, extracellular	7.0	30	Powder chitin, Glucose, yeast ex, KH_2PO_4, K_2HPO_4, $MgSO_4$, $(NH_4)_2SO_4$, NaCl, $CaCl_2$.	239 U mL^{-1}	Sun et al., 2014
Scopulariopsis brevicaulis	SmF, extracellular	7.0	29	3,6-O-carboxymethylchitin, sucrose, NaNO3, K_2HPO_4, KCl, peptone, $MgSO_4$, $MnSO_4$, $CoCl_2$	36 U mL^{-1}	Cai et al., 2013
Gongronella butleri	SmF, intracellular	N.S.	N.S.	Glucose, yeast extract, peptone, $(NH_4)_2SO_4$, K_2HPO_4,NaCl, $MgSO_4$,$CaCl_2$	51.2 U	Maw et al., 2002

A flow chart for the bioproduction of chitin, chitosan, and their oligosaccharides from natural resources by using enzymes and microorganisms is shown in Figure 1.4. The objective of the present study is concerned with the production of chitinase and CDA.

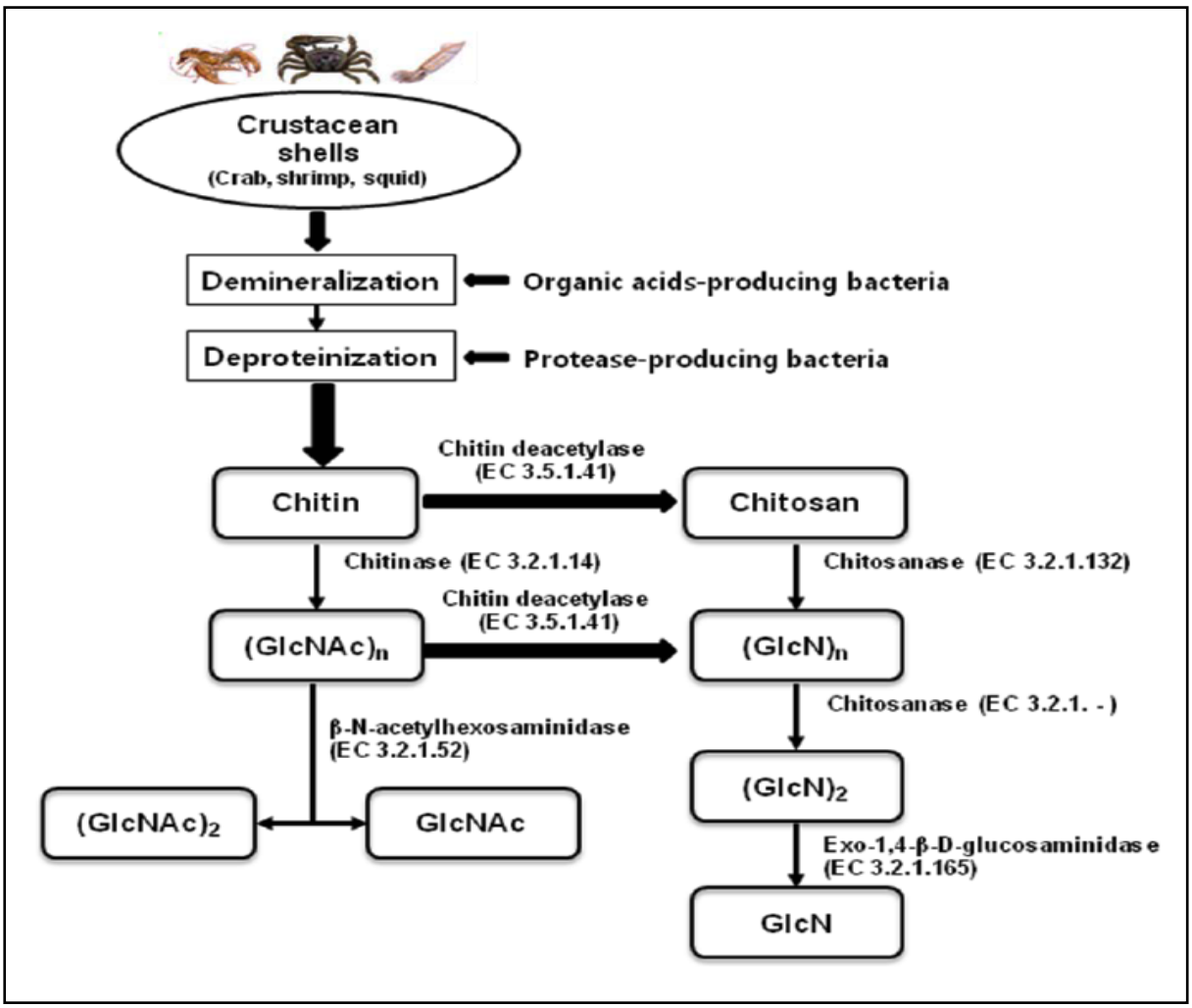

Fig. 1.4. Biological process for the production of chitin and chitosan (Jung and Park, 2014).

1.9. Statistical Media optimisation

Medium components and culture conditions have significant influence on extracellular enzyme production and are different for each microorganism. Bioprocess parameter optimisation plays a crucial role in the development of any fermentation process, since it significantly influences the economy and efficiency of the process. The conventional approach of optimizing one-factor-at-a-time though gives an initial understanding of the effect of individual bioprocess parameters on production of the desired product; it cannot provide the information on mutual interactions between the variables on the desired outcome. On the other hand, statistical experimental designs offer an organised plan for optimization of variables by experimentation and can eliminate the limitations of one-factor-at-a-time approach (Baş and Boyaci, 2007). Statistical data analysis involves a limited number of experiments where the interactions among several experimental variables can be visualized simultaneously. Further computational analysis leads to the prediction of data in the areas not directly covered by experimentation.

1.9.1. Plackett-Burman design

Plackett-Burman experimental design (PBD) was developed in 1946 by statisticians, R. L. Plackett and J. P. Burman as an efficient screening method to identify the active factors using as few experimental runs as possible, hence making the process economical (Plackett and Burman, 1946). The PBD type is a two level fractional factorial screening design for studying N-1 variables using N runs, where N is a multiple of 4. In these designs, main effects have a complicated confounding relationship with two-factor interactions and the main effects are aliased with two-way interactions. Therefore, these designs are used to study main effects when it can be assumed that two-way interactions are negligible (Park, 2007). In practical use, two-level full or fractional factorial designs, and PBDs are often used to screen for the important factors that influence process output measures or product quality. These designs

are useful for fitting first-order models (which detect linear effects) and can provide information on the existence of second-order effects (curvature) when the design includes centre points (Park, 2007).

1.9.2. Response Surface Methodology

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques (design of experiments (DOE)) useful for developing, improving, and optimizing processes. It is often used to refine models after determination of important factors using factorial designs; especially where there is curvature in the response surface (Singh and Sodhi, 2014; Myers et al., 2016). The most extensive applications of RSM are in the particular situations where several input variables potentially influence some performance measure or quality characteristic of the process. This performance measure or quality characteristic is called the response. The input variables are sometimes called independent variables, and they are subject to the control of the scientist or engineer (Myers et al., 2016). The field of RSM consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and optimization methods for finding the values of the process variables that produce desirable values of the response (Myers et al., 2016).

The difference between a response surface equation and the equation for a factorial design is the addition of the squared (or quadratic) terms that allows modelling the curvature in the response, making them useful for (Minitab Inc., 2010):

- Understanding or mapping a region of a response surface. Response surface equations model how changes in variables affect a response of interest.
- Finding the levels of variables that optimize a response.
- Selecting the operating conditions to meet specifications.

There are two main types of response surface designs (Singh and Sodhi, 2014):

(a) Central Composite design

Central Composite designs (CCD) can fit a full quadratic model. They are often used when the design plan calls for sequential experimentation because these designs can include information from a correctly planned factorial experiment (Minitab Inc., 2010). It has a few steps, viz., initial determination of the optimum region for the variables, behaviour of the response in the optimum region, estimation of the optimal condition and verification (Khuri & Cornell, 1987; Myers and Montgomery, 1995).

A CCD is the most commonly used response surface designed experiment. CCDs are a factorial or fractional factorial design with centre points, augmented with a group of axial points (also called star points) that allows estimating the curvature. A central composite design can be used to efficiently estimate first- and second-order terms and model a response variable with curvature by adding center and axial points to a previously-done factorial design. CCDs are especially useful in sequential experiments because it can be built on previous factorial experiments by adding axial and centre points. When possible, CCD has the desired properties of orthogonal blocks and rotatability (Minitab Inc., 2010).

(b) Box-Behnken design

Box-Behnken designs (BBD) usually have fewer design points than CCDs, thus, they are less expensive to run with the same number of factors. They can efficiently estimate the first- and second-order coefficients; however, they can't include runs from a factorial experiment. BBDs always have three levels per factor, unlike CCDs, which can have up to five. Also, unlike CCDs, BBDs never include runs where all factors are at their extreme setting, such as all of the low settings (Minitab Inc., 2010).

1.9.3. Taguchi design

A Taguchi design is a designed experiment that allows choosing a product or process that functions more consistently in the operating environment (Taguchi, 1986). Taguchi designs recognize that not all the factors that cause variability can be controlled and these are called noise factors. Taguchi designs try to identify controllable factors (control factors) and thus minimize the effect of the noise factors (Minitab Inc., 2010). During experimentation, the noise factors are manipulated to force variability to occur and then determine optimal control factor settings that make the process or product robust, or resistant to variation from the noise factors. A process designed with this goal will produce more consistent output. A product designed with this goal will deliver more consistent performance regardless of the environment in which it is used (Roy, 2001).Taguchi designs use orthogonal arrays (OA), which estimate the effects of factors on the response mean and variation. An OA means the design is balanced so that factor levels are weighted equally. Because of this, each factor can be assessed independently of all the other factors, so the effect of one factor does not affect the estimation of a different factor. This can reduce the time and cost associated with the experiment when fractionated designs are used. OA designs concentrate primarily on main effects (Minitab Inc., 2010).

1.9.4. Application of statistical designs in bioprocess optimisation for microbial chitinase and CDA production

Bioprocess optimisation using statistical methods has been applied for achieving highest possible chitinase production. Media optimisation using PBD was carried out for *Bacillus subtilis* (Narasimhan & Shivakumar, 2012) and *Pantoea dispersa* (Gohel et al., 2006a) cultures and a 10-fold and 4.21-fold increase in chitinase production were obtained, respectively. Similarly, by using a combination of PBD and RSM methods, the chitinase yield increased by 2.56-fold for a *Paenibacillus* sp. and by 15.5-fold for *Chitinolyticbacter*

meiuanensis (Hao et al., 2012). With the use of similar statistical methods of optimisation, the level of chitinase production by *Bacillus cereus* (Ghorbel-Bellaaj et al., 2012), *Bacillus pumilus* (Tasharrofi et al., 2011), *Sanguibacter antarticus* (Han et al., 2011) and *Serratia marsescens* (Sudhakar & Nagarajan, 2011) increased considerably. The optimization of nutrient levels for chitinase production by *Enterobacter* sp. NRG4 in SSF was carried out using RSM and a 2.4-fold increase in chitinase production was observed (Dahiya et al., 2005). A similar RSM optimisation approach resulted in a 2-fold increase of chitinase, and a 9.1-fold increase of NAG production by SSF for a *Vibrio* sp. (Suresh, 2012). In statistical optimisation experiments using fungal cultures, similar results were obtained. Sudhakar and Nagarajan (2011b) used a combination of PBD and CCD for obtaining maximum chitinase production by *Trichoderma harzianum*. Ghanem et al. (2010) achieved a 1.81-fold increase in chitinase production by *Aspergillus terreus* using PBD and BBD. Similar bioprocess optimisation using CCD was done and 1.81-, 3- and 7.71-fold increases were observed in the chitinase production by *Trichoderma* virens (Wasli et al., 2009), *Penicillium ochrochloron* (Patil & Jadhav, 2014) and *Basidiobolus ranarum* (Mishra et al., 2012) respectively. By using PBD and CCD, 2-fold and 4.12-fold enhancements in CDA production was attained when the media components were used at their optimum levels for a *Penicillium oxalicum* strain (Pareek et al., 2014b) and *Rhodococcus erythropolis* strain (Sun et al., 2014), respectively. Similarly, using PBD and Taguchi design, media composition was optimised and maximum CDA production for a *Rhizopus japonicus* strain (Zhang et al., 2014).

1.10. Purification and characterisation of microbial chitinases

The purification of microbial chitinases to homogeneity is carried out by different methods such as fractional ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, chitin-affinity chromatography, isoelectric focusing (IEF), etc. Many chitinolytic microorganisms produce more than one kind of chitinase, i.e., different isoforms having varying molecular weights (Li, 2006; Singh et al., 2009). Several purified microbial chitinases have been characterized for their molecular weight, isoelectric point (pI), optimal pH, optimal temperature, thermostability, pH stability, effect of different metal ions and inhibitors, and antifungal activity.

Molecular masses of bacterial and fungal chitinases are generally found within the range of 30 to 80 kDa (Table 1.8) with few of them having higher or lower masses. The bacterial chitinases show a broad range of pI ranging from 3.0–9.0 (Table 1.8) while the isoelectric pH of the fungal chitinases ranges within 3.0–8.0 (Li, 2006).

Most of the purified bacterial and fungal chitinases have an optimal pH around 5.0-7.0 range and show moderate to high activity within the pH 4.0–9.0 range (Table1.8). Similarly, the pH stability of the bacterial and fungal chitinases varies within the range of pH 4.0–9.0. However, a few microbial chitinases showed moderate to high activity in comparatively broader pH range of 2.0-14.0 (Nawani et al., 2002; Toharisman et al., 2005; Jung et al., 2006; Laribi-Habchi et al., 2015).

The optimum temperature for most bacterial and fungal chitinase activity is within 40-60°C and show moderate activity within the range of 30-70°C (Table 1.8). Also, the thermal stability is generally upto 50°C. However a few exceptions include chitinases from *Bacillus* sp. (Toharisman et al., 2005; Laribi-Habchi et al., 2015) and *Rhodothermus marinus* (Hobel et al., 2005), which exhibit high optimum temperature of 70-80°C. Activity of the chitinase

produced by a marine psychrotolerant *Vibrio* sp. at 5°C was 40% of the highest activity observed at 35°C (Bendt et al., 2001).

Metal ions play an important role in the biological catalysis by forming complexes with the enzymes and maintaining or disrupting the three dimensional structure and configuration (Andreini et al., 2008). Metal ions, which generally enhance microbial chitinase activity are Ca^{2+}(Guo et al., 2008; Lee et al., 2009; Annamalai et al., 2010; Natsir et al., 2010; Dai et al., 2011; Ma et al., 2012; Nguyen et al., 2012; Chen et al., 2013; Garcia-Fraga et al., 2014), Mg^{2+} (Lee et al., 2007; Novotna et al, 2008; Han et al., 2009; Lee et al., 2009; Natsir et al., 2010; Dai et al., 2011; Jiang et al., 2012; Kopparapu et al., 2012) and Mn^{2+}(Yong et al., 2005; Han et al., 2009; Lee et al., 2009; Zarei et al., 2011). The major inhibitor of chitinase activity is Hg^{2+} since it reacts with -SH groups found in cysteine residues in the protein chain and disrupts the tertiary structure. It strongly inhibits chitinases from different genera, including *Aeromonas* (Lien et al., 2007), *Bacillus* (Natsir et al., 2010), *Microbiospora* (Nawani et al., 2002), *Rhodothermus* (Hobel et al., 2005), *Serratia* (Kim et al., 2007), *Stenotrophomonas* (Jankiewicz et al., 2012), *Streptomyces* (Kim et al., 2003), *Aspergillus* (Jung et al., 2006), *Thermomyces* (Guoet al., 2008) and *Penicillium* (Lee et al., 2009). The other major inhibitory metal ions, which show strong inhibitory action on chitinases include Cu^{2+} (Jung et al., 2006; Zhu et al., 2007; Annamalai et al., 2010; Chang et al., 2010; Ma et al., 2012; Chen et al., 2013; Pradeep et al., 2014), Fe^{2+} (Kim et al., 2007; Guo et al., 2008; Wang et al., 2008; Lee et al., 2009; Pradeep et al., 2014), Mn^{2+} (Wang et al., 2008; Garcia-Fraga et al., 2014; Pradeep et al., 2014), Ag^{2+} (Lien et al., 2007; Zhu et al., 2007; Guo et al., 2008; Liu et al., 2010; Ma et al., 2012; Nagpure & Gupta, 2013), Zn^{2+} (Kim et al., 2007; Lee et al., 2009; Jankiewicz et al., 2012; Ma et al., 2012; Nguyen et al., 2012; Prakash et al., 2013) and Co^{2+} (Wang et al., 2008; Jiang et al., 2012; Nguyen et al., 2015). EDTA, which is a chelating agent capable of binding metal ions in solution has been reported to reduce the activity of chitinases produced by

Bacillus sp. (Dai et al., 2011), *Micrococcus* sp. (Annamalai et al., 2010), *Massilia timonae* (Adrangiet al., 2010) and *Thermomyces lanuginosus* (Guo et al., 2008). But, it had no significant effect on chitinases produced by a *Bacillus thuringensis* (Liu et al., 2010), *Microbiospora* (Nawani et al., 2002), *Streptomyces* sp. (Han et al., 2009; Nagpure & Gupta, 2013) and *Aspergillus fumigatus* (Jung et al., 2006). Interestingly, an activity enhancing effect was observed on the chitinase produced by a *Penicillium* sp. (Lee et al., 2009).

1.11. Purification and Characterisation of microbial CDAs

Similar to chitinases, the purification of microbial CDAs to homogeneity is carried out by different methods such as fractional ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography. Most purified microbial CDAs have been characterized for various parameters, including molecular weight, isoelectric point (pI), optimal pH, optimal temperature, thermostability, pH stability and effect of different metal ions and inhibitors. Molecular masses of bacterial and fungal CDAs generally range from 20 to 80 kDa (Table 1.9) with few of them having higher or lower masses. The isoelectric pH for a CDA from *Aspergillus nidulans* was determined to be 4.6 (Wang et al., 2010).

Most of the purified bacterial and fungal CDAs have an optimal pH around 4.0-9.0 range (Table 1.9). The optimum temperature for most bacterial and fungal chitinase activity is within 50-60°C (Table 1.9). There are no reports of CDAs, which show optimal activity at extreme pH and temperatures as in the case of chitinases.

As stated above, metal ions play an important role in biological catalysis by forming complexes with the enzymes and maintaining or disrupting the three dimensional structure and configuration (Andreini et al., 2008). Metal ions, which generally inhibit microbial CDA activity are Ca^{2+} (Araki and Ito, 1975; Kafetzopouos, 1993; Martinou, 2002), Mg^{2+}

(Kafetzopouos, 1993; Martinou, 2002), Mn^{2+} (Araki and Ito, 1975; Kafetzopouos, 1993; Tsigos and Bouritis, 1995; Tokayasu et al., 1996; Kim et al., 2008), Cu^{2+} (Kafetzopouos, 1993; Tsigos and Bouritis, 1995; Tokayasuet al., 1996; Martinou, 2002; Wang et al., 2010; Pareek et al., 2012), Fe^{3+} (Gao et al., 1995), Zn^{2+} (Kafetzopouos, 1993; Tsigos and Bouritis, 1995; Martinou, 2002; Wang et al., 2010; Pareek et al., 2012), Co^{2+} (Araki and Ito, 1975; Kafetzopouos, 1993; Tokayasu et al., 1996; Wang et al., 2010), Ag^{2+} (Ohishi et al., 1997; Kim et al., 2008) and Hg^{2+}(Ohishi et al., 1997; Kim et al., 2008). EDTA reduces the activity of CDA (Araki and Ito, 1975; Martinou, 2002; Wang et al., 2010). Co^{2+} increased the activity of CDAs from *Colletotrichum lindemuthianum* (Tsigos and Bouritis, 1995) and *Saccharomyces cerevisiae* (Tokuyasu et al., 1996). Cu^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}, Mg^{2+} and Ca^{2+} activated the CDA produced by a *Penicillium oxalicum* strain (Pareek et al., 2012).

Acetate inhibited most microbial CDAs at various concentrations (Araki and Ito, 1975; Kafetzopouos, 1993; Tsigos and Bouritis, 1995; Tokayasu et al., 1996; Kim et al., 2008; Wang et al., 2010; Pareek et al., 2012).

Table 1.8. Characterisation of microbial chitinases.

Organism	*pH*		Temperature (°C)		Molecular Mass (kDa)	Sp. Activity (U/mg)	pI	Reference
	Opti-mum	Range	Opti-mum	Range				
Aeromonas sp.	5.0	4.0-7.0	50	20-60	36	27.34	N.S.	Lien et al., 2007
Alcaligenes faecalis	8.0	6.0-11.0	37	35-65	36	81.52	N.S.	Annamalai et al., 2011
Anaeromyces mucronatus	6.5	2.5 to 8	40	20 to 50	N.S.	50.2 pkat/mg	N.S.	Novotná et al., 2008
Aspergillus fumigatus	6	3 to 6	50-60	35 to 70	104	9.19	N.S.	Jung et al., 2006
Aspergillus niger	6.0-6.5	4.5-7.0	40	30-50	43	22.5	N.S.	Brzezinska & Jankiewicz, 2012
Bacillus sp.	7.0	6.5-7.5	60	55-65	79, 71, 48, 43 and 22	1.46	N.S.	Natsir et al., 2010
Bacillus sp.	6.5	4.5-9.0	60	45-75	80.8	62.4	N.S.	Dai et al., 2011
Bacillus cereus	6.0	4.5-8.0	50	40-60	65	78.9	N.S.	Wang et al., 2012
	6.0	3.5-8.0	50-60	40-60	63	45.5	N.S.	
Bacillus cereus	6.0	4.5-7.5	60	40-60	91	142.1	N.S.	Chen et al., 2012
Bacillus cereus	6.5	4.5-9.0	65	50-75	30	74.35	N.S.	Hammami et al., 2013
Bacillus licheniformis	6.0 & 8.0	4.0-10.0	55	40-70	72	22.5	4.6	Kudan & Pichyangkura, 2009
Bacillus licheniformis	6.0	4.0-8.0	60	50-70	66	0.294	N.S.	Songsiriritthigul et al., 2010
Bacillus pumilus	8.0	N.S.	70	50-80	64	2.51	N.S.	Bhattacharya et al., 2016
Bacillus subtilis	9.0	5.0-9.0	50	30-50	14	879	N.S.	Shivakumar et al.,2014
Bacillus thuringiensis	6.5	5.0-8.0	55	30-60	74.5	N.S.	5.75	Barboza-Corona 2008
Burkholderia cepacia	4.5	3.5-7.0	50	30-65	34	440	5.9	Ogawa, 2002
Chitinibacter sp.	6.8	6.0-8.5	40	N.S.	130	N.S.	N.S.	Gao et al., 2015
Citrobacter freundii	8.0	5.5-9.5	40	25-60	64	140.55	N.S.	Meruvu & Donthireddy, 2014
Glaciozyma antarctica	4.0	3.0-4.5	15	5-30	39	N.S.	9.65	Ramli et al., 2011
Gliocladium catenulatum	5.0-6.0	4 to 10	60	20 to 70	51	12.17	N.S.	Ma et al., 2012
Halobacterium salinarum	7.3	6.5-8.0	40	30-45	66.48	513.6	N.S.	Garcia-Fraga et al., 2014
Massilia timonae	5.0	4.0-6.0	55	40-60	56	557.1	8.5	Adrangi et al., 2010
	5.0	4.0-7.0	60	50-60	64	1236.4	8.5	
Microbiospora sp.	3.0	2.0-10.0	60	40-70	35	4.6	N.S.	Nawani et al., 2002
Micrococcus sp.	8.0	6.0-11.0	45	35-70	33	93.02	N.S.	Annamalai et al., 2010
Oerskovia xanthineolytica	7.5	3.0-9.0	50	30-70	23	50.0	N.S.	Waghmare and Ghosh, 2010
	8.0	3.0-9.0	55	30-70	66	90.0	N.S.	
Orpinomyces sp.	6.5	2.5 to 8	50	40 to 60	N.S.	95.6 pkat/mg	N.S.	Novotná et al., 2008
Paenibacillus sp.	5.0	4.0-8.0	50	40-60	56.56	492.4	N.S.	Shibasaki et al., 2014

Organism	*pH*		Temperature (°C)		Molecular Mass (kDa)	Sp. Activity (U/mg)	pI	Reference
	Opti-mum	Range	Opti-mum	Range				
Paenibacillus pasadensis	10.0	9.0-11.0	37	N.S.	35	564.22	N.S.	Loni, et al., 2014
Paenibacillus thermoaerophilus	4.0	4.0-7.0	60	40-70	48	1.32	N.S.	Ueda & Kurosawa, 2014
Paecilomyces thermophila	4.5	4 to 6	50	35 to 55	43.7	N.S.	N.S.	Kopparapu et al., 2012
Penicillium sp.	5	3 to 8	40	30 to 60	47	12.3	N.S.	Lee et al., 2009
Penicillium aculeatum	5.5	4.5 to 6	50	40 to 65	82.6, 33.9 and 29.1	8	N.S.	Binod et al., 2005
Penicillium ochrochloron	7.0	6.0-8.0	40	10-50	64	2360	N.S.	Patil et al., 2013
Pseudomonas sp.	6.0	4.0-7.5	50	30-65	68	22.2	N.S.	Wang et al., 2008
Pseudoalteromonas sp.	8.0	5.0-12.0	20	4-30	113.5	42.17	N.S.	Wang et al., 2014
Rhodothermus marinus	4.5-5	4.0-6.5	70	60-80	39	7.6	5.13	Hobel et al., 2005
Rhizopus oryzae	5.5-6.0	5 to 8.5	60	50 to 70	50	65.2	N.S.	Chen et al., 2013
Sanguibacter	4.6	3.0-9.0	37	25-70	58	4.92	4.2	Lee et al., 2007
Serratia sp.	8.0	5.0-12.0	40	20-70	57	1.44	6.3	Kim et al., 2007
Serratia marcescens	6.0	5.0-8.0	55	30-70	62	6.42	N.S.	Babashpour et al., 2012
Streptomyces sp.	8.0	6.0-12.0	50	30-60	34	2.95	N.S.	Han et al., 2009
Streptomyces sp.	10	6.0-11.0	70	30-90	44	N.S.	3.6	Prakash et al., 2013
Streptomyces sp.	12.5	8.0-14.0	60	50-80	41	1811.2	N.S.	Pradeep et al., 2014
Streptomyces griseus	5.0-7.0	4.0-9.0	45-50	20-65	31.8	N.S.	N.S.	Hoster et al., 2005
Streptomyces rimosus	8.0	N.S.	40	N.S.	50	25	N.S.	Brzezinska et al., 2013a
Streptomyces roseolus	6.0	3.0-10.0	60	30-70	40	30	N.S.	Jiang et al., 2012
Streptomyces sporovirgulis	8.0	N.S.	40	N.S.	27	24.8	N.S.	Brzezinska et al., 2013b
Streptomyces venezuelae	7.5	4.5-9.0	35	20-50	66	26.67	N.S.	Mukherjee & Sen, 2006
Streptomyces violaceusniger	5.0	3.0-10.0	50	30-70	56.5	45.86	N.S.	Nagpure & Gupta, 2013
Streptomyces violascens	6.0	4.0-8.0	37	20-50	65	1.43	N.S.	Gangwar et al., 2016
Thermoascus aurantiacus	8.0	6 to 10	50	40 to 60	48	26.19	N.S.	Li et al., 2010
Thermomyces lanuginosus	4.5	3 to 9	55	50 to 60	48	35.51	N.S.	Guo et al., 2008
Trichoderma harzianum	5.0	2.0-8.0	20	10-60	42	N.S.	N.S.	Sharma et al., 2014
Trichoderma reesei	5.0	6 to 8	55	30 to 55	46	71.5	N.S.	Ike et al., 2006
Trichothecium roseum	5.0	4 to 8	40	20 to 40	39	64.1	N.S.	Li et al., 2004
Vibrio sp.	8.0	5.5-8.5	35	10-50	80	0.11	4.2	Bendt et al., 2001

(N.S.- Not specified)

Table 1.9. Characterisation of microbial CDAs.

Organism	pH		Temperature (°C)		Molecular Mass (kDa)	Sp. Activity (U/mg)	Reference
	Optimum	Range	Optimum	Range			
Absidia coerulea	5.0	N.S.	50	N.S.	75	11.37	Gao et al., 1995
Absida coerulea	5.8	N.S.	50	N.S.	N.S.	0.33	Win and Stevens, 2001
Absida corymbifera	6.5	4.5-9.0	55	30-80	N.S.	N.S.	Zhao et al., 2010
Aspergillus nidulans	7.0	N.S.	50	N.S.	27	25	Alfonso et al.,1995
Aspergillus nidulans	8.0	6.0-9.0	50	50-70	26.9	4.17	Wang et al., 2010
Colletotrichum lindemuthianum	8.5	N.S.	50	N.S.	150	0.219	Tsigos and Bouriotis, 1995
Colletotrichum lindemuthianum	12	7-12	60	35-65	32	18.4	Tokuyasu et al.,1996
Colletotrichum lindemuthianum	8.0	N.S.	60	N.S.	25	72	Srestha et al., 2004
Flammulina velutipes	7.0	N.S.	60	N.S.	31	N.S.	Yamada et al., 2008
Metarhizium anisopliae	8.5	N.S.	N.S.	N.S.	70, 37 & 26	N.S.	Nahar et al., 2004
Mortierella sp.	5.5	5.0-8.0	60	35-65	50 & 59	3.75	Kim et al., 2008
Mucor rouxii	4.5	N.S.	50	N.S.	80	2.96	Kafetzopou-los et al., 1993
Mucor rouxii	5.5	4.5-7.0	30	N.S.	N.S.	17.8	Araki & Ito, 1975
Mucor rouxii	4.5	N.S.	50	N.S.	80	N.S.	Hunt et al., 2008
Penicillium oxalicum	9.0	6.0-9.0	50	50-60	53	55.38	Pareek et al., 2012
Rhizopus circinans	5.5-6.0	N.S.	37	N.S.	75	965	Gauthier et al.,2008
Rhizopus nigricans	N.S.	N.S.	N.S.	N.S.	100	N.S.	Jeraj et al., 2006
Saccharomyces cerevisiae	8.0	N.S.	50	N.S.	43	771	Martinou et al., 2002
Scopulariopsis brevicaulis	7.5	N.S.	55	N.S.	55	2.58	Cai et al., 2013
Uromyces viciae-fabae	5.5-6.0	N.S.	N.S.	N.S.	48.1, 30.7, 25.2, 15.2, 12.7	N.S.	Deising and Siegrist, 1995
Vibrio alginolyticus	8.5-9.0 (CDA_1); 8.0-8.5 (CDA_2)	7.0-11.0 (CDA_1, CDA_2)	45 (CDA_1), 40 (CDA_2)	30-50 (CDA_1, CDA_2)	48 (CDA_1), 46 (CDA_2)	32.2 (CDA_1), 35.0 (CDA_2)	Ohishi et al., 1997

(N.S.- Not specified)

Objectives of the study

Bioproduction of chitooligosaccharides with enzymes and microorganisms has been studied earlier. However, the yield of bioproduction is still lower and the cost is higher than traditional chemical methods. Crude rather than pure enzyme preparations of chitinase and CDA were preferred for this purpose for the practical production of the chitooligosaccharides and their deacetylation. But for understanding the physicochemical properties of the enzyme, for their optimised and efficient use, their characterisation is necessary.

With the above in background, the objectives of this study were as below.

1. Isolation and screening of various chitinase and CDA enzyme producing microbial strains from environmental samples.
2. Optimization of different process parameters to improve the enzyme production from the selected strains.
3. Purification and characterization of both the two enzymes.
4. Application studies of the chitinase as biocontrol agent and as an effective chitin degrader.
5. Determination of degree of deacetylation of the CDA.

Organization of the thesis

The thesis comprises seven chapters. Chapter 1 is introductory. In chapter 2, the isolation and screening of microbial strains for the production of chitinase enzyme is discussed. The optimization of theprocess and nutritional parameters by statistical methods for the enhanced production of the chitinase is reported. The chapter also deals with bioreactor studies for the production of enzyme. Chapter 3 deals with the purification and characterization of the chitinase enzyme. In chapter 4, the isolation and screening of microbial strains for the production of CDA enzyme is discussed. The optimization of the process and nutritional parameters by statistical methods for the enhanced production of the CDA by both SmF and SSF methods are presented. Chapter 5 deals with the purification and characterization of the CDA enzyme. Chapter 6 incorporates the description of the study on the application of the chitinase and CDA enzymes. Finally, in chapter 7, the results are summarized and conclusions are drawn based on the present study.

Chapter 2

Isolation of Microbial Culture and Optimisation of Bioprocess Conditions for Chitinase Production

2.1. Introduction

A host of challenges are associated with microbial production of enzymes at the industrial scale. Significant technological sophistication is required to maximise the enzyme production at minimum possible cost. The enzyme production process can be divided into following phases.

1. Selection of the suitable microbial strain.
2. Strain improvement.
3. Selection of production methodology.
4. Optimization of the culture medium and production conditions.
5. Down-stream processes for enzyme isolation and purification.
6. Formulation/stabilization of enzyme.

From application point of view, chitinase is a low value- high volume enzyme; hence, it is very important to have an organism that can produce large amounts of enzyme using a cost- effective bioprocess. Microbial sources of chitinases are preferred over the othersbecause their production levels are high and cost could be minimized; furthermore, the enzyme is produced in a more predictable and controllable manner. Usually the chitinase producing microorganisms are isolated from the soils, especially those contaminated by chitinous wastes and those from marine environments. The screening and isolation is carried out on a chitin containing medium, which acts as the selection pressure. Mostly microbial strains of the genus *Bacillus, Streptomyces, Aeromonas, Aspergillus, Penicillium* and *Trichoderma* have been reported as chitinase producers, which have been isolated from soil or marine environments. Generally, chitinase produced from microorganisms is inducible in nature and the presence of chitin in the production medium enhances the chitinase yield to a great extent. Colloidal chitin has been considered as the best inducer of chitinase enzyme in

comparision to other sources of chitin (Narayana and Vijayalakshmi, 2009; Zarei et al., 2010; Tasharrofi et al., 2011; Hao et al., 2012; Kuddus and Ahmad, 2013).

Cellulosimicrobium cellulans was grown in chitinase production medium in a 5 L fermentor with aeration of 3 vvm and the yield was 4.38 U/mL after 144 h (Fleuri et al., 2009). In an attempt to develop a continuous chitinase production process from *Paenibacillus* sp. by Kao et. al. (2007), a bioreactor coupled to a microfilter membrane-based cell recycling column was applied and the chitinase activity could be stably maintained at a high level and harvested at a constant rate. In the current study production of chitinase using a *Streptomyces* isolate has been carried out using SmF method. The optimization of the nutritional media requirements as well as other physical factors including aeration, pH, and incubation temperature of the culture have been done using one-factor-at-a-time approach as well as statistical approach. Lab-scale fermentor studies for chitinase production were also carried out in a 2.5L bench-top-lab- scale fermentor using the optimised conditions.

2.2. Materials and Methods

2.2.1. Preparation of colloidal chitin

The colloidal chitin was prepared by the method of Hsu and Lockwood (1975) with minor modifications. Forty gram of flaked chitin was dissolved in 400 ml of concentrated HCl by stirring for 1h. The chitin was precipitated as a colloidal suspension by adding it slowly to 4-L of water at 4°C. The suspension was repeatedly washed by centrifugation until the pH of the suspension was about 5.0. The colloidal chitin thus prepared was stored at 4°C for subsequent use.

2.2.2. Chitinase assay

Extracellular chitinase activity was determined using the protocol suggested by Jayamurthy et al. (2014) with some minor modifications; 0.5 mL of enzyme sample was incubated with 1.0

mL 0.1 M citrate-phosphate buffer, pH 5.5 and 0.5 mL of 1% (w/v) colloidal chitin prepared in the same buffer. The mixture was kept in a water bath at 50°C for 1 h along with appropriate substrate and enzyme blanks. The reaction was arrested by addition of 3.0 mL 3, 5 di-nitrosalicylic acid (DNS) reagent (Miller, 1959), followed by heating for 10 min in a boiling water bath. DNS is reduced to give a coloured orange-red product (Figure 2.1) which is directly proportional to the amount of reducing sugar.

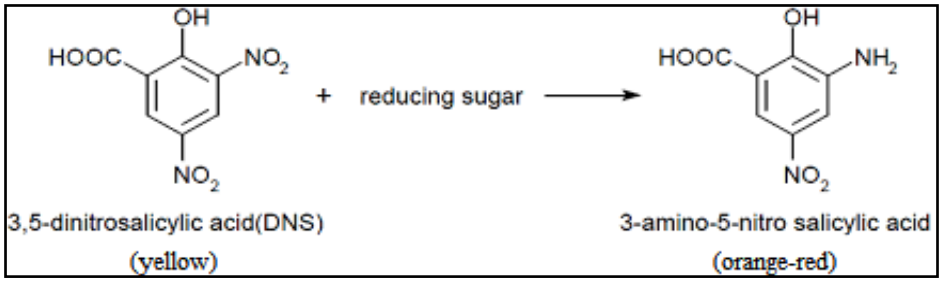

Fig. 2.1. Conversion of DNS to coloured product during DNS assay for detecting reducing sugar.

After cooling to room temperature (30°C), the coloured solution was centrifuged at 11,000 ×g for 5 min at room temperature and absorption of the supernatant was measured at 540 nm along against the control (blank) using a UV-Visible spectrophotometer (Shimadzu).

A standard graph using different concentrations of NAG was generated using this DNS assay and the equation obtained was used for calculating the unknown concentration of NAG after the chitinase assay. The equation obtained was: $y=4.453x-0.404$ ($R^2 = 0.997$); where y is the DNS assay reading at 540nm and x is the unknown NAG concentration.

One unit of the enzyme activity was defined as the amount of enzyme that catalysed the release of 1 μmol mL^{-1} min^{-1} of reducing sugar under the above mentioned assay conditions and was denoted as **U.**

Enzyme activity in U = $(X*10^6*4)/(221.21*60)$

Where X= concentration of NAG in mg mL^{-1}; 10^6=conversion of moles to micromoles; 4=dilution factor; 221.21= molecular weight of NAG; 60= time in minutes

2.2.3. Isolation of cultures

Twenty-six soil samples were collected from different regions along the coast near Kochi, Cherthala, Alappuzha, Kollam and Trivandrum in Kerala state, India (Figure 2.2). One gram of the each sample was suspended in 100 ml sterile saline individually and incubated on a rotary shaker at 30^{o}C, 200 rpm for 2h. The samples were then serially diluted and plated on chitinase selective agar medium containing (g L^{-1}): yeast extract, 0.05; NaCl, 2.0; K_2HPO_4, 0.8; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.5; and $CaCl_2.2H_2O$, 0.5, agar, 15 and colloidal chitin 1% (w/v), and incubated at 30°C for 96h and the colonies, which appeared n the plates were isolated on the basis of the hydrolysis zones formed around them. A total of 60 chitinolytic bacteria were isolated. One of the isolates, P6B2, which showed biggest clear zone diameter in comparison to the others, was chosen for further studies. This isolate was maintained on chitin agar slants with periodic sub-culturing for further use.

Fig. 2.2. Places of collection of environmental samples in Kerala for isolation of chitinase producing microorganisms (Map not to scale).

2.2.4. Microscopical studies

The microbial culture was observed under a clinical light microscope (Leica DM2000, Wetzlar, Germany) and scanning electron microscope (SEM). For SEM analysis, the 24 h old culture sample was fixed in a mixture of 2.5% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde in freshly prepared 0.1 M phosphate buffer for 2 h on clean glass coverslips. After being gently washed with distilled water, fixed samples were dehydrated through a graded ethanol wash (a series of 20-40-60-80 and 100% ethanol for 5 min each), and then the sample was mounted on a stub using double sided carbon tape and dried overnight in a desiccator. For sporulated culture, sample was directly picked from the agar media plate containing mature culture and mounted. The dried samples were sputter coated with gold using SC7620 sputtercoater device and analysed under SEM at an operating voltage of 30 kV (Zeiss Evo-18, Carl Zeiss, Germany).

2.2.5. Isolation of genomic DNA from the bacterial culture

The bacterial culture was inoculated in liquid medium of the above mentioned composition in 250 mL Erlenmeyer flasks and was incubated at 30°C, 200 rpm for 48h. Two millilitres of the culture broth was centrifuged at 11000 ×g at 4°C for 10 min, the supernatant was discarded and the bacterial pellet was frozen at -20°C overnight, then suspended in 1.0 mL TES buffer (6.7% (w/v) sucrose, 10 mM EDTA and 50 mM Tris-HCl, pH 8.0) and washed. It was further suspended in 300 µL STET buffer (8.0% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA and 50 mM Tris-HCl, pH 8.0) containing 50 mg mL^{-1} lysozyme and incubated at 37°C for 1h. Fifty microlitres of 20% (w/v) SDS was added and the solution was vortexed for 60 sec. It was further incubated at 37°C for 10 min, followed by incubation at 60°C for 10 min. One hundred microlitres of TE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0) was then added to the solution. The polysaccharides and proteins were precipitated by adding

equal volume of Phenol:Chloroform:Isoamyl alcohol mixture (25:24:1). The clear aqueous fraction was transferred with a wide bore tipped pipette into a fresh tube. The DNA was precipitated by adding 2/3 volume of ice-cold iso-propanol to the aqueous fraction. The solution was gently mixed by inversion, placed at -20°C for 1h and the DNA was recovered by centrifugation at 15,000 ×g and 4°C for 10 min. The DNA precipitate was washed twice with 70% ethanol and was allowed to air dry after which it was suspended in 50 µL TE buffer. RNAse was added to a final concentration of 10µg mL^{-1} and incubated at 37°C for 30 min. The above mentioned purification procedure was repeated. The purified DNA was stored at -20°C until further use.

2.2.6. Identification of isolate by 16S rDNA sequencing and phylogenetic analysis

The 16S rDNA region was amplified from the genomic DNA using universal forward and reverse primers: 16SrRNAf (5'- GGT-CTG-AGA-GGA-TGA-TCA-GT3') (Alm et al., 1996) and 16SrRNAr (5'- TTA-GCT-CCA-CCT-CGC-GGC- 3') (Widmer et al., 1998). The PCR conditions were maintained as an initial 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min; and a final extention step at 72°C for 10 min. This 16SrRNA amplicon was further analysed for its sequence data by dye terminator sequencing method (3500 Genetic Analyser, Hitachi, Tokyo, Japan). The identification of the phylogenetic neighbours was initially carried out by the BLAST (Altschul et al., 1997) and megaBLAST (Zhang et al., 2000) programs against the database of type strains with validly published prokaryotic names (Chun et al., 2007). The 50 sequences with the highest scores were then selected for the calculation of pairwise similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org) (Chun et al., 2007). The evolutionary history was inferred by using the Maximum Likelihood method based on

the Tamura-Nei model (Tamura and Nei, 1993). Evolutionary analyses were conducted using MEGA 5 software (Tamura et al., 2011).

2.2.7. Production of chitinase

The *Streptomyces* sp. was inoculated in a chitin yeast extract-salts (CYS) production medium with the following composition (g L^{-1}): colloidal chitin, 4.0; glucose, 2.0; yeast extract, 2.0; peptone, 2.0; NaCl, 2.0; K_2HPO_4, 0.8; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.5; and $CaCl_2.2H_2O$, 0.5. In all the following optimisation experiments, 1% (v/v) of 24 h grown inoculum culture of the *Streptomyces* sp. was inoculated in 50 mL CYS medium in 250 mL Erlenmeyer flasks. It was then incubated at 30°C and 200 rpm with appropriate modifications as set by the specific conditions of the experiment. For every 24 h intervals up to 96 h, culture sample was collected, centrifuged at 11,000 ×g for 10 min at 4^oC and the supernatant was used for the chitinase assay.

2.2.8. Optimisation of chitinase production-Single parameter studies

2.2.8.1. Effect of incubation time

The culture was inoculated in CYS medium and incubated at 30°C upto 96 h in a rotary shaker at 200 rpm. Samples were withdrawn after periodic intervals of 12 h, centrifuged as above and enzyme assay was performed using the culture supernatants.

2.2.8.2. Effect of initial pH and incubation temperature

The effect of initial pH value on chitinase production was investigated by varying the initial pH of the CYS culture medium (using 1N HCl or 1N NaOH) from 4.0 to 12.5 with a difference of 0.5 pH. The effect of temperature on enzyme production was determined by incubating the inoculated CYS medium at different temperatures (26, 28, 30, 32, 34, 37°C).

Flasks were inoculated, incubated and then contents were centrifuged as above. Chitinase assay was performed using the culture supernatants as per the above mentioned protocol.

2.2.8.3. Effect of colloidal chitin concentration

To determine the effect of colloidal chitin concentration on the induction and production of chitinase enzyme, it was added at increasing concentrations of 0, 1, 2, 3, 4, 5 and 6 g L^{-1} to the CYS medium. Flasks were inoculated, incubated and then contents were centrifuged as above. Chitinase assay was performed using the culture supernatants as per above mentioned protocol.

2.2.8.4. Effect of variation of nitrogen source

The effect of different sources of inorganic and organic nitrogen sources on chitinase production was determined by individually supplementing various nitrogen sources such as urea, ammonium nitrate, ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrate, diammonium hydrogen phosphate, dihydrogen ammonium phosphate, peptone, yeast extract, beef extract, casein enzyme hydrolysate and corn steep liquor (CSL) to the CYS medium. The nitrogen sources were supplemented such that the final nitrogen content in the medium would be at 2.0 g L^{-1}). A control flask, wherein no nitrogen source was added was also maintained. Flasks were inoculated, incubated and then contents were centrifuged as above. Chitinase assay was performed using the supernatants following the same protocol.

2.2.8.5. Effect of addition of metal ions

Influence of various metal ions on chitinase production was determined by supplementing CYS medium with different divalent metal cations such as Co^{2+}, Ca^{2+}, Mg^{2+} Mn^{2+}, and Zn^{2+} in their chloride form and Fe^{2+} and Cu^{2+} in its sulphate form. The metal ions were added at

final concentrations of 5 mM and 10 mM to the production medium. A control flask, wherein no metal ion was added was also maintained. Flasks were inoculated, incubated and then contents were centrifuged as above. Chitinase assay was performed using the supernatants following the same protocol.

2.2.8.6. Effect of addition of surfactants

For studying the effect of addition of surfactants on the production of chitinase, the CYS medium was supplemented with 0.5% (w/v) of SDS, CTAB and 0.5% (v/v) of Triton X-100, Tween 20, Tween 80, Tween 60 and Tween 80. A control flask, wherein no surfactant was added was also maintained. Flasks were inoculated, incubated and then contents were centrifuged as above. Chitinase assay was performed using the supernatants following the same protocols.

2.2.9. Optimisation of chitinase production-Statistical optimisation

2.2.9.1. Screening of parameters affecting chitinase production by Plackett-Burman statistical design

The identification of essential medium constituents to improve chitinase production by *Streptomyces* sp. was carried out using PBD. The important physical and nutritional parameters affecting the enzyme production were screened in a design with six variables at two levels: a higher level designated as +1, and a lower level designated as -1. The actual and coded values tested for each parameter are given in Table 2.1. The parameters tested were concentrations of glucose, colloidal chitin, yeast extract, peptone, ammonium nitrate (NH_4NO_3) and sodium chloride (NaCl) in the CYS medium. A total of 20 experimental runs were carried out as shown in Table 2.2.

Table 2.1. Values for the Plackett-Burman experimental design for chitinase production.

Code	Parameter Name	Low level (-1) g L^{-1}	High level (+1) g L^{-1}
X_1	**Glucose**	0.5	2.0
X_2	**Colloidal Chitin**	2.0	5.0
X_3	**Yeast Extract**	1.0	4.0
X_4	**Corn Steep Liquor**	1.0	4.0
X_5	**NH_4NO_3**	0.5	2.0
X_6	**NaCl**	1.0	4.0

Table 2.2. Plackett-Burman experimental design matrix chitinase production.

Std. Run Order	X_1	X_2	X_3	X_4	X_5	X_6	Chitinase (U)
1	-1	+1	-1	+1	+1	+1	38.41
2	-1	-1	-1	+1	-1	+1	41.68
3	-1	+1	+1	-1	+1	+1	31.54
4	-1	-1	+1	-1	+1	-1	26.83
5	+1	+1	-1	+1	+1	-1	35.02
6	-1	-1	+1	+1	-1	+1	43.80
7	-1	+1	+1	-1	-1	-1	48.32
8	+1	+1	-1	-1	+1	+1	38.00
9	-1	-1	-1	-1	-1	-1	29.02
10	+1	+1	-1	-1	-1	-1	50.96
11	+1	-1	+1	-1	+1	+1	30.84
12	+1	-1	+1	+1	+1	+1	35.30
13	-1	-1	-1	-1	+1	-1	24.58
14	-1	+1	-1	+1	-1	+1	43.21
15	-1	+1	+1	+1	+1	-1	50.61
16	+1	-1	-1	-1	-1	+1	39.21
17	+1	+1	+1	-1	-1	+1	56.74
18	+1	-1	+1	+1	-1	-1	42.45
19	+1	+1	+1	+1	-1	-1	53.53
20	+1	-1	-1	+1	+1	-1	31.77

The number of positive and negative signs per trial was $(k + 1)/2$ and $(k - 2)/2$, respectively. Each row represented a trial, and each column represented an independent (assigned) variable. The effect of each variable was determined by the following equation:

$$\mathbf{E(X_i)= [\Sigma(M_i+)-\Sigma(M_i-)]/n}$$

where, $E(X_i)$ was the concentration effect of the tested variable, M_i+ and M_i- represented chitinase production from the higher and lower levels respectively for each parameter, and n

was the total number of trials. All experiments were conducted in duplicates, and the mean value of chitinase activity was taken as the response. Chitinase assay was performed with the crude supernatant obtained, as per the protocol mentioned earlier. The analysis of variance (ANOVA) was performed on the data to determine the significance of the fitted model and to test the significance of the effect of individual parameters on the chitinase production. The most significant parameters affecting the chitinase production were identified.

2.2.9.2. Optimization of significant parameters by Taguchi Experimental Design

The significant parameters identified by the PBD were used in Taguchi Experimental design for further fine tuning the parameters for chitinase production. A matrix was designed with the appropriate orthagonal array (OA) for the selected factors and their levels and OA L_{16} (which indicated 16 experimental trials) was selected for the above controlled factors with four levels of factor variation as shown in Table 2.3.

Table 2.3. Taguchi design matrix for chitinase production- colloidal chitin and yeast extract.

Run No.	CC (g L^{-1})	YE (g L^{-1})	Chitinase (U)
1	8	4	64.43
2	8	6	68.55
3	8	8	85.22
4	8	10	88.28
5	10	4	59.16
6	10	6	66.73
7	10	8	77.57
8	10	10	67.03
9	12	4	60.45
10	12	6	71.65
11	12	8	97.25
12	12	10	91.16
13	14	4	66.94
14	14	6	75.64
15	14	8	104.98
16	14	10	100.29

Another Taguchi Design was performed using CSL as a nitrogen source, in place of yeast extract, being a comparatively low cost organic nitrogen source along with being a good

supplement of micronutrients and growth factors to the medium. A similar matrix was designed as above with the appropriate orthogonal array for the selected factors and their levels as shown in Table 2.4.

Table 2.4. Taguchi design matrix for chitinase production- colloidal chitin and corn steep liquor.

Run No.	CC (g L^{-1})	CSL(g L^{-1})	Chitinase (U)
1	6	6	56.50
2	6	8	63.96
3	6	10	77.41
4	6	12	45.69
5	8	6	65.62
6	8	8	66.67
7	8	10	78.9
8	8	12	75.24
9	10	6	83.51
10	10	8	92.18
11	10	10	101.65
12	10	12	96.35
13	12	6	70.68
14	12	8	98.59
15	12	10	75.28
16	12	12	66.69

In order to validate the experimental model using yeast extract and CSL, three verification experiments were performed using the statistically optimized medium.

2.2.10. Fermentor Studies

Lab-scale fermentor studies for chitinase production were carried out in a 2.5L bench-top-lab- scale fermentor (Minifors, Infors-HT, Switzerland). The working volume was 1L and the medium consisted of the optimised composition obtained from the statistical optimisation studies. The temperature was maintained at 32°C, the impeller was run at 200, 250, 300 and 350 rpm and the aeration was maintained between 0.5-1.0 vvm of air. Each fermentation batch was carried out for 96h and periodic sampling was carried out to check chitinase activity. Separate experiments were carried out using yeast extract and CSL as nitrogen sources.

2.3. Results and Discussion

2.3.1. Location, sampling and isolation of cultures

A total of 160 chitinolytic bacteria were isolated from 26 soil samples collected from different regions along the coast Kerala state, India (Table 2.5). The isolation was based on the clearance zones formed around the colonies on the chitin-agar medium (Fig. 2.3).

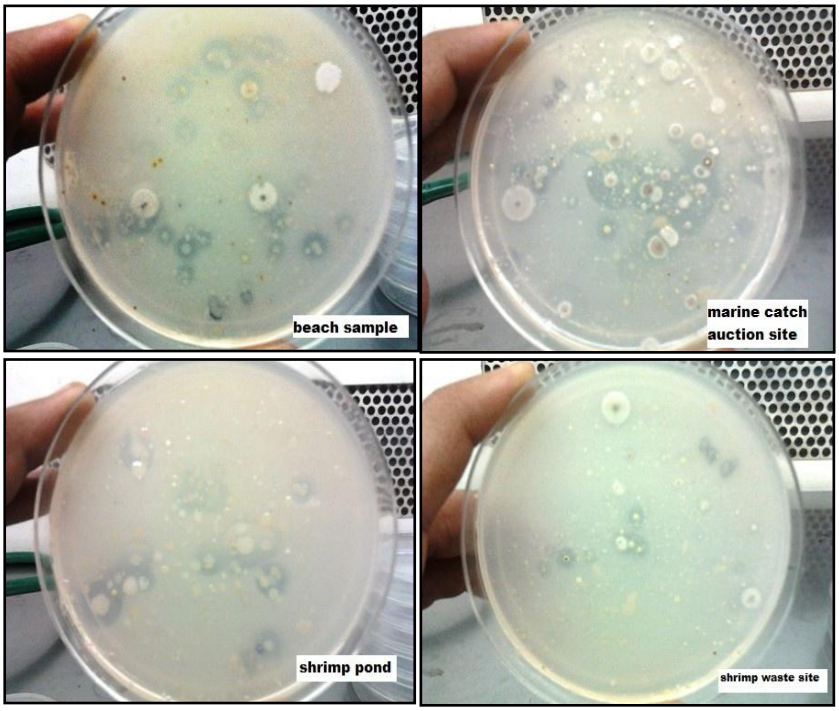

Fig. 2.3. Isolation of chitinase producing microorganisms on chitinase agar plates.

Table 2.5. Isolate code names of the 160 strains isolated for chitinase production.

P1B1	P2B10	P6B7	P5B7	P1D5	P2G6	P4G3	V5 10 2
P1B2	P2B11	P6B8	P5B8	P1D6	P2G7	P4G4	V5 10 3
P1B3	P2B12	P6B9	P5B9	P1D7	P2G8	P4G5	V5 10 4
P1B4	P3B1	P6B10	P5B10	P1D8	P2G9	P4G6	V5 10 5
P1B5	P3B2	P6B11	P1C1	P2D1	P2G10	P4G7	V5 10 6
P1B6	P3B3	P4B1	P1C2	P2D2	P2G11	P4G8	V5 10 7
P1B7	P3B4	P4B2	P1C3	P2D3	P3D1	P4G9	V5 10 8
P1B8	P3B5	P4B3	P1C4	P2D4	P3D2	P4G10	V5 10 9
P1B9	P3B6	P4B4	P1C5	P2D5	P3D3	P5G1	V5 10 10
P1B10	P3B7	P4B5	P1C6	P2D6	P3D4	P5G2	V5 100 1
P1B11	P3B8	P4B6	P1C7	P2D7	P3D5	P5G3	V5 100 2
P2B1	P3B9	P4B7	P1C8	P2D8	P3D6	P5G4	V5 100 3
P2B2	P3B10	P4B8	P1C9	P2D9	P3D7	P5G5	V5 100 4
P2B3	P3B11	P4B9	P1C10	P2D10	P3D8	P5G6	V5 100 5
P2B4	P6B1	P5B1	P1C11	P2D11	P3D9	P5G7	V5 100 6
P2B5	P6B2	P5B2	P1C12	P2G1	P3D10	P5G8	V5 100 7
P2B6	P6B3	P5B3	P1D1	P2G2	P3D11	P5G9	V5 100 8
P2B7	P6B4	P5B4	P1D2	P2G3	P3D12	P5G10	V5 100 9
P2B8	P6B5	P5B5	P1D3	P2G4	P4G1	P5G11	V5 100 10
P2B9	P6B6	P5B6	P1D4	P2G5	P4G2	V5 10 1	V5 100 11

The highest-producing strain was identified as a *Streptomyces* sp. named P6B2 based on its phenotypic characteristics; round irregular-edged colonies, slow-growing, initially white and turning greyish-brown on maturity and having a powdery surface (Figure 2.4).

2.3.2. Microscopical studies

Phenotypically the strain was Gram positive (Figure 2.4), filamentous (Fig. 2.4C and 2.5), spore forming (Figure 2.5) and aerobic in nature.

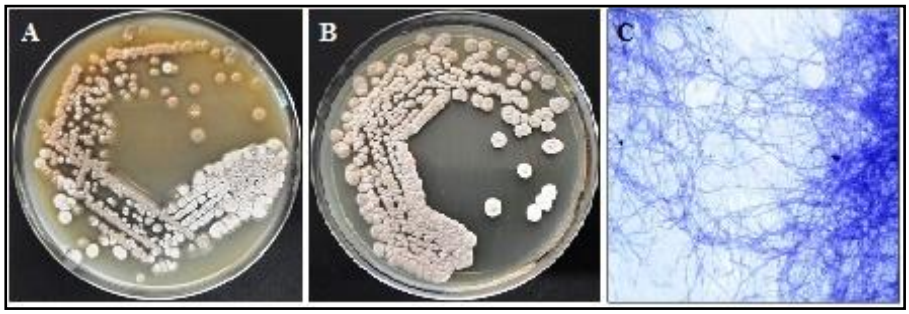

Fig.2.4. *Streptomyces* sp. colonies on (A) chitin agar (B) *Streptomyces* agar and (C) Grams stained microscopy mount (100X).

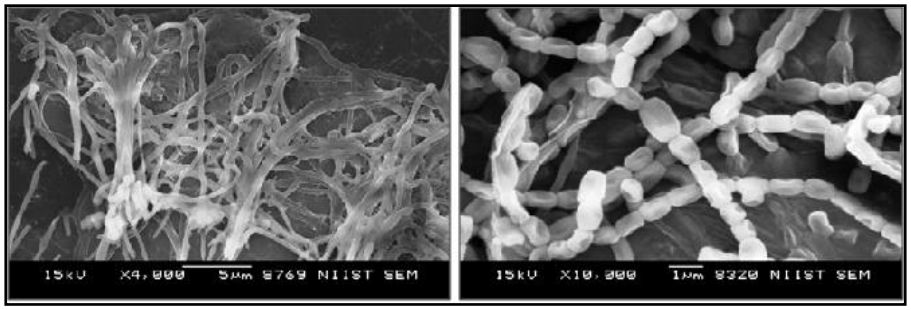

Fig. 2.5. SEM images of *Streptomyces* sp. filamentous cells (left) and spores (right).

2.3.3. Identification of isolate by phylogenetic analysis

The isolate P6B2 was identified as a *Streptomyces* sp. bearing highest homology to the type strain *Streptomyces enissocaesilis* strain NRRL B-16365 as confirmed by 16S rDNA analysis using NCBI-BLAST and the MEGA6 software and sequence alignment data obtained from EzTaxon server. Figure 2.6 shows the gel picture showing PCR amplified 16S rDNA product run on an agarose gel. Figure 2.7 displays the phylogenetic tree generated based on 16S rDNA sequences of *Streptomyces* strains, which were similar to the present isolate as

obtained from the BLAST results. The *Bacillus cerus* strain (ATCC 11778) was used as the out-group.

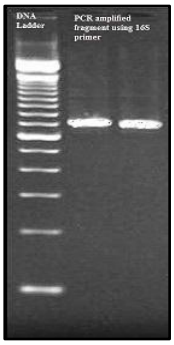

Fig. 2.6. Gel picture showing PCR amplified 16S rDNA product of ~ 700 bp.

Sequencing Result for 16S rDNA region

ACATGCAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTC
TGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGATCCTCGCAGGCATCTGCGAGGTTCGAAAGCTCCGGCGGTGC
AGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCG
ACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCC
TGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTA
CCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGG
CTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGG
CGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTA
CGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCG
AAGAACCTTACCAAAGGCTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCT
TGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTA
TGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGT
CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGG
A

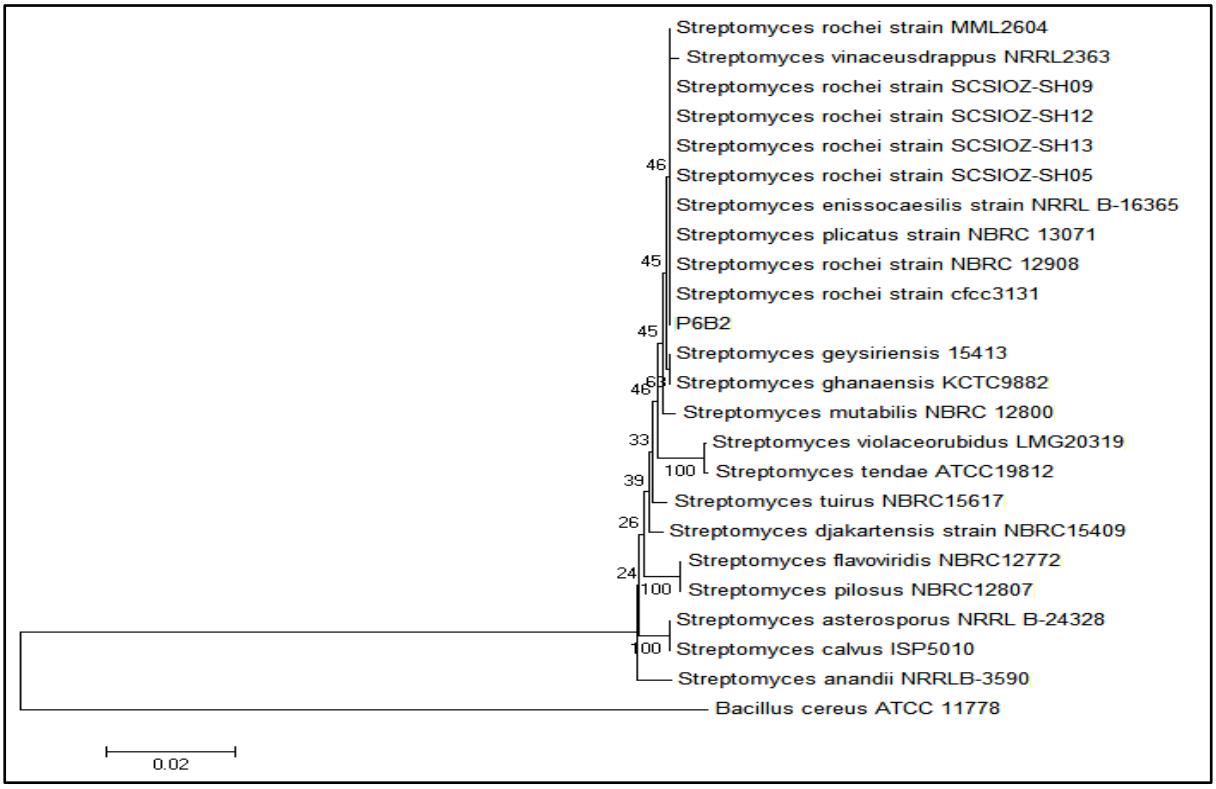

Fig. 2.7. Phylogenetic tree with species level identification of *Streptomyces* sp. isolate.

2.3.4. Optimisation of chitinase production-Single parameter studies

2.3.4.1. Effect of incubation time

The study of effect of incubation time on chitinase production by *Streptomyces* sp. P6B2 showed that the highest level of chitinase was produced within 60 h of incubation, after which the level of chitinase enzyme in the fermentation broth decreased gradually with time (Fig. 2.8). A significant increase in the chitinase production was observed after 24 h of incubation. Similar to present results, maximum chitinase production was observed in 60 h by a strain of *Streptomyces* sp. ANU 6277 by Narayana and Vijayalakshmi, (2009). The main reason for decreased production after 60 h could be the depletion of nutrients in the fermentation medium. It might also be due to the production of inhibitory products in the medium, resulting in the inactivation of secretary machinery of the enzymes. A few variations in this trend were reported for *Serratia marcescens* (Zarei et al., 2010) and two *Aeromonas* sp. (Al-Ahmadi et al., 2008; Kuddus and Ahmad, 2013) where the maximum production was observed in 48 h of incubation. In several other studies, the maximum yield of chitinase was found after 72 h of incubation. e.g., by *Streptomyces halstedii* (Joo, 2005) and *Serratia marcescens* (Chakrabortty et al., 2012), and after 96 h by *Bacillus laterosporus* (Shanmugaiah, 2008).

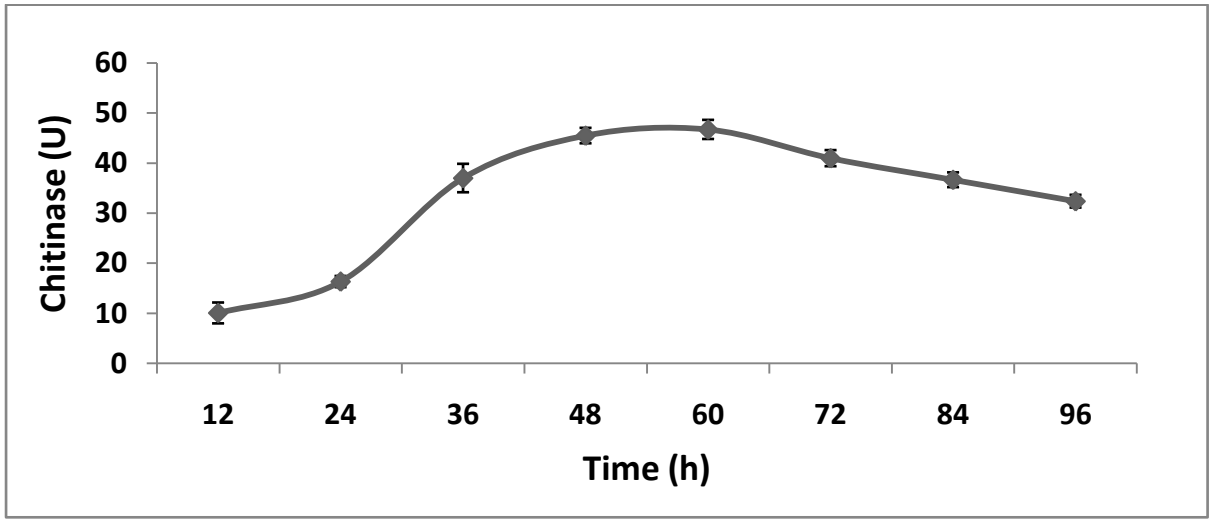

Fig. 2.8. Effect of incubation time on chitinase production.

2.3.4.2. Effect of initial pH of production medium

Among the tested pH from 4.0 to 12.5, an initial pH of 8.0 gave the best chitinase production for the *Streptomyces* sp. isolate P6B2. The level of enzyme production remained almost constant until an initial medium pH of 11.0 after which it decreased rapidly (Fig. 2.9). Similarly, there was decreased enzyme production in the medium below pH 8.0. The pH of the media plays an important role in the growth of the organism as well as on the production of chitinase. In similarity to the *Streptomyces* sp. P6B2, optimal chitinase production at pH 8.0 was reported for *Serratia marsescens* (Xia et al., 2011), two *Aeromonas* sp. (Al-Ahmadi et al., 2008; Kuddus and Ahmad, 2013) and *Bacillus laterosporus* (Shanmugaiah, 2008). A few cultures, including *Paenibacillus* sp. (Singh, 2010), *Streptomyces* sp. ANU 6277 (Narayana and Vijayalakshmi, 2009) and *Serratia marcescens* (Chakrabortty et al., 2012) showed an optimal pH of 7.0 for chitinase production, while a *Paenibacillus* sp. had a pH optima of 5.0 (Singh, 2010).

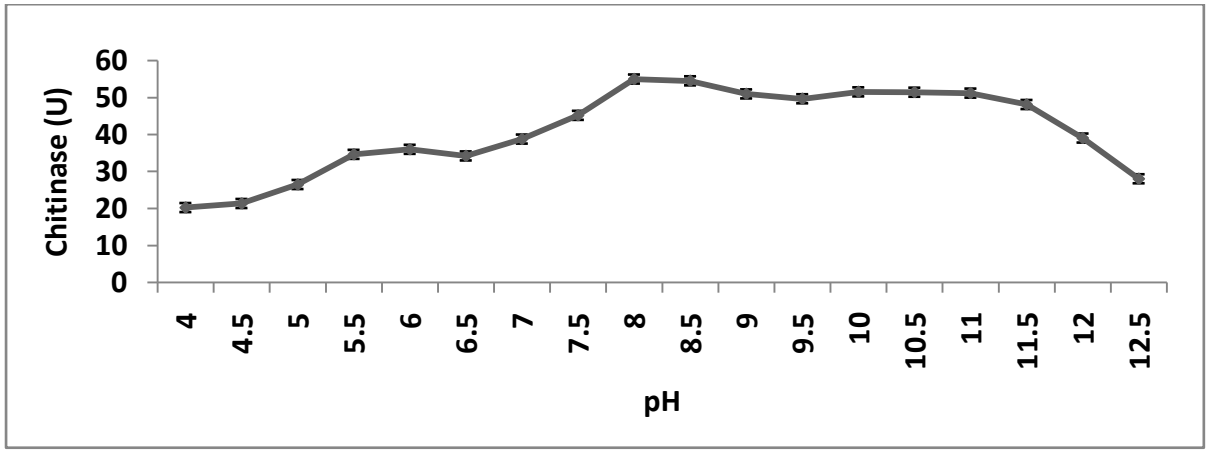

Fig. 2.9. Effect of initial pH of production medium on chitinase production.

2.3.4.3. Effect of incubation temperature

To evaluate the optimum growth temperature for chitinase production, the *Streptomyces* sp. isolate P6B2 was grown at 26–37°C. Chitinase production was maximum at 30°C (Fig. 2.10). The enzyme production progressively decreased at temperatures above and below 30°C. A profound effect of temperature is observed on various biological processes, including the

growth of microbes and microbial enzyme production. Thus, the change in incubation temperature is an important factor affecting the fermentation process. General observations suggest that optimal temperature for chitinase production by different microbes is between 30 and 35°C. An optimal temperature of 30°C was observed for chitinase production by *Serratia marcescens* (Xia et al., 2011; Chakrabortty et al., 2012), *Bacillus pumilis* (Tasharrofi et al., 2011) and *Aeromonas* sp. (Al-Ahmadi et al., 2008), which was in agreement with that of *Streptomyces* sp. P6B2. An optimal temperature of 35°C for chitinase production was observed for *Streptomyces* sp. ANU 6277 (Narayana and Vijayalakshmi, 2009) and *Bacillus laterosporus* (Shanmugaiah, 2008). Two *Aeromonas* isolates had been identified to grow at an optimum temperature at 37°C (Kuddus and Ahmad, 2013). Singh has reported that the optimum temperature for the enzyme production by *Paenibacillus* sp. as 50°C (Singh, 2010).

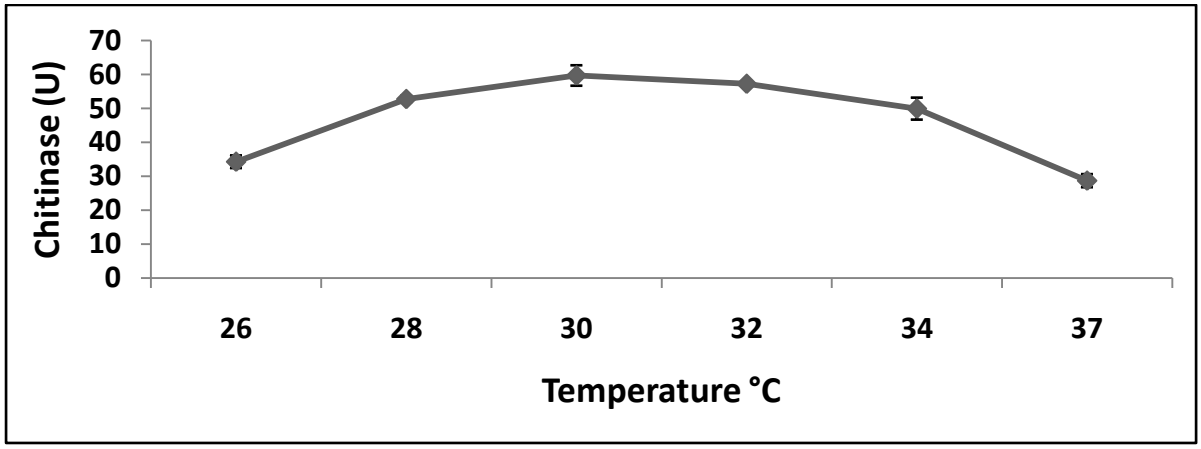

Fig. 2.10. Effect of incubation temperature on chitinase production.

2.3.4.4. Effect of colloidal chitin concentration

A minimal level of chitinase production (16.79 U) by *Streptomyces* sp. isolate P6B2 was observed even without the addition of colloidal chitin in the CYS medium. However, with the addition of increasing concentration of colloidal chitin, there was marked increase in the production of chitinase enzyme. At colloidal chitin concentrations of 5.0 and 6.0 g L^{-1}, the chitinase production level remained almost the same (50.17 ± 1.37 U), which was maximum.

Streptomyces sp. P6B2 showed the production of chitinase even without the addition of any colloidal chitin to the growth medium; hence the culture could be a constitutive producer. But with the addition of colloidal chitin to the medium, the production of chitinase enhanced. Probably after the consumption and depletion of glucose in the medium, chitin was the carbon source left for further utilisation by the culture. Optimal colloidal chitin concentrations reported for maximal production of chitinase were 2.0 g L^{-1} for *Paenibacillus* sp. (Meena et al., 2014), 3.0 g L^{-1} for *Bacillus laterosporus* (Shanmugaiah, 2008) and *Aeromonas* sp. (Kuddus and Ahmad, 2013), 7.5 g L^{-1} for *Aeromonas* sp. (Al-Ahmadi et al., 2008) and 10 g L^{-1} for *Serratia marcescens* (Chakrabortty et al., 2012).

2.3.4.5. Effect of variation of nitrogen source

Among the various nitrogen sources supplemented in the CYS medium individually, the organic nitrogen sources yeast extract and corn steep liquor were the most effective in inducing the production of chitinase by *Streptomyces* sp. isolate P6B2. It was observed that the production of chitinase doubled in comparison to the control as shown in the Figure 2.11. Among the inorganic nitrogen sources, ammonium nitrate gave the highest yield of chitinase enzyme for *Streptomyces* sp. isolate P6B2 with a 40% increased yield in comparision to the control. However dihydrogen ammonium phosphate suppressed the chitinase production to nearly 20% of the control value as seen in Figure 2.11. Yeast extract has been reported to show an enhancing effect on chitinase production in cases of *Streptomyces* sp. ANU 6277 (Narayana and Vijayalakshmi, 2009), *Serratia marcescens* (Chakrabortty et al., 2012), *Bacillus laterosporus* (Shanmugaiah, 2008) and *Paenibacillus sabina* (Patel et al., 2007). There are only a few reports wherein a positive effect of CSL on chitinase production has been reported, e.g., by a *Bacillus subtilis* (Narasimhan & Shivakumar, 2012). Otherwise CSL did not aid in chitinase production as reported by Hao et al. (2012) for *Chitiolyticbacter*

meiyuanensis. Peptone has been reported to improve chitinase production by *Aeromonas* sp. (Al-Ahmadi et al., 2008) and *Alcaligenes xylosoxydans* (Vaidya et al., 2001). In contrast to the present study, the inorganic nitrogen source, viz., urea enhanced chitinase production by *Paenibacillus* sp. (Singh, 2010) and *Chitiolyticbacter meiyuanensis* (Hao et al., 2012) while ammonium sulphate enhanced the production by *Aeromonas* sp. (Al-Ahmadi et al., 2008) and *Serratia marcescens* (Xia et al., 2011).

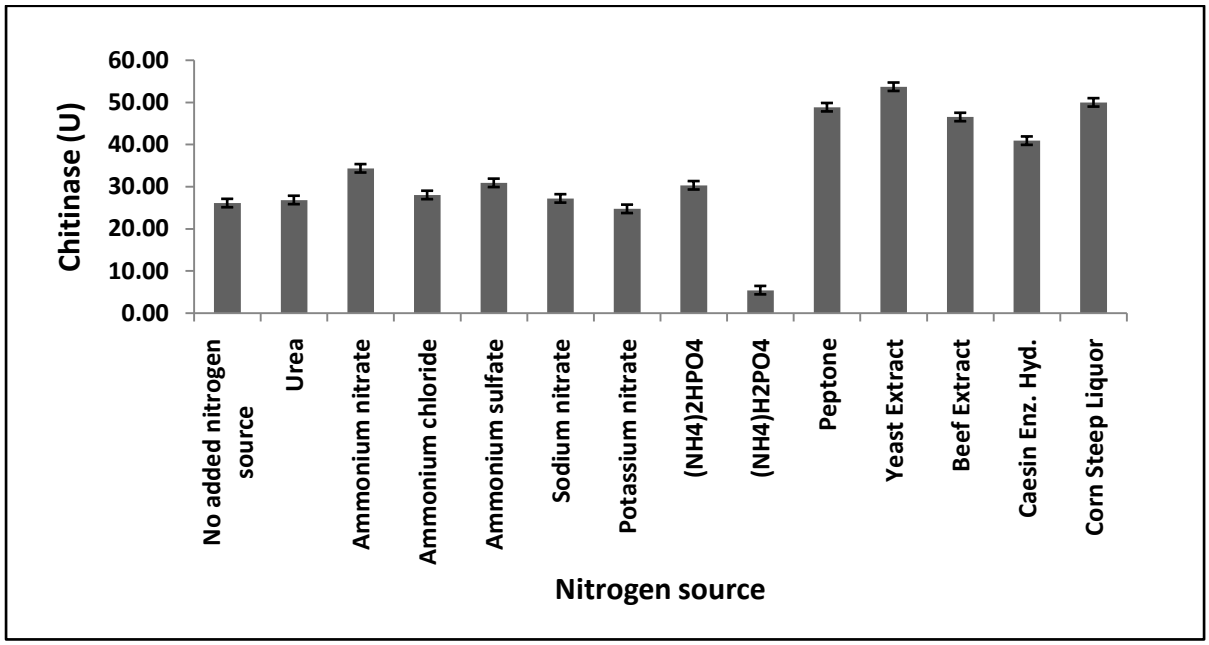

Fig. 2.11. Effect of nitrogen source variation on chitinase production.

2.3.4.6. Effect of addition of metal ions

The results showed that chitinase production was enhanced by the addition of $CaCl_2$ and $MgCl_2$ in the culture media of *Streptomyces* sp. isolate P6B2 as given in Figure 2.12. There was a significant reduction in the production when $CoCl_2$, $CuSO_4$ and $MnCl_2$ were added to the medium. Metal ions play an important role in biological catalysis by forming complexes with the enzymes and maintaining the three dimensional structure and configuration (Andreini et al., 2008). The effect of metal ions enhancing the chitinase production in *Streptomyces* sp. P6B2 was in similarity with that for $CaCl_2$ reported for *Paenibacillus sabina* (Patel et al., 2007) and *Pantoea dispersa* (Gohel et al., 2006) and $MgCl_2$ reported for

Aeromonas sp. (Al-Ahmadi et al., 2008) and *Pantoea dispersa* (Gohel et al., 2006). But as opposed to the results obtained in the current study, in another *Aeromonas* sp. isolate, $MnCl_2$ and $CuSO_4$ increased while $CaCl_2$ and $MgCl_2$ decreased the chitinase production (Kuddus and Ahmad, 2013). In the case of *Paenibacillus* sp., only $FeCl_3$ had a significant positive effect on chitinase production (Singh, 2010).

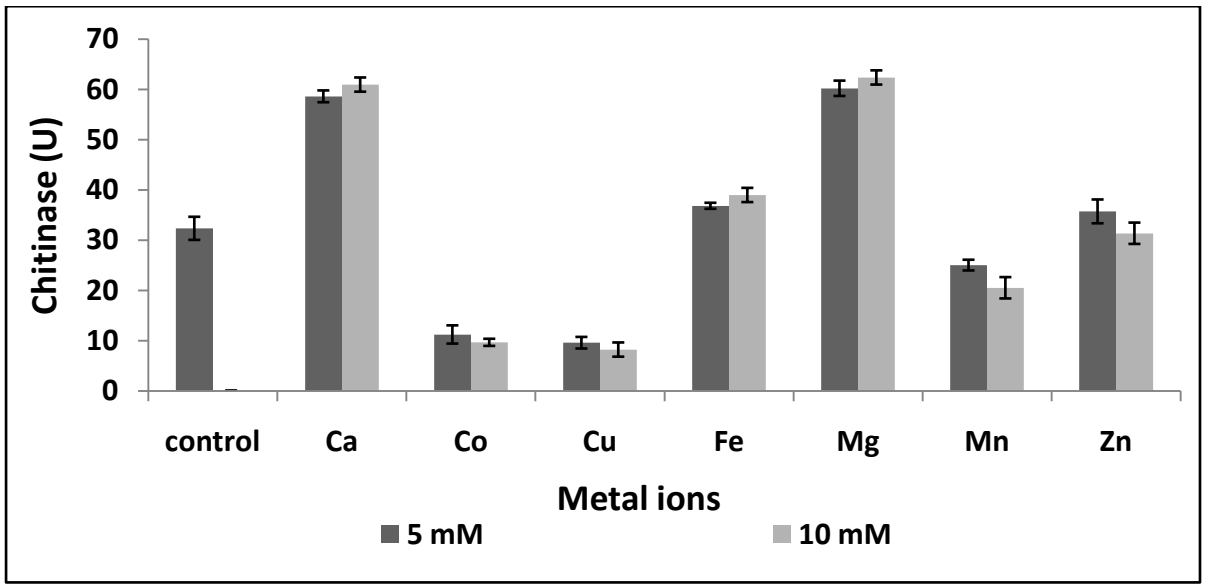

Fig. 2.12. Effect of metal ion addition on chitinase production.

2.3.4.7. Effect of addition of surfactants

Non-ionic surfactants such as Tween 20, Tween 40, Tween 60 and Tween 80 increased chitinase production by *Streptomyces* sp. isolate P6B2, while the ionic surfactants such as SDS and CTAB and the non-ionic surfactant Triton X-100 decreased the chitinase production significantly as shown in the Figure 2.13. An alteration in the porosity of the cell membrane can be caused by the surfactants at low concentrations without the total disruption of the cells, causing an increased release of intracellular metabolites including enzymes into the extracellular environment. Though only a few reports were available, results similar to that of *Streptomyces* sp. P6B2 were reported for *Alcaligenes xylosoxydans* (Vaidya et al., 2001), *Aeromonas* sp. (Al-Ahmadi et al., 2008) and *Paenibacillus* sp. (Singh, 2010).

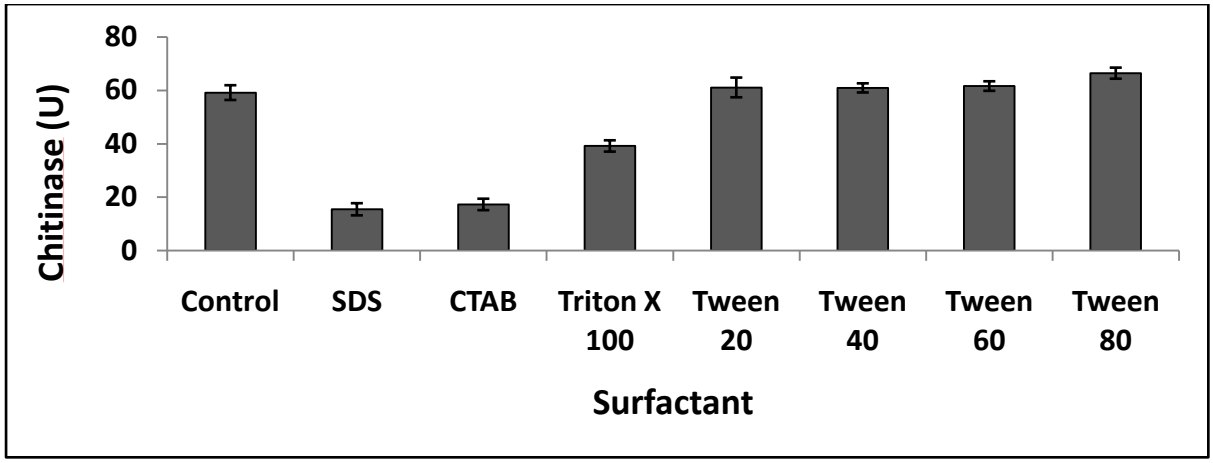

Fig. 2.13. Effect of surfactant addition on chitinase production.

2.3.5. Optimisation of chitinase production-Statistical optimisation

2.3.5.1. Screening of parameters affecting chitinase production by Plackett-Burman statistical design

The concentrations of the medium components were optimised using statistical methods. Plackett-Burman screening experimental design was employed to determine the influence of independent variables on the production of chitinase by *Streptomyces* sp. P6B2. The components were screened at the confidence level of 95% on the basis of their effects (either positive or negative). If the component showed at or above 95% confidence level and its effect was negative, it indicated that the component was effective for chitinase production but the amount required was lower than the indicated low concentration in Plackett-Burman experiment. Here, similar observations were seen for the result for NH_4NO_3. But even with the use of decreased concentration of NH_4NO_3, there was no significant increase in the chitinase production, hence excluded from further study (results not shown). Among the variables that were studied, colloidal chitin (X_2), yeast extract (X_3) and CSL (X_4) concentrations were considered highly significant (Fig. 2.14 and Fig. 2.15).

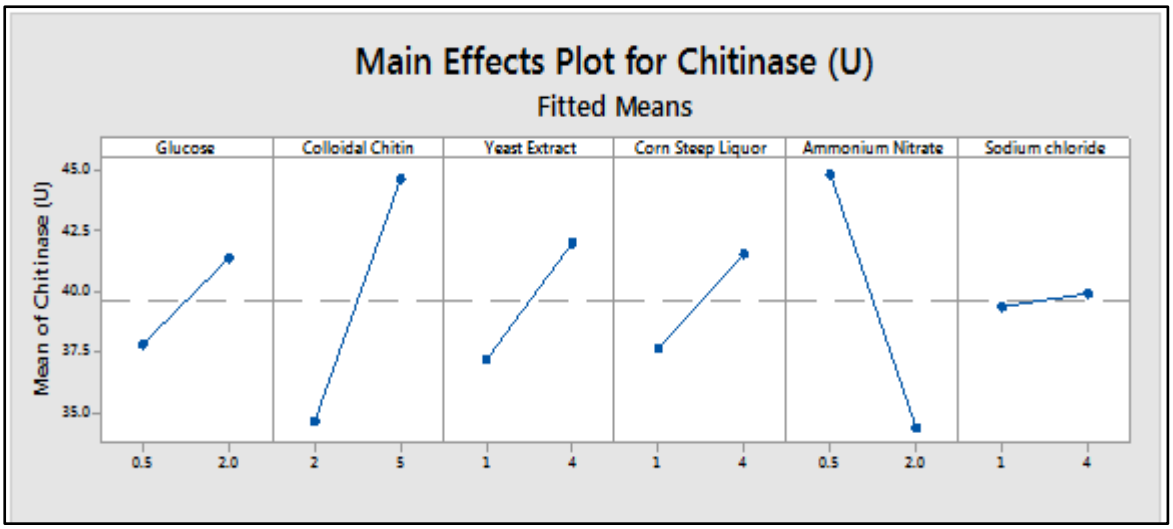

Fig. 2.14. Main effects plot showing effect of high and low level concentrations of factors influencing chitinase production obtained using Packett-Burman design.

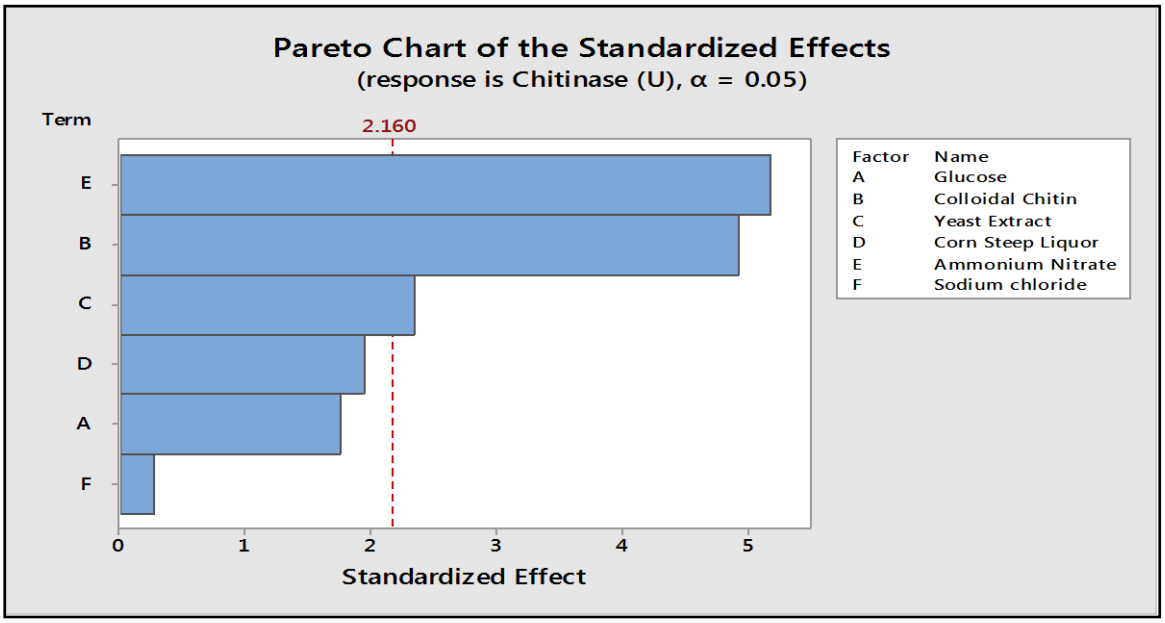

Fig. 2.15. Pareto plot showing significance of factors influencing chitinase production obtained using Packett-Burman design.

There are reports describing the use of the PBD in medium optimization for chitinase production with several microorganisms, including *Serratia marcescens* (Xia et al., 2011), *Chitiolyticbacter meiyuanensis* (Hao et al., 2012), *Paenibacillus sabina* (Patel et al., 2007), *Bacillus subtilis* (Narasimhan & Shivakumar, 2012) and *Pantoea dispersa* (Gohel et al., 2006). In the present study, colloidal chitin and yeast extract concentrations were highly

significant and CSL concentration was considered significant from the model (Figure 2.15). Glucose was beneficial in enhancing chitinase production by helping initial biomass development but was not highly significant. Hence, its concentration was kept constant at 2.0g L^{-1} in all further studies. The above results showed that the use of PBD for the screening of the medium components, which had significant influence on chitinase production, proved to be advantageous.

The three significant variables selected based on the results of PBD (colloidal chitin, yeast extract and CSL) were optimized using Taguchi design individually for yeast extract and CSL. Increasing concentrations of colloidal chitin, yeast extract and CSL were used on the basis of the Plackett-Burman design. As given in Table 2.3, the highest chitinase production (105.98 ± 1.57 U) was obtained using 14 g L^{-1} colloidal chitin and 8 g L^{-1} yeast extract as nitrogen source. The main effects plot and contour plot of effect of colloidal chitin and yeast extract was shown in Figure 2.16 and 2.17 respectively.

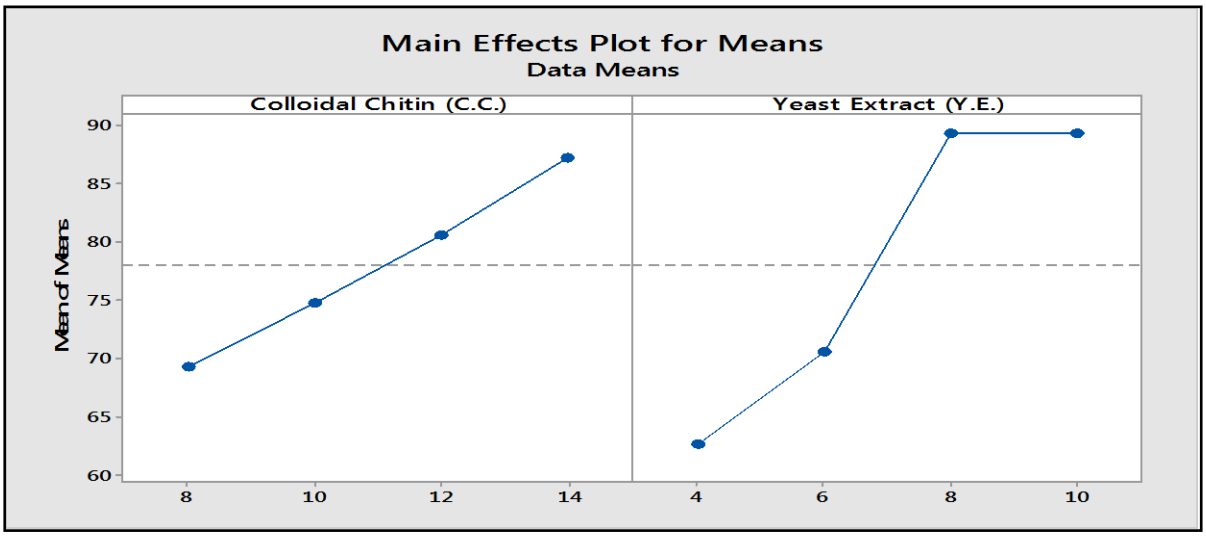

Fig. 2.16. Main effects plot showing optimal concentrations of colloidal chitin and yeast extract for chitinase production obtained using Taguchi method.

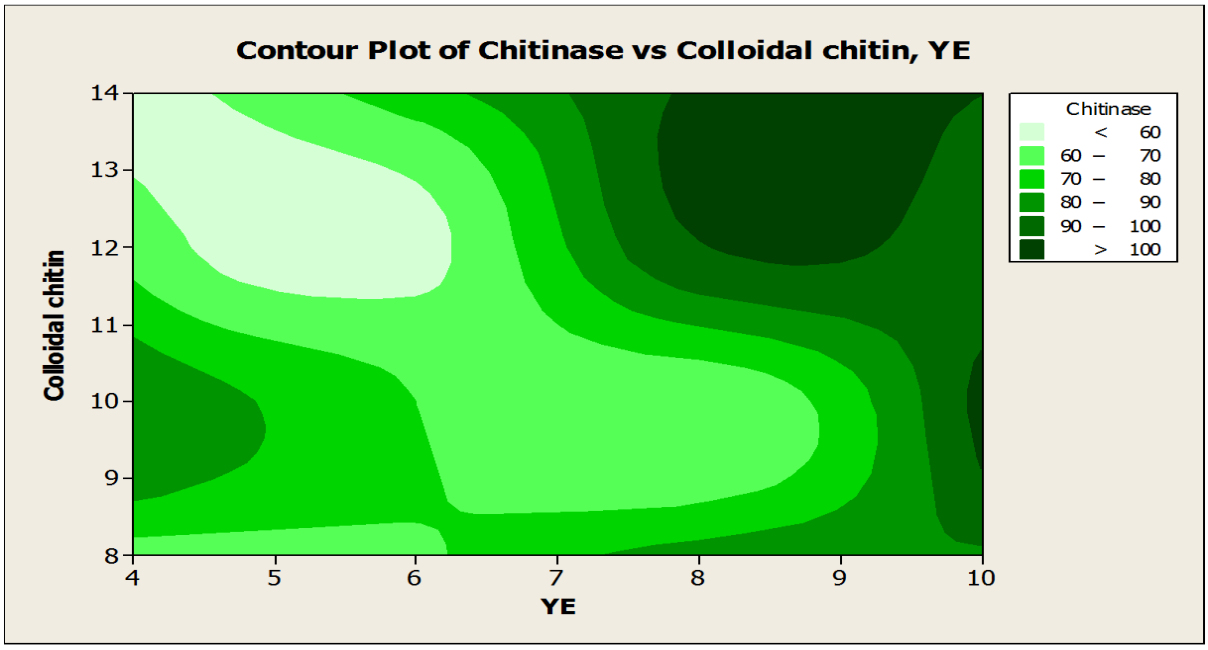

Fig. 2.17. Contour plot showing optimal concentrations of colloidal chitin and yeast extract for chitinase production obtained using Taguchi method.

The experiment where yeast extract was replaced with CSL as nitrogen source showed a highest chitinase production of 102.95± 1.96 U under the optimum conditions, i.e., 10 g L^{-1} colloidal chitin and 10 g L^{-1} CSL (Table 2.4). The main effects plot and contour plot of effect of colloidal chitin and CSL was shown in Figure 2.18 and 2.19 respectively.

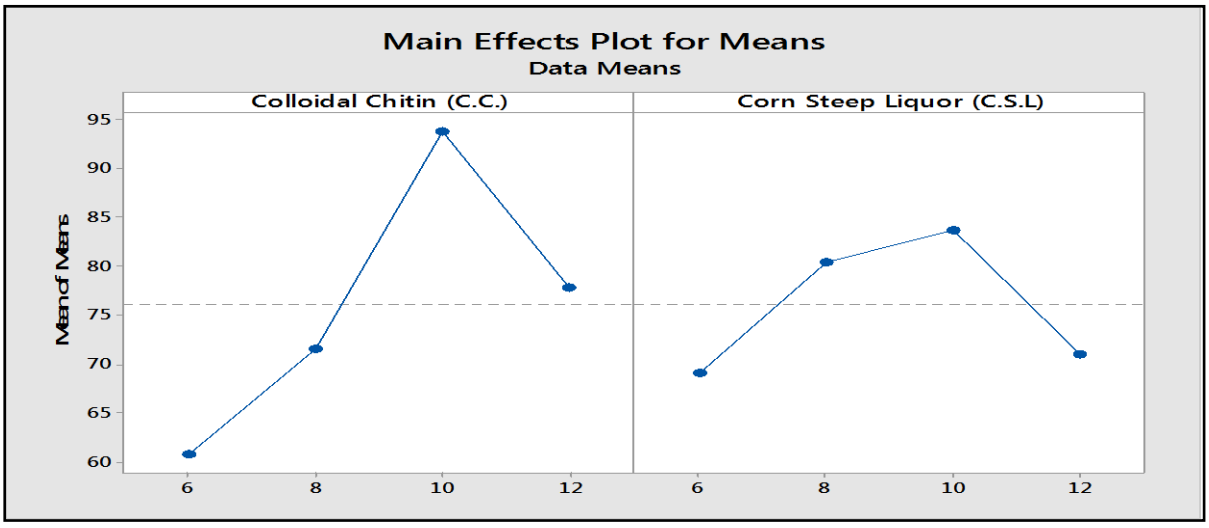

Fig. 2.18. Main effects plot showing optimal concentrations of colloidal chitin and CSL for chitinase production obtained using Taguchi method.

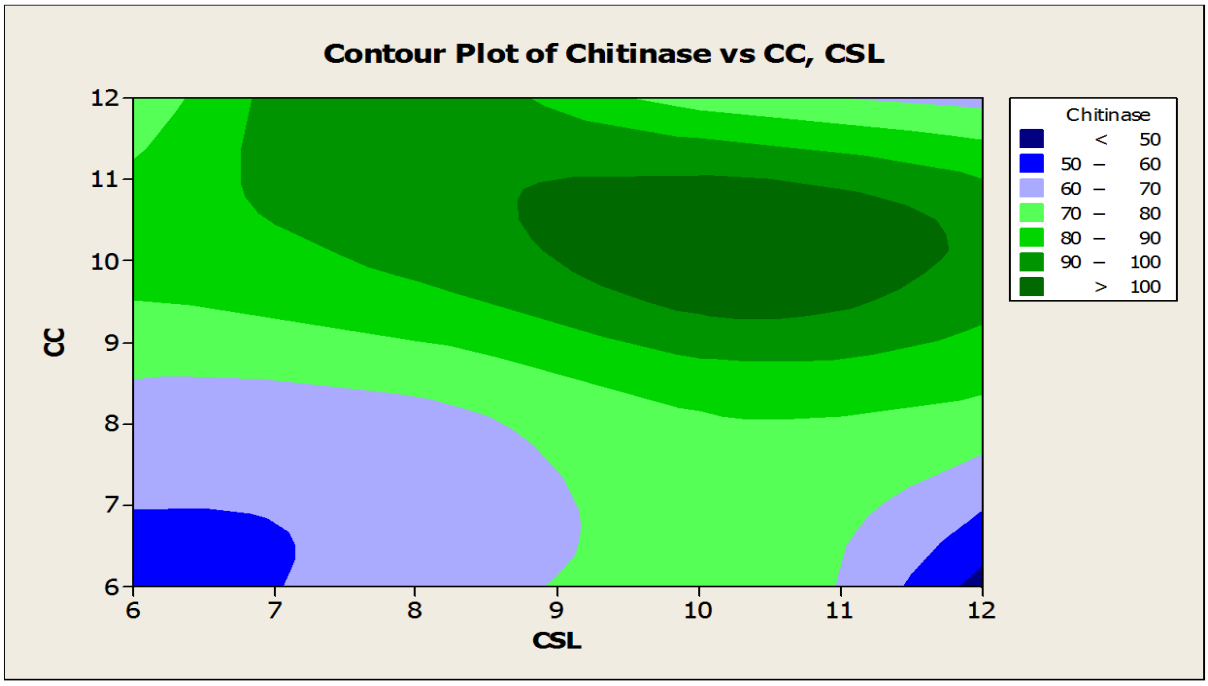

Fig. 2.19. Contour plot showing optimal concentrations of colloidal chitin and CSL for chitinase production obtained using Taguchi method.

The value of chitinase production obtained from the validation experiments, which were performed in triplicate using the optimised condition from the Taguchi design, using yeast extract was 102.28 ± 2.13 U; which was about 97% of the value obtained from the design. Similarly, for the validation experiments performed in triplicate using CSL, a chitinase production of 98.59 ± 2.25 U was obtained, which was 96% of the value obtained from the design.

The main advantages of Taguchi design method in statistical media optimisation are the reduction in number of experiments by orthogonal arrays (OAs) in comparison to the factorial design, and reduction in the sensitivity of the system to sources of variation using the signal-to noise (S/N) ratio to analyze the results, thus resulting in better performance (Zarei et al. 2010). Use of Taguchi design for optimisation of process parameters in chitinase production has been reported by other researchers such as Zarei et al. (2010) and Xia et al. (2011). Even though the chitinase enzyme production for *Streptomyces* sp. P6B2 was slightly

lesser in comparision to yeast extract, the use of CSL could have an added advantage of cost reduction when considering large-scale production.

By using the statistical method of optimisation, a 2.36-fold increase in the chitinase production by *Streptomyces* sp. P6B2 was observed in comparison to the initial unoptimised medium. The use of statistical optimisation methods led to increase in chitinase production by 2.41-fold for *Alcaligenes xylosoxydans* (Vaidya et al., 2001), 4.21-fold for *Pantoea dispersa* (Gohel et al., 2006a), 2.74-fold for *Paenibacillus sabina* (Patel et al., 2007), 15.5-fold for *Chitiolyticbacter meiyuanensis* (Hao et al., 2012), 11-fold for *Bacillus subtilis* (Narasimhan & Shivakumar, 2012), and 6.9-fold for *Bacillus pumilus* (Rishad et al., 2015).

2.3.6. Fermentor studies

Figure 2.20 shows the fermentation of the *Streptomyces* sp. isolate P6B2 in a 2.5 L fermentor with 1 L working volume. The medium used was the optimised production medium and fermentation was carried out at 32°C and aeration of 0.5-1.5 vvm was maintained according to the variation in the dissolved oxygen levels. Four different experiments were carried out each at 200, 250, 300 and 350 rpm. The pH of the medium was maintained at 8.0 during the course of the fermentation. The maximum chitinase production of 78.59 U was obtained after 60h of fermentation. The increase in the impeller speed from 200 to 300 rpm increased chitinase production, indicating that dissolved oxygen directly influenced the chitinase production. But when the impeller speed was further increased to 350 rpm, there was a drop in the chitinase production as seen in Figure 2.21. This could be due to the high shear force experienced by the bacterial cells, which were filamentous in nature, and hence get damaged. But in comparison to the shake-flask study, though the maximum chitinase yield was obtained after 60 h, the production was less, in that we could obtain a maximum of 105 U in the shake flask. When CSL was used as the nitrogen source, the maximum chitinase

production of 75.29 U was obtained after 60h of fermentation, which was comparable with the production, obtained using yeast extract.

Fig. 2.20. Batch fermentor setup (1L) for chitinase production.

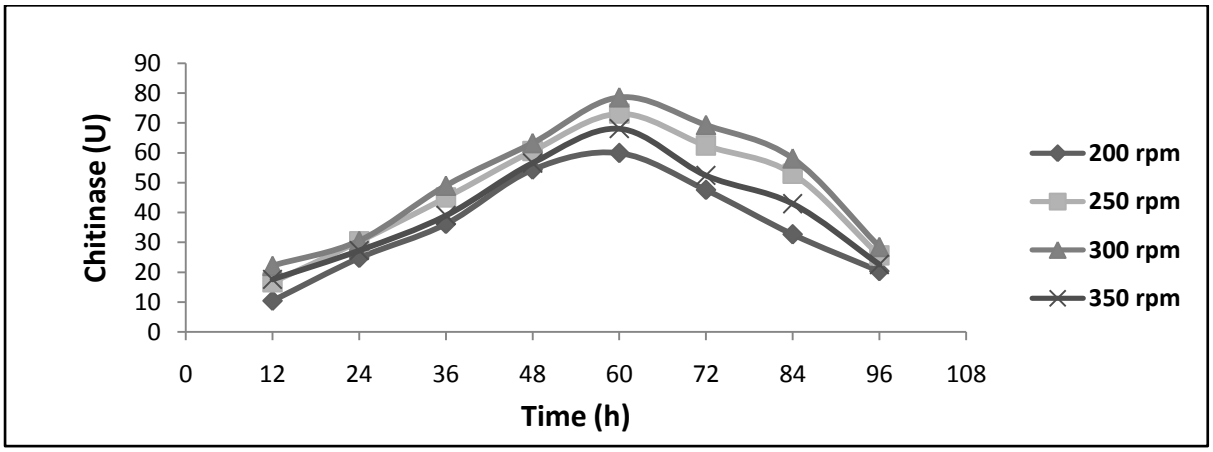

Fig. 2.21. Bioreactor studies for chitinase production using yeast extract as nitrogen source.

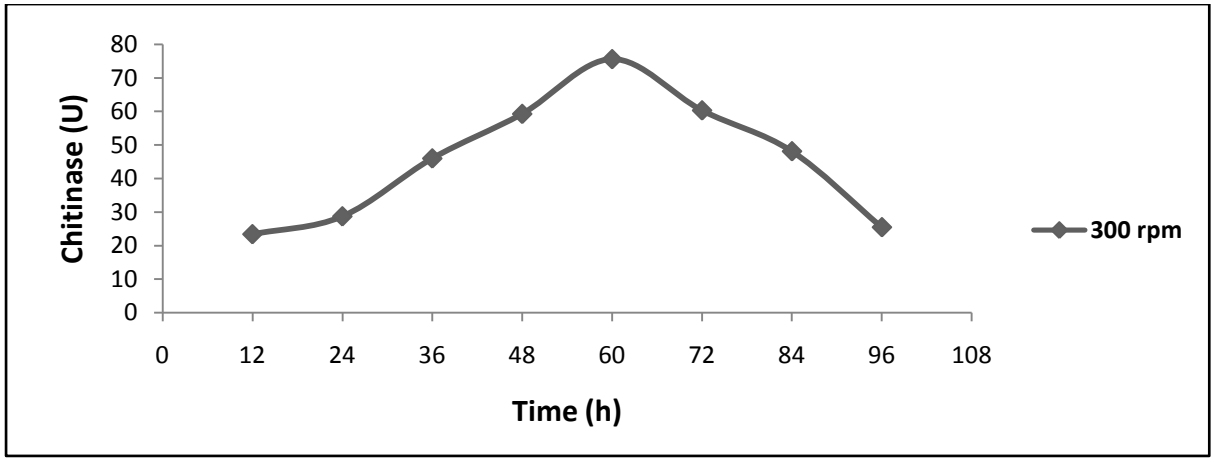

Fig. 2.22. Bioreactor studies for chitinase production using CSL as nitrogen source.

As described by Liu et al. (2003) for chitinase production by *Verticillium lecanii* in 5- and 30-L bioreactors, increased aeration affected the growth of the microorganism positively. Kao et al. (2007) found highest chitinase production from *Paenibacillus* sp. CHE-N1 in a 5-L fermentor at 34.3°C, 200 rpm and at an aeration rate of 3 vvm, in comparision with yield using 1 and 2 vvm. In a study by Fleuri et al. (2009), it was observed that increased aeration resulted in chitinase production in a shorter time, as in aeration values of 1.5 vvm and 3 vvm, highest chitinase yields were obtained after 168 h and 144 h of incubation, respectively. Submerged fermentation of *Lecanicillium muscarium* was carried out in a 2-L bench-top CSTR bioreactor in order to optimise the production of chitinolytic enzymes and the effect of stirrer speed and aeration rate combination was studied (Fenice et al., 2012). Highest activity (383.7 ± 7.8 U/L) was recorded at 1 vvm and 300 rpm with observed shear effect caused at higher stirrer speed and, partially, by high aeration rates. About 78% higher chitinase production was obtained in comparision to batch mode of operation. Fed-batch fermentation was attempted by Rao et. al. (2013) using a mixed culture of *Vibrio harveyi* and *Vibrio alginolyticus* with colloidal chitin as substrate over a period of three days. The colloidal chitin was added on a daily basis at 2% (w/v) to the fermentation medium in a biostat.

2.4. Summary and Conclusions

From the 160 chitinase producing microbial strains isolated by enrichment technique from the natural environment, an isolate P6B2 was selected and identified as a *Streptomyces* sp. bearing highest homology to the type strain *Streptomyces enissocaesilis* strain NRRL B-16365 using 16S rDNA identification method. The single parameter method followed by statistical approach showed significant results for optimizing the process parameters for the chitinase production in SmF using the *Streptomyces* sp. isolate and allowed rapid screening of a large number of variables. The identification of important process variables by the PBD experiments and the optimization of their levels by the Taguchi design helped to improve the chitinase production from 45U to 105 U. Use of corn steep liquor which is comparatively cheaper, as nitrogen source gave comparable results as that of yeast extract, and hence it could make the bioprocess economically more viable. When the process was carried out in a fermentor, a maximum chitinase production of 78.59 U was obtained in 60h.

Chapter 3

Purification and Characterisation of Chitinase

3.1. Introduction

During fermentation, a variety of proteins including enzymes are produced intracellularly as well as secreted into the growth medium by the microorganisms. These proteins vary from each-other with respect to size, shape, charge, hydrophobicity, solubility and biological activity. For better understanding of the structure and functions of the protein of interest, it has to be separated from the rest of the proteins. This led to the development in techniques for the isolation, purification and characterization of enzymes, so as to effectively study their mechanisms of action as well as other physicochemical properties, which may be useful in their industrial application. The classical enzyme purification procedures employed include centrifugation, ultrafiltration, selective precipitation and chromatographic procedures. A variety of techniques have been used in the purification of chitinases; ammonium sulphate precipitation, alcohol precipitation, ion exchange chromatography (IEC), gel permeation chromatography (GPC), etc. Affinity chromatography is a very specific and powerful method for purification of chitinases since it exploits the specific binding of the chitinase to its substrate, i.e. chitin, by way of chitin binding domains (CBD) and has been used with varying degrees of success by Jeuniaux (1966). Many chitinolytic microorganisms produce more than one kind of chitinase, i.e., different isoforms having varying molecular weights (Li, 2006; Singh et al., 2009). Microbial chitinases have been characterized for different parameters such as molecular weight, isoelectric point (pI), optimal pH, pH stability, optimal temperature, thermostability, effect of different metal ions and inhibitors, and antifungal activity. Some of them exhibit appreciable activity at extremes of temperature and pH, as well as in the presence of compounds which are usually inhibitory to enzyme activity, as mentioned in Chapter 1. Such chitinases may have valuable industrial or environmental applications. This chapter deals with the purification and characterisation of various

physicochemical properties of the extracellular chitinase produced by the isolated *Streptomyces* sp. (Chapter 2).

3.2. Materials and Methods

3.2.1. Preparation of colloidal chitin and chitinase assay

The colloidal chitin was prepared as mentioned in Chapter 2, section 2.2.1 and chitinase activity was determined as mentioned in section 2.2.2. The assay procedure was suitably modified according to the requirements of the experiments.

3.2.2. Microbial culture and chitinase production conditions

A *Streptomyces* sp. was isolated and identified, and the bioprocess parameters for maximum chitinase production by the same were optimised as mentioned in Chapter 2. The culture was inoculated in the optimised medium and after 60 h of incubation, the culture broth was harvested, centrifugation was done at 9500 ×g at 4°C for 10 min and the supernatant that consisted of the crude chitinase was further used for enzyme purification and characterisation studies.

3.2.3. Enzyme purification and determination of molecular weight and isoelectric point

Crude enzyme obtained as above was first purified by ammonium sulphate precipitation fractionation method, followed by DEAE anion-exchange chromatography and further by gel permeation chromatography. Two hundred millilitres cell-free supernatant was precipitated with ammonium sulphate (70%). The pellets obtained after centrifugation at 11000 ×g at 4°C for 15 min were dissolved in citrate-phosphate buffer (0.1 M, pH 5.5) and dialyzed overnight in the same buffer. IEC was done using a DEAE-methyl acrylate copolymer (Toyopearl DEAE-650, Tosoh Bioscience, Japan) column (30cm × 1cm). The column was packed with

overnight-swollen resin in 0.1 M citrate phosphate buffer (pH 5.5) and eluted stepwise with 0.1 to 0.8 M NaCl at a flow rate of 1.0 mL min^{-1}. The protein fractions (1.0 mL) were analysed for chitinase activity and those with positive activity were pooled and concentrated. This concentrated fraction was then further applied to a GPC column (50cm × 1.7cm). The column was packed with overnight-swollen resin (Toyopearl HW50, Tosoh Bioscience, Japan) in 0.1 M citrate buffer (pH 5.5). The same buffer was passed through the column at a flow rate of 1.0 mL min^{-1} to separate the proteins. The protein fractions (1.0 mL) were analysed for chitinase activity. The total protein content was determined for all the fractions by Bradford's method using bovine serum albumin (BSA) as the standard protein (Bradford, 1976).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli (1970) using vertical slab gel electrophoresis unit. A 14% separating and 5% stacking gel were used for PAGE analysis. The molecular mass of purified enzyme was determined by using protein molecular mass-standards (Precision-Plus) obtained from Biorad, USA. After SDS-PAGE, gel was stained by silver staining method.

GPC was used for determining the molecular mass of the chitinase. The separation was carried out using the GPC column as mentioned above with 50 mM Tris-HCl, pH 7.5 with 100 mM KCl; as equilibration buffer at a flow rate of 1.0 mL min^{-1}. One mL samples were collected for analysis. The elution volume (V_e) of the standard protein markers {Cytochrome C (12.4 kDa); Carbonic anhydrase (29 kDa); Bovine serum albumin (66 kDa); Alcohol dehydrogenase (150 kDa); β amylase (200 kDa); Blue dextran (2000 kDa)} obtained from Sigma Aldrich, USA; and the void volume (V_o) were determined. A semilog plot projecting the standard curve of molecular mass vs. V_e/V_o for each respective protein standard was plotted (Gel filtration Marker Kit, Sigma Aldrich, USA).

Chitin zymography (activity staining) was performed using a method developed by Gohel et al., (2005) with some modifications. Native polyacrylamide gel electrophoresis (NATIVE-PAGE) with a 12% separating gel was used. After loading the purified chitinase samples, electrophoresis was carried out. Further, the gel was washed with deionized water and was incubated at 50°C in 0.1 M citrate-phosphate buffer (pH 5.5), containing 1% (w/v) chito-oligosaccharide mixture (TCI, Japan) for 2 h. The gel was then again washed with deionized water and stained with 0.01% (v/v) Calcofluor white M2R in 0.1 M citrate-phosphate buffer (pH 5.5) for 30 min, and further destained with water. The lytic zones were photographed under the UV-transilluminator in a Chemidoc MP imaging system (Biorad, USA). Similarly, activity staining was done using 4-Nitrophenyl N-acetyl-β-D-glucosaminide and 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide which were chromogenic and fluorogenic substrates respectively for β-N-acetyl-glucosaminidase activity. The isoelectric point (pI) of the chitinase was determined by using IPG strips (ReadyStrip^{TM}, Biorad, USA) and the isoelectric focusing was carried out in Protean IEF Cell (Biorad, USA). For determining the pI, the pI calibration kit from Amersham (GE Healthcare Lifesciences, UK) was used. Gel permeation chromatography purified chitinase fraction was used for the pI determination.

3.2.4. Effects of pH, temperature, salinity, and addition of metal ions, solvents and denaturing agents on enzyme activity

The chitinase purified by DEAE-anion exchange chromatography was used for enzyme characterisation studies, including enzyme physicochemical properties and kinetics. The effect of pH was determined by incubating the purified chitinase over a pH range of 1.0–11.0 at 50°C. The buffer systems used were, HCl-KCl (100 mM, pH 1.0-2.0); citrate-phosphate (100 mM, pH 3.0–8.0); Tris-HCl (100 mM, pH 8.0–9.0) and carbonate-bicarbonate (100 mM, pH 9.0–11.0). The pH stability was tested by pre-incubation of the purified chitinase in

buffers with different pH from 1.0 to 11.0 at standard assay temperature for 2 h. The residual enzyme activity was tested at optimum pH. Optimum temperature for the chitinase activity was determined over a temperature range of 20 to 80°C at the optimum pH determined earlier (pH 6.0). Temperature stability was tested by the pre-incubation of the enzyme at temperatures ranging from 20 to 80°C for 3 h, and further assay was carried out at optimum temperature. A shelf-life study of the crude chitinase enzyme filtered through a 0.22 μ nylon membrane filter was carried out. The enzyme solution was dialysed in 0.1 M citrate phosphate buffer (pH 6.0) and was then incubated at 4°C and 30°C, respectively. Samples were assayed on a daily basis to determine the reduction in the activity of the enzyme upon storage at the respective temperatures. All further characterisation studies were carried out under conditions of optimal pH and temperature. The effects of metal ions on enzyme activity were assayed after the addition of metal ions Ag^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{2+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, Sn^{2+} and Zn^{2+} in the form of either their chloride or phosphate salts to the reaction mixtures. EDTA was added as a negative control. The metal ions and EDTA were added to the reaction mixture so as to achieve different final concentrations of 10 and 20 mM. The enzyme activity at various levels of salinity was recorded over a range of 0.25 to 5.0% (w/v) NaCl concentration in the assay mixture. The effects of addition of various solvents on enzyme activity was studied by the addition of acetic acid, acetone, butanol, chloroform, DMSO, ethanol, hexane, isoamyl alcohol, isopropanol, methanol and toluene at final concentrations of 10 and 20% in the assay mixture. The effects of various protein denaturants on the chitinase activity were also studied. Urea was added at final concentration of 200 mM; reducing agents such as β-mercaptoethanol, dithiothreitol were added at final concentrations of 10 mM and surfactants, viz. Sodium dodecyl sulphate (SDS), Tween (20, 40, 60, 80) and Triton X-100 were added at final concentrations of 1% to the assay mixture. The residual chitinase activity was measured after each characterisation experiment. The

chitinase activity was expressed as the percent relative activity. Appropriate controls were maintained wherever required during the above studies.

3.2.5. Substrate specificity and enzyme kinetics

The purified chitinase was incubated with various substrates such as colloidal chitin, powder chitin, powder chitosan, glycol chitosan, powder cellulose, carboxymethyl cellulose (CMC) and soluble starch. These were added to the assay mixture individually at a concentration of 1% (w/v) under standard assay conditions and the degree of substrate hydrolysis was analysed wherein the quantity of the reducing sugar released was determined by DNS method as mentioned earlier. The effect of substrate concentration on chitinase activity was studied using colloidal chitin as substrate. Colloidal chitin with final concentration ranging from 2.0 to 50.0 mg mL^{-1} was used, and the enzyme activity was then determined by the standard assay protocol. The kinetic constants K_m and V_{max} were determined from the Lineweaver–Burk plot.

3.3. Results and Discussion

3.3.1. Enzyme purification

The crude enzyme obtained after the fermentation process using the *Streptomyces* sp. was purified using ammonium sulphate precipitation, DEAE based ion exchange chromatography (Fig. 3.1) and gel permeation chromatography (Fig. 3.2) consecutively. The chitinase purification carried out has been summarised in Table 3.1. The chitinase was 21.1-fold purified with a final specific activity of 273.71 U mg^{-1}, and a recovery yield of 2.24%. Many chitinases produced by different bacterial cultures have been purified using ammonium sulphate precipitation method followed by multiple column chromatographic steps (Han et al., 2009; Wang et al., 2010; Dai et al., 2011; Rabeeth et al., 2011; Brzezinska et al., 2013).

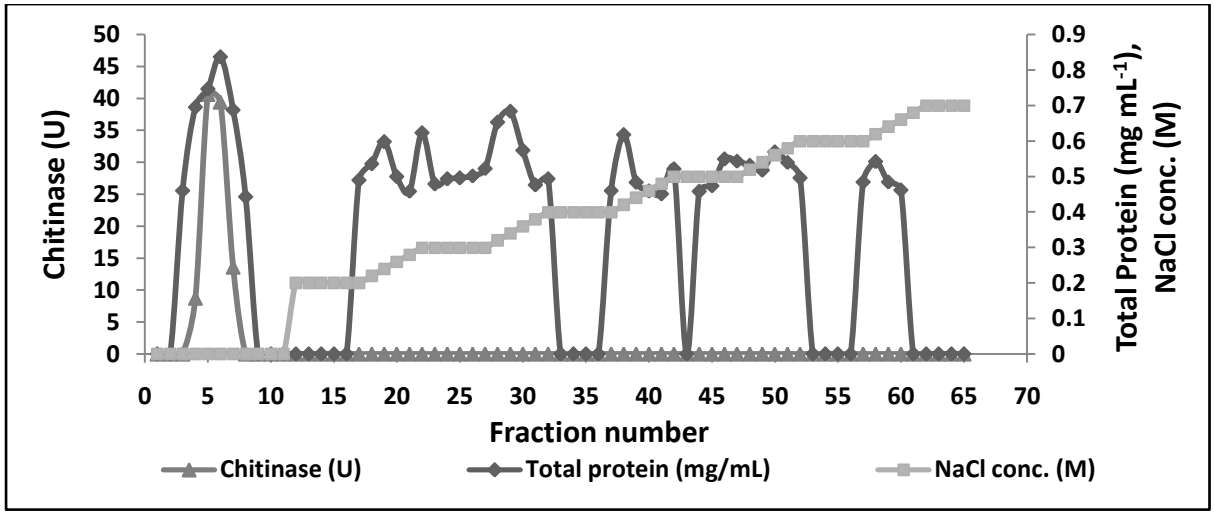

Fig. 3.1. Chitinase- Ion exchange chromatography elution profile.

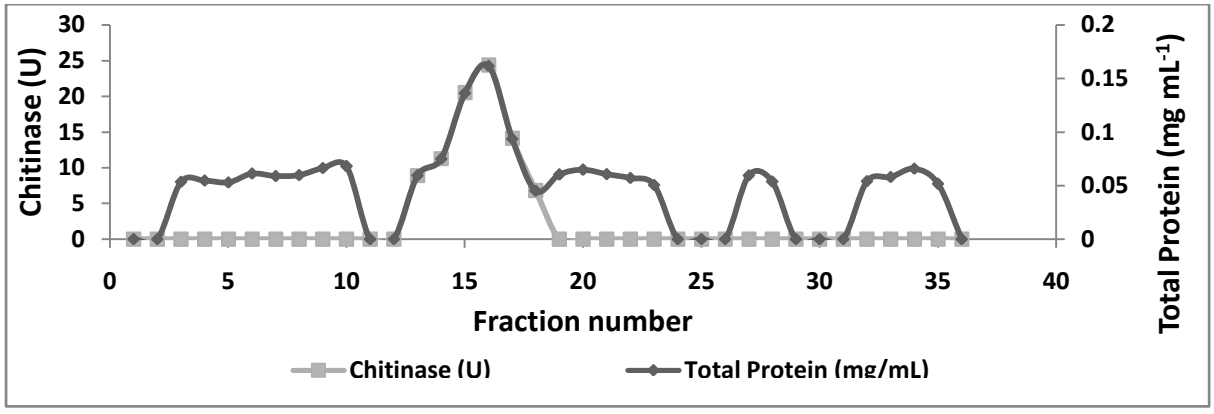

Fig. 3.2. Chitinase- Gel permeation chromatography elution profile.

Table 3.1. Purification of *Streptomyces* sp. chitinase.

Purification Step	Volume (mL)	Total Proteins (mg)	Specific Activity (U mg^{-1})	Total Activity (U)	Purification (Fold)	Yield % (Total Activity)
Culture filtrate	100	418.0	12.97	5421.46	0	100
$(NH_4)_2SO_4$ precipitation (60%)	50	102.50	23.53	2411.83	1.82	44.49
DEAE-Anion exchange chromatography	10	5.58	72.51	404.61	5.59	7.46
Gel permeation chromatography	5	0.45	273.71	121.80	21.10	2.24

A comparison of specific activity, yield and fold of purification achieved after the last purification step of the chitinase from the *Streptomyces* sp. in the present study with that of other bacterial cultures has been portrayed in Table 3.2.

Table 3.2. Comparison of specific activity, yield and fold of purification achieved after the last purification step of chitinases from various bacterial sources.

Organism	Specific activity (U mg^{-1})	Yield %	Purification fold	Reference
Streptomyces sp.	273.71	2.24	21.10	Present study
Bacillus sp.	62.4	15	5.6	Dai et al., 2011
Bacillus licheniformis	22.5	35	11.5	Kudan and Pichyangkura, 2009
Brevibacillus formosus	94.21	37.7	6.1	Meena et al., 2014
Micrococcus sp.	93.02	19.95	10.33	Annamalai et al., 2010
Pseudomonas sp.	0.175	9	25	Wang et al., 2010
Streptomyces sp.	2.95	1.09	6.15	Han et al., 2009
Streptomyces sp.	506.55	17.15	9.84	Margino et al., 2010
Streptomyces halstedii	527	37	182	Joo et al., 2005
Streptomyces roseolus	30	34	23	Jiang et al., 2012
Streptomyces violascens	1.43	33.67	13.75	Gangwar et al., 2016

3.3.2. Gel electrophoresis and chitinase molecular weight estimation

The molecular mass of the *Streptomyces* sp. chitinase was estimated approximately to be 40 kDa by SDS-PAGE and was seen on the gel as a single band purified after carrying out gel permeation chromatography. Also, the gel permeation chromatography based molecular weight determination method using protein standards (Table 3.3) confirmed this observation.

The standard semi-log plot used for calculations is as observed in Figure 3.3. The molecular weight calculated by this method was 39.72 kDa.

Table 3.3. Protein standards used for determination of molecular weight by GPC.

Protein Std	Molecular weight	Ve
Blue Dextran	2000	**Vo** = 31
B Amylase	200	44
Alcohol Dehydrogenase	150	45
BovineSerum Albumin	66	48
Carbonic Anhydrase	29	51
Cytochrome C	12.4	55

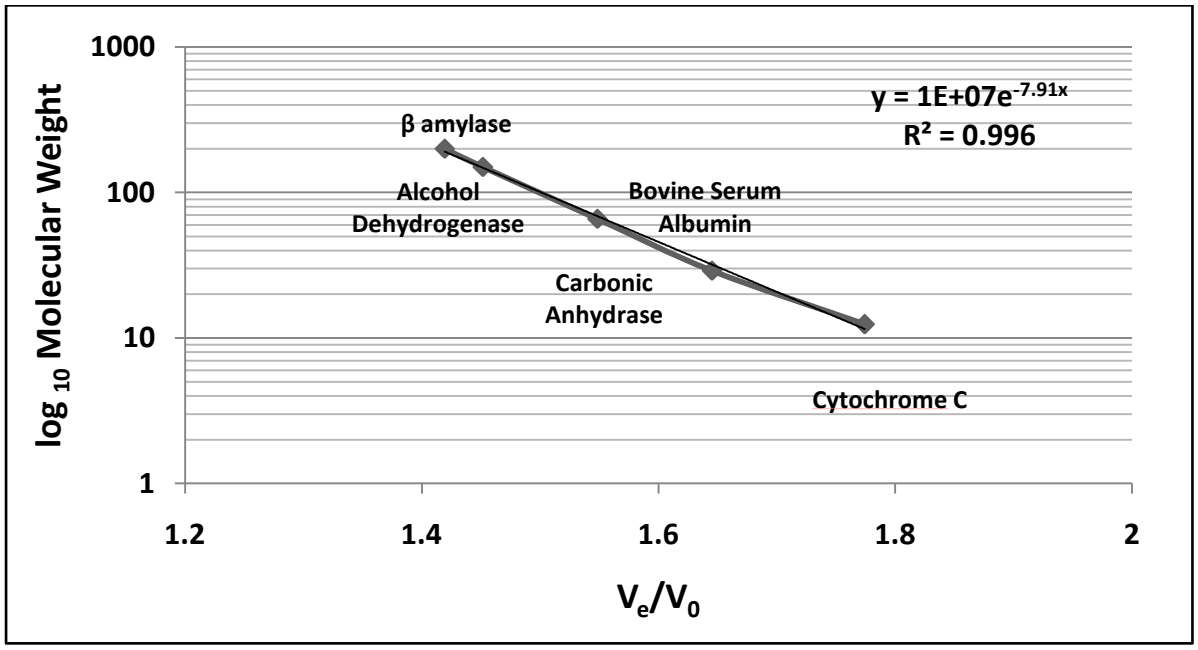

Fig. 3.3. Protein standards semi-log plot for determining unknown protein molecular weight.

In accordance with the activity staining results, the chitinase enzyme showed a single clear band by zymography, and confirmed that it was a chitinase and only a single protein with chitinase activity was produced with no isozymes. The analysis of the purified enzyme by SDS-PAGE and NATIVE-PAGE-activity staining is shown in Figure 3.4 and Figure 3.5 respectively.

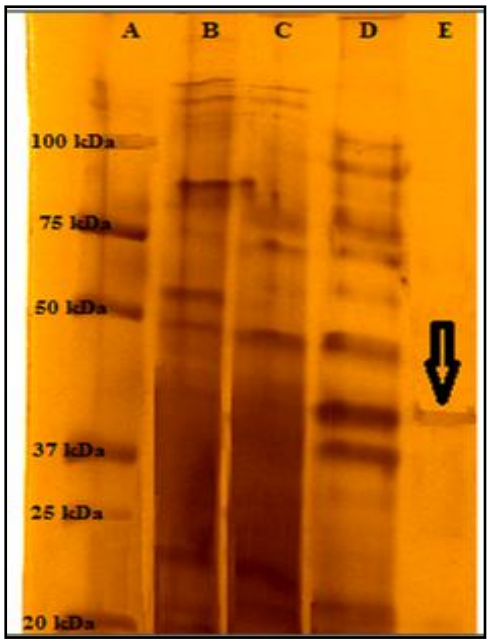

Fig. 3.4. SDS-PAGE analysis of *Streptomyces* sp. chitinase enzyme. Lane A: Protein standard molecular weight marker, Lane B: Crude enzyme, Lane C: Ammonium sulphate precipitation, Lane D: Ion exchange chromatography, Lane E: Gel permeation chromatography. The arrow indicates the single 40 kDa band of the purified enzyme.

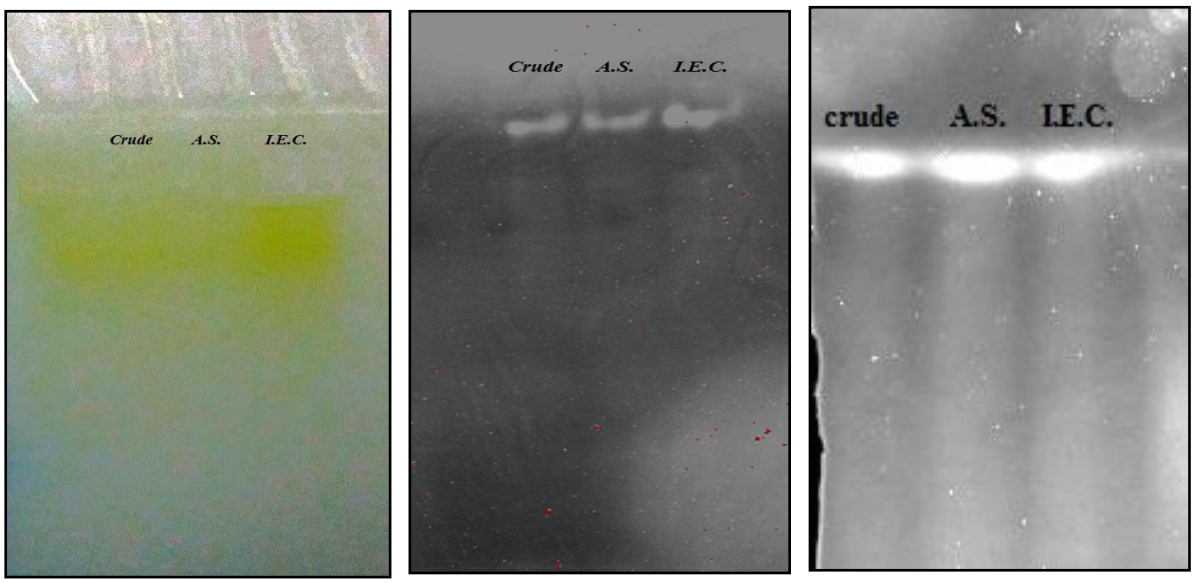

Fig. 3.5. *Streptomyces* sp. chitinase activity staining using different substrates: 4-Nitrophenyl N-acetyl-β-D-glucosaminide (left); chitooligosaccharides stained using Calcofluor white M2R (middle) and 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (right). Crude, ammonium sulphate precipitation purified fraction (A.S.) and ion exchange chromatography (I.E.C.) were loaded.

Molecular masses of bacterial chitinases generally range from 30 to 80 kDa (Chapter 1, Table 1.8). The molecular weight of the chitinase reported in this study was similar to that reported for two *Streptomyces* strains (Jiang et al., 2012 and Pradeep et al., 2014). The pI of the *Streptomyces* sp. chitinase in the present study was 6.9. The pI values of the standard proteins and the standard graph obtained are provided in Table 3.4 and Figure 3.6, respectively. According to earlier reports bacterial chitinases show a broad range of pI ranging from 3–9 (Hobel et al., 2005; Yong et al., 2005; Adrangi et al., 2010; Kudan and Pichyangkura 2009; Prakash et al., 2013).

Table 3.4. pI values of protein standards of broad range pI determination kit (pH 3-10).

Protein	pI at 24 °C±1.5°C
Amyloglucosidase	3.50
Methyl red (dye)	3.75
Soyabean trypsin inhibitor	4.55
β-Lactoglobulin A	5.20
Bovine carbonic anhydrase B	5.85
Human carbonic anhydrase B	6.55
Horse myoglobin-acidic band	6.85
Horse myoglobin-basic band	7.35
Lentil lectin-acidic band	8.15
Lentil lectin-middle band	8.45
Lentil lectin-basic band	8.65
Trypsinogen	9.30

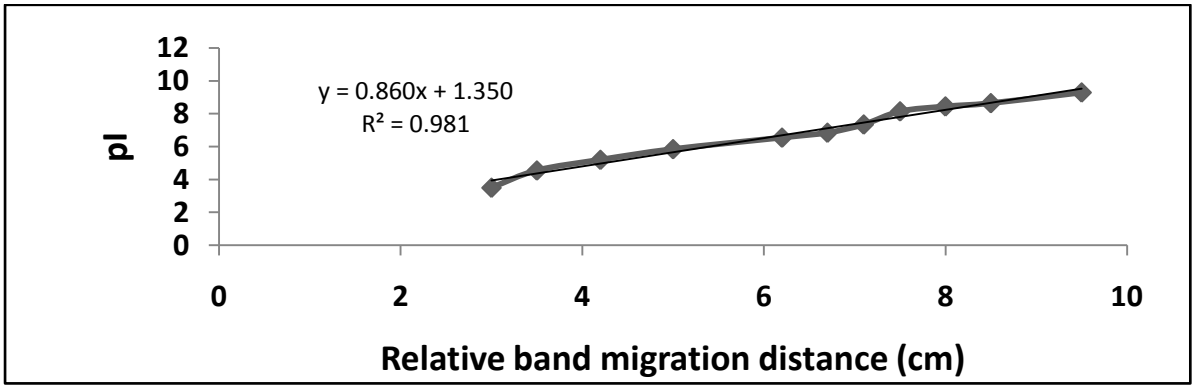

Fig. 3.6. Protein standard graph for determination of pI of unknown protein.

3.3.3. Effect of pH and temperature on the activity and stability of the purified chitinase

pH and temperature play an important role in determining the enzyme activity and stability. The chitinase from the *Streptomyces* sp. was active from pH 2.0 to 7.0, exhibiting maximum activity at pH 2.0 and pH 6.0 as observed in Figure 3.7. Beyond pH 7.0, there was a rapid decrease in the activity, indicating that the enzyme was active within the acidic range. Similarly, the stability was also exhibited in the acidic pH range as seen in Figure 3.8. The enzyme, when incubated at basic pH, might have been denatured irreversibly, hence, a reduction in its activity was observed.

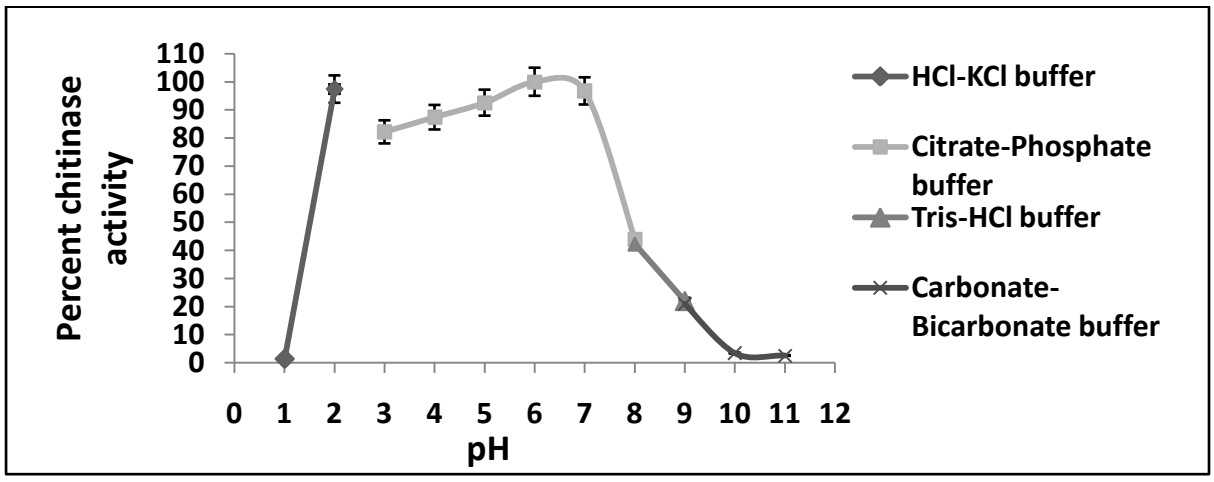

Fig. 3.7. Determination of pH optimum of chitinase.

Most of the purified bacterial chitinases show moderate to high activity as well as stability within the pH range of 4.0–10.0 (Kudan and Pichyangkura, 2009; Adrangi et al., 2010; Margino et al., 2010; Rabeeth et al., 2011; Babashpour et al., 2012; Meena et al., 2014; Laribi-Habchi et al., 2015). But, the chitinase in this study was comparable to the acidic chitinase reported for a *Microbispora* sp. showing highest activity at pH 3.0 (Nawani et. al. 2002). Also it is more stable in acidic media than the chitinase from *Rhodothermus marinus* (Hobel et al., 2005), *Sanguibacter* sp. (Yong et. al. 2005) and *Streptomyces roseolus* (Jiang et al., 2012). A *Streptomyces* sp. chitinase was reported to be active in the alkaline range from

pH 8.0-14.0 with optimal pH of 12.5 (Pradeep et al., 2014). Chitinases from *Aeromonas* sp. (Sutrisno et al., 2001) and *Bacillus thuringiensis* (Kudan and Pichyangkura, 2009) also demonstrated the phenomenon of two pH optima like the *Streptomyces* sp.

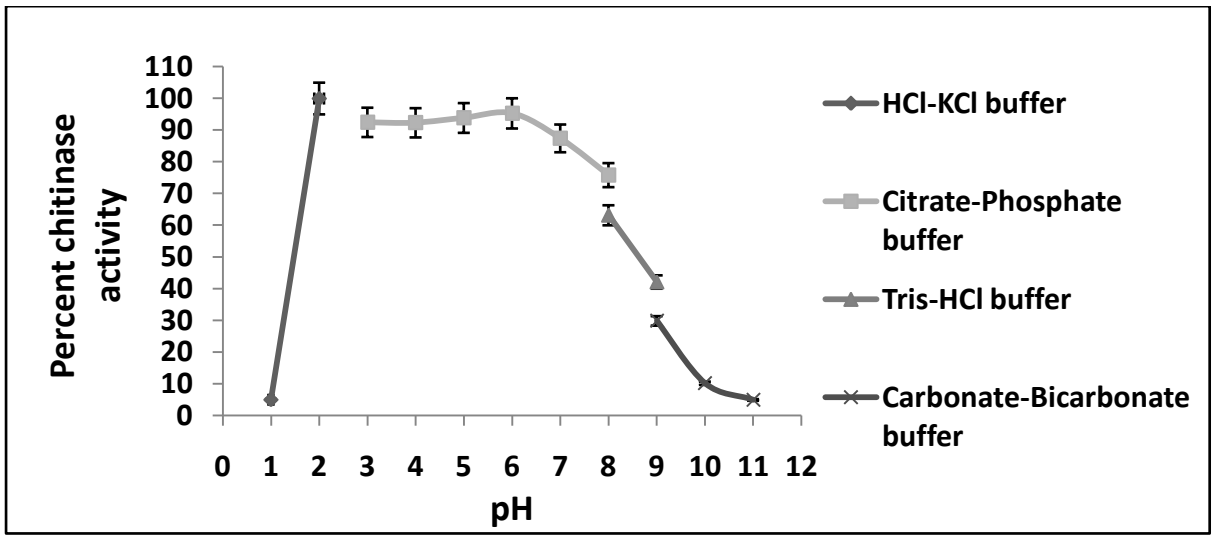

Fig. 3.8. Determination of pH stability of chitinase.

Chitinase produced by the *Streptomyces* sp. exhibited a temperature optimum at 50°C. At temperatures below and above 50°C, the enzyme activity reduced progressively and significantly (Fig. 3.9). The activity dropped to below 50 % of the highest activity at 30°C and 60°C. The chitinase exhibited high stability up to 40°C even after three hours of incubation (Fig. 3.10). The residual enzyme activity reduced to around 60% after 3 h when incubated at 50°C, showing the reduced stability of the enzyme at 50°C. At temperatures above 50°C, the enzyme stability was significantly reduced as seen in Figure 3.10. The $t_{1/2}$ value for 50°C was calculated to be 234 minutes while the same for 60 °C was calculated to be 148 minutes. The optimum temperature for most bacterial chitinases has been reported within 40-60°C and show moderate activity within the range of 30-70°C, while the thermal stability is generally observed up to 50°C (Rabeeth et al., 2011; Brzezinska et al., 2013; Kudan and Pichyangkura, 2009; Pradeep et al., 2014; Meena et al., 2014). The temperature

optimal and stability of the chitinase of the present study was similar to the chitinase from a *Streptomyces* sp. (Han et al., 2009) and a *Pseudomonas* sp. (Wang et al., 2010). Chitinases from *Bacillus licheniformis* (Toharisman et al., 2005), *Rhodothermus marinus* (Hobel et al., 2005) and a *Streptomyces* sp. (Prakash et al., 2013) exhibit high optimum temperature of 70°C.

Many industrial processes are operated at extremes of pH (either acidic or alkaline) and at elevated temperatures that make the enzyme necessary to suit such process requirements. It must also be capable of withstanding such harsh conditions for prolonged periods of processing time. The other advantage of carrying out conversions at increased temperatures (50°C and above) is that, it significantly reduces microbial contamination of the substrate being processed. During the shelf life studies it was found that the crude chitinase retained 90% of its activity for up to three weeks at 4°C, and retained more than 50% of its total activity for up to eleven weeks. But, at room temperature (30°C) the chitinase retained more than 50% of its total activity for up to only six days. This may be due to the presence of proteases in the crude supernatant. A chitinase produced by a *Streptomyces* sp. retained 100% of its activity for up to twelve weeks at 4°C while at room temperature (25°C) the shelf-life of the chitinase was eight weeks (Pradeep et al., 2014).

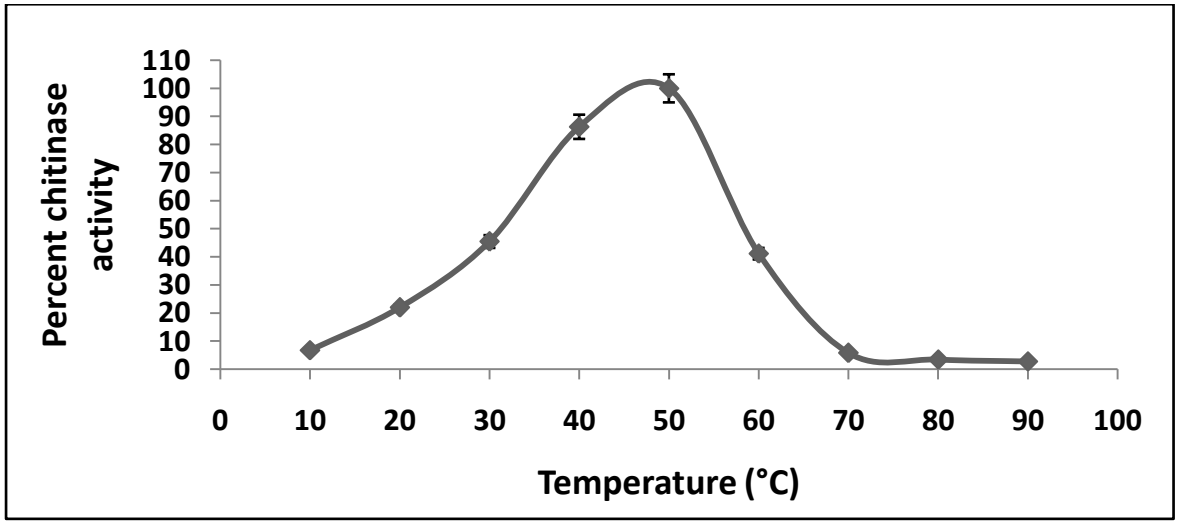

Fig. 3.9. Determination of temperature optimum of chitinase.

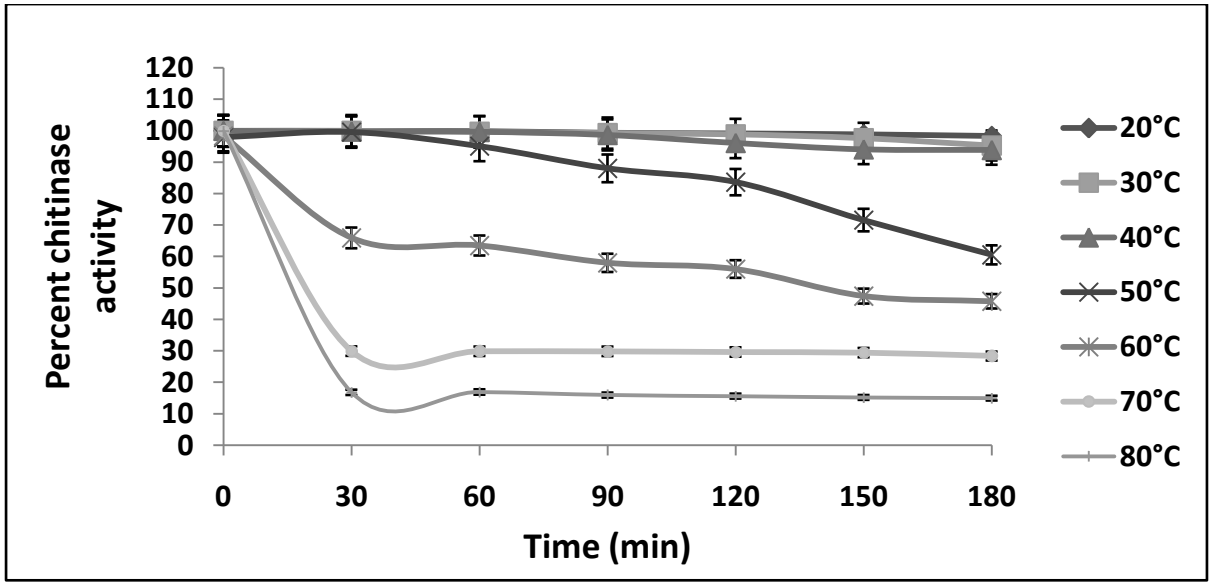

Fig. 3.10. Determination of temperature stability of chitinase.

3.3.4. Effect of metal ion addition on the activity of the purified chitinase

Metal ions play a significant role in biological catalysis by forming complexes with the enzymes and maintaining or disrupting the 3-dimensional structure and configuration (Andreini et al., 2008). The influence of metal ions on the activities of chitinase produced by the *Streptomyces* sp. is shown in Figure 3.11.

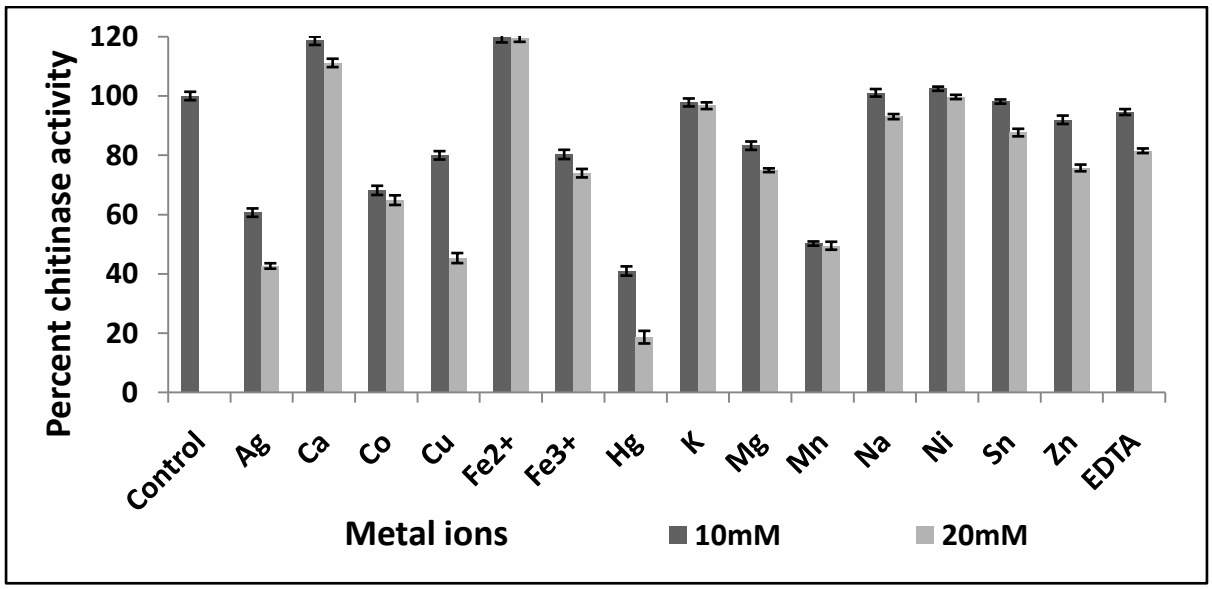

Fig. 3.11. Effect of addition of metal ions on chitinase activity.

The activity was enhanced by 15% in the presence of Ca^{2+} and Fe^{2+}. It was inhibited moderately to strongly by Ag^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+}. The activity was least affected by K^{+}, Na^{+}, Ni^{2+} and Sn^{2+}. Also, there was no significant inhibition of activity in the presence of EDTA. As observed for the *Streptomyces* sp. in this study, Ca^{2+} was reported by several authors to enhance chitinase activity, including Annamalai et al. (2010), Natsir et al. (2010), Dai et al. (2013) and Gao et al., (2015). Cu^{2+} ions are known to catalyze the auto-oxidation of cysteines to form intra-molecular disulfide bridges or the formation of sulphenic acid. Cu^{2+} has been reported as a moderate to strong inhibitor of chitinases produced by different bacterial genera including *Bacillus* (Natsir et al., 2010; Waghmare and Ghosh, 2010), *Chitinibacter* (Gao et al., 2015), *Streptomyces* (Pradeep et al., 2014; Jiang et al., 2012), *Micrococcus* (Annamalai et al., 2010), *Pseudomonas* (Wang et al., 2010) and *Stenotrophomonas* (Jankiewicz et al., 2012). The major inhibitor of chitinase activity was Hg^{2+} since it reacted with -SH groups found in cysteine residues in the protein chain and disrupted the tertiary structure. It strongly inhibited chitinases from different genera, including *Bacillus* (Waghmare and Ghosh, 2010; Bhattacharya et al., 2016), *Massilia timonae* (Adrangi et al., 2010), *Microbiospora* (Nawani et al., 2002), *Rhodothermus* (Hobel et al., 2005), *Stenotrophomonas* (Jankiewicz et al., 2012) and *Streptomyces* (Nagpure and Gupta, 2013; Prakash et al., 2013). The other major inhibitory metal ions, which show strong inhibitory action on chitinases include Fe^{2+} (Pradeep et al., 2014; Wang et al., 2010), Fe^{3+} (Bhattacharya et al., 2016), Mn^{2+} (Pradeep et al., 2014; Wang et al., 2010), Ag^{2+} (Nagpure and Gupta, 2013; Liu et al., 2010), Zn^{2+} (Jankiewicz et al., 2012; Prakash et al., 2013) and Co^{2+} (Jiang et al., 2012; Yong et al., 2005). As opposed to this Mn^{2+}, moderately enhanced the activity of chitinase obtained from a *Sanguibacter* sp. (Yong et al., 2005) and a *Streptomyces* sp. (Han et al., 2009), while Zn^{2+} slightly enhanced the chitinase activity of *Bacillus* sp. (Dai et al., 2013), *Chitinibacter* sp. (Gao et al., 2015) and a *Streptomyces* sp.

(Han et al., 2009). Cu^{2+} enhanced the chitinase activity of *Chitinibacter* sp. (Gao et al., 2015) and a *Streptomyces* sp. (Gangwar et al., 2016). EDTA, which is a chelating agent capable of binding metal ions in solution has been reported to reduce the activity of chitinases produced by *Bacillus* sp. (Dai et al., 2013), *Micrococcus* sp. (Annamalai et al., 2010), *Pseudomonas* sp. (Wang et al., 2010), *Streptomyces* sp. (Pradeep et al., 2014) and *Massilia timonae* (Adrangi et al, 2010). But it had no significant effect on chitinases produced by a *Microbiospora* sp. (Nawani et al., 2002), *Bacillus thuringensis* (Liu et al., 2010), *Streptomyces* sp. (Han et al., 2009; Nagpure and Gupta, 2013; Jiang et al., 2012).

3.3.5. Effect of salinity on the activity of the purified chitinase

Marine micro-organisms usually are tolerant to higher salinity levels. Salt-tolerant enzymes may play significant roles in the industrial processes that operate in high-salt conditions. Since the *Streptomyces* sp. was isolated from coastal environment samples (Chapter 2), it was expected that the chitinase expressed would be salt tolerant. The activity of the chitinase remained constant up to 1% NaCl concentration. After that, it gradually reduced to give 50% residual activity at 5% NaCl concentration (Fig. 3.12). A *Streptomyces* sp. produced chitinase that showed highest activity upto 45 g% psu salinity (Han et al., 2009).

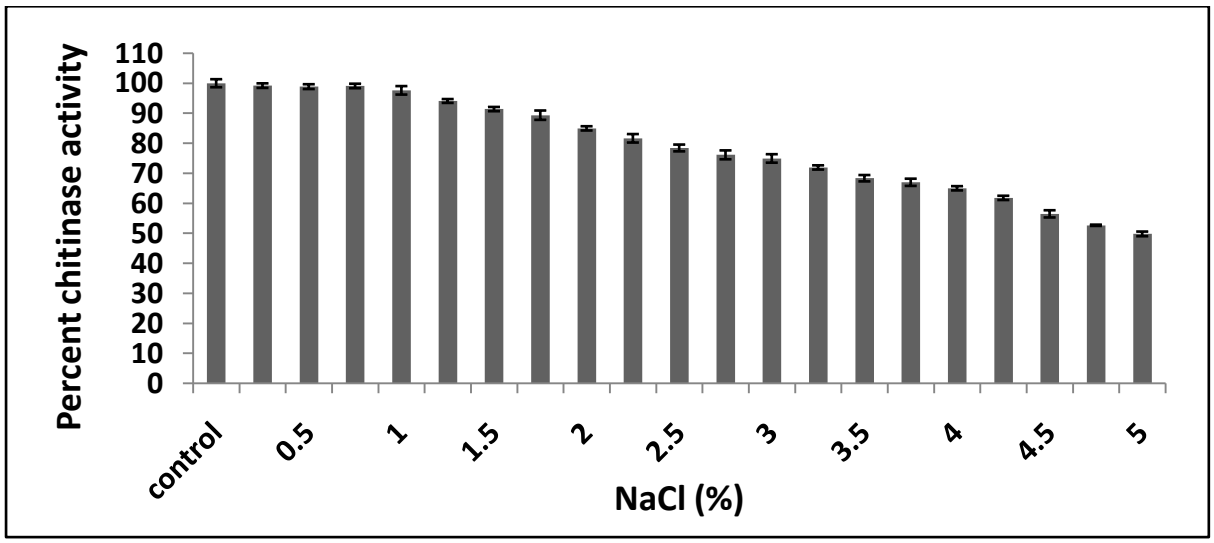

Fig. 3.12. Effect of salinity on chitinase activity.

3.3.6. Effects of solvent addition on the activity of the purified chitinase

The effect of solvent addition on the activity of chitinase obtained from the *Streptomyces* sp. is shown in Figure 3.13. It was found that acetic acid strongly inhibited the chitinase activity reducing it to 17% of the original activity. Most of the solvents such as butanol, chloroform, DMSO, ethanol, isoamyl alcohol, isoamyl alcohol, isopropanol and methanol moderately reduced the chitinase activity. But in the presence of hexane and toluene, there was a small increase in enzyme activity. It indicated that hydrophobic interactions were essential for enzyme activity. A *Bacillus licheniformis* chitinase was reported unstable toward organic solvents such as butanol, 2-propanol, ethanol, and DMSO at a concentration of 5%, (Toharisman et al., 2005). A *Streptomyces violascens* chitinase activity was reported to be inhibited by chloroform and acetone while enhanced in the presence of ethanol (Gangwar et al., 2016).

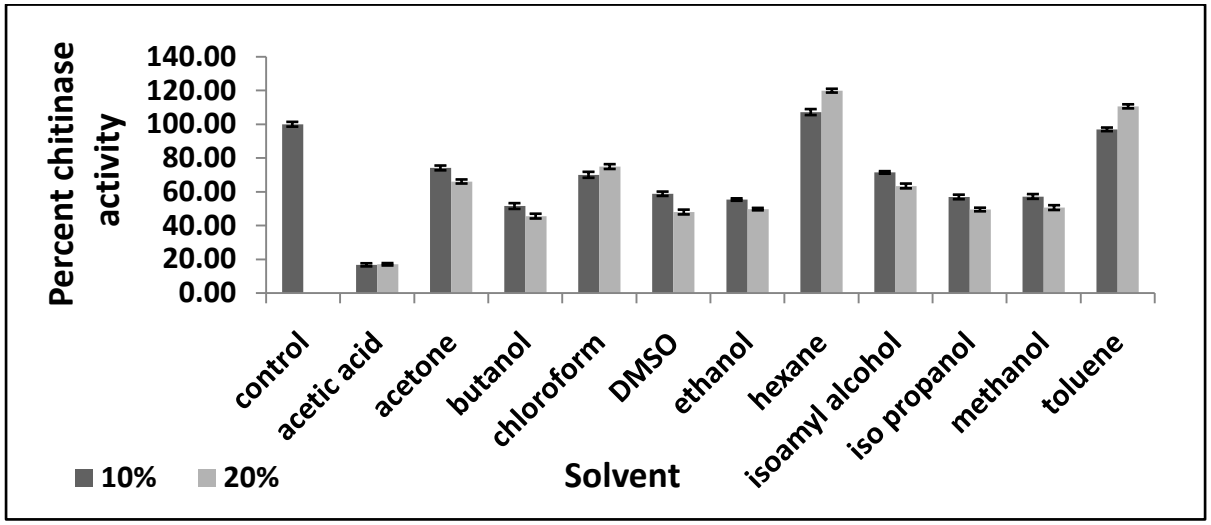

Fig. 3.13. Effect of solvent addition on chitinase activity.

3.3.7. Effect of denaturing agent addition on the activity of the purified chitinase

The influence of denaturing agents on the activity of chitinase obtained from the *Streptomyces* sp. is as shown in Figure 3.14. In the case of surfactants, chitinase activity was insignificantly affected by Tween (20, 40, 60 and 80) and Triton-X 100 but, was affected by

SDS that caused a 27% reduction in its original activity. Urea caused a decrease in activity to 86% of the original activity. β-mercaptoethanol and dithiothreitol significantly reduced the chitinase activity to 23 and 20% of the original activity respectively. Urea inhibited the activity of chitinases produced by two *Streptomyces* sp. (Han et al., 2009; Prakash et al., 2013). The activity of a *Streptomyces* sp. chitinase was moderately reduced by the presence of β-mercaptoethanol (Pradeep et al., 2014) but was enhanced as reported by Rabeeth et al. (2011) for another *Streptomyces* sp. Dithiothreitol also inhibited chitinases from *Streptomyces* sp. (Prakash et al., 2013) and *Bacillus* sp. (Dai et al., 2013). There was no effect of SDS on the activity of chitinases produced by a *Bacillus thuringiensis* (Liu et al., 2010), *Bacillus licheniformis* (Toharisman et al., 2005) and *Streptomyces griseus* (Rabeeth et al., 2011), while the presence of SDS increased the activity of the chitinases produced by a *Pseudomonas* sp. (Wang et al., 2010). But SDS strongly inhibited the chitinase a *Streptomyces* sp. as reported by Han et al. (2009) and Gangwar et al., (2016). Tween 20 and Triton X-100 did not exhibit any effect on the activity of *Bacillus licheniformis* (Toharisman et al., 2005).

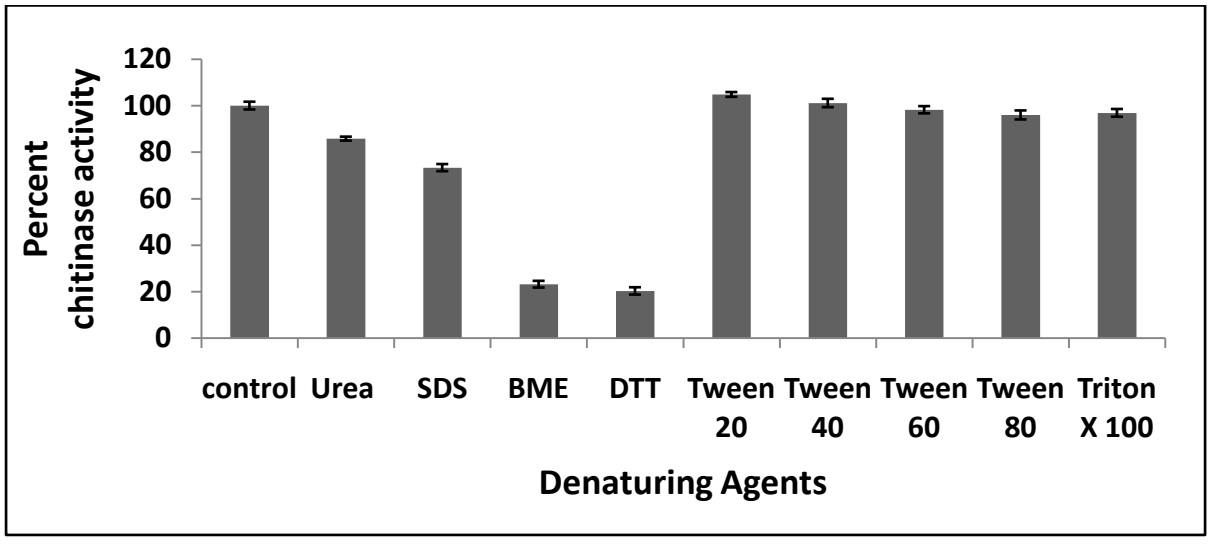

Fig. 3.14. Effect of denaturing agent addition on chitinase activity. Final concentrations: Urea at 200 mM; β-mercaptoethanol (BME) and dithiothreitol (DTT) at 10 mM; and surfactants Sodium dodecyl sulphate (SDS), Tween (20,40, 60, 80) and Triton X-100 at 1%.

3.3.8. Substrate specificity and kinetic parameters

The purified *Streptomyces* sp. chitinase was assayed using different substrates and it showed highest activity towards colloidal chitin. A comparatively lower activity was observed in the presence of powdered chitin, while it showed very feeble activity towards powdered chitosan. It showed no activity towards cellulose, carboxymethyl cellulose and starch (Fig. 3.15). Similar was observed in case of *Streptomyces roseolus* (Jiang et al., 2012) and a *Bacillus* sp. (Dai et al., 2011) where the chitinase did not show any activity towards chitosan, cellulose, carboxymethyl cellulose and starch. The chitinase of *Streptomyces violascens* was able to efficiently hydrolyze colloidal chitin and chitopentose and showed reduced activity with chitosan and chitin powder, but it failed to hydrolyse amylose, cellobiose, cyclodextran, raffinose and starch. (Gangwar et al., 2016). A chitinase produced by *Bacillus licheniformis* was highly active for substrates such as colloidal chitin, glycol chitin, chitosan and colloidal chitosan (Toharisman et al., 2005). In a curious case for a chitinase secreted by a *Rhizopus oryzae* strain, among the selected substrates, along with colloidal chitin, soluble starch was also efficiently hydrolyzed, supposing that the purified chitinase might have by-function of amylolysis (Chen et al., 2013). But cellulose, α-crystalline chitin, chitosan, CMC, agarose, avicel and glycol chitosan were not hydrolyzed.

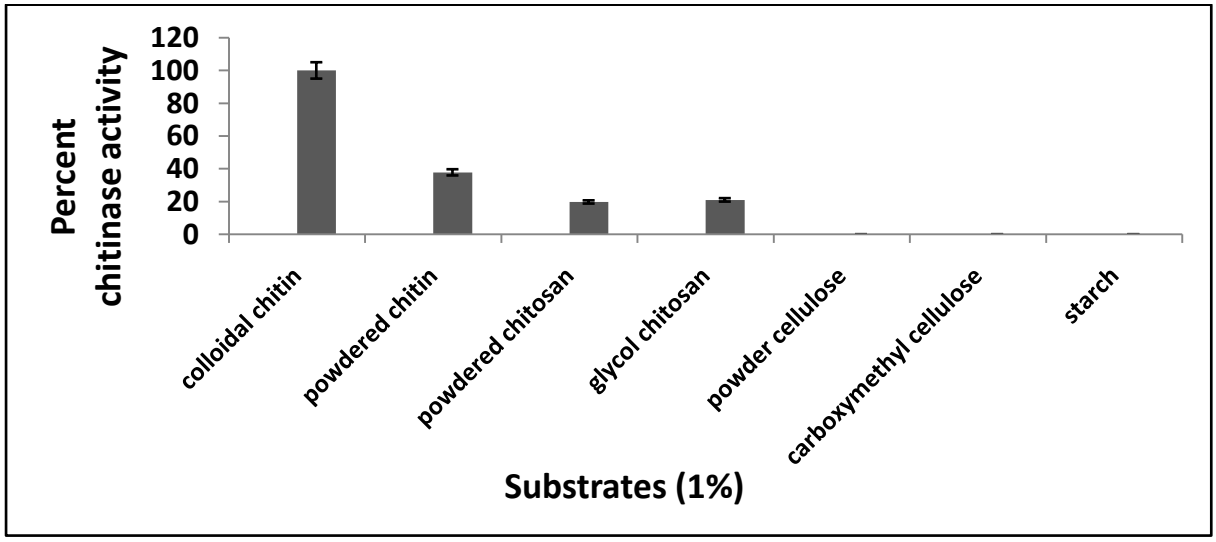

Fig. 3.15. Substrate specificity of chitinase enzyme.

The Michaelis-Menten kinetic constants K_m and V_{max} of the *Streptomyces* sp. chitinase was determined using a Lineweaver–Burk Plot to be 6.74 mg mL^{-1} and 102.95 U mL^{-1} using different concentrations of colloidal chitin (2 to 50 mg mL^{-1}). The effect of substrate concentration on chitinase enzyme is depicted in Figure 3.15, while the double reciprocal - Lineweaver–Burk plot is depicted in Figure 3.16. The K_m values were similar to that of a *Sanguibacter* sp. whose K_m was reported to be 6.95 mg ml^{-1} for colloidal chitin (Yong et al., 2005). Many of the reported K_m values for colloidal chitin were lower than that for the *Streptomyces* sp. (Joo et al., 2005; Han et al., 2009; Kudan and Pichyangkura, 2009; Rabeeth et al., 2011; Nagpure and Gupta, 2013; Bhattacharya et al., 2016; Gangwar et al., 2016), suggesting that the affinity of the enzyme for the substrate obtained in this study was different from that of chitinase from other microorganisms. V_{max} values of 6.6 U mg^{-1}, 7.03 U mg^{-1}, 180 U ml^{-1} and 2.6 U ml^{-1} using colloidal chitin as substrate were reported for different *Streptomyces* sp. by Nagpure and Gupta (2013), Kudan and Pichyangkura (2009), Rabeeth et al., (2011) and Gangwar et al., (2016), respectively. Yong et al., (2005) reported a V_{max} value of 10.53 U min^{-1} mg^{-1} for a *Sanguibacter* sp.; Babashpour et al., (2012) reported a V_{max} value of 10.53 U for *Serratia marcescens* and Bhattacharya et al., (2016) reported a V_{max} value of 38.23 U for *Bacillus pumilus* using colloidal chitin as substrate.

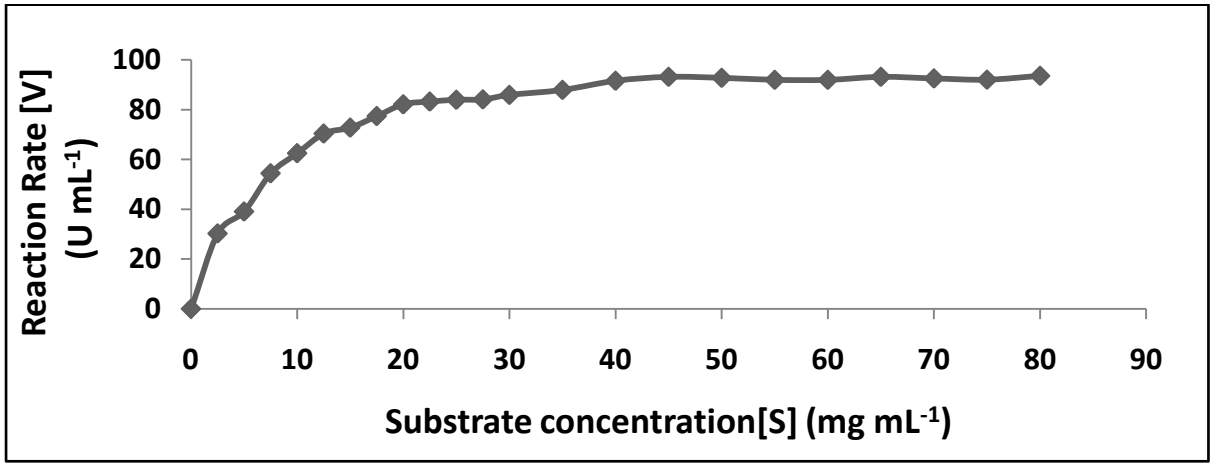

Fig. 3.16. Effect of substrate concentration on chitinase enzyme.

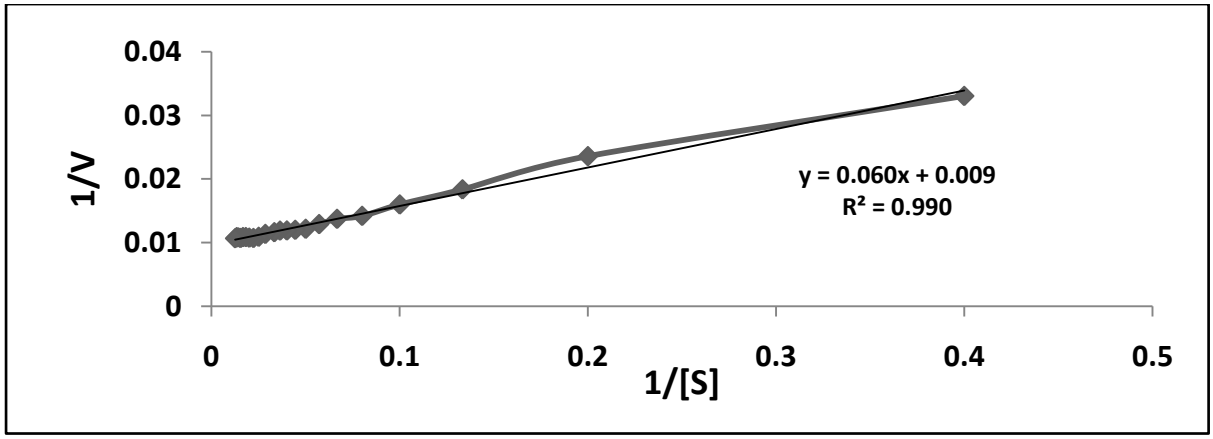

Fig. 3.17. Double reciprocal - Lineweaver–Burk Plot for chitinase enzyme.

3.4. Summary and Conclusions

Purification of *Streptomyces* sp. chitinase enzyme was carried out using methods such as ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography. A 21-fold purification was achieved with 2.24% final yield. Characterization of chitinase enzyme was done with respect to parameters such as effect of pH, temperature, salinity, addition of metal ions, solvents and denaturing agents. The purified enzyme had the optimal temperature and pH as 50°C and 2.0 and 6.0 (dual pH optima), respectively. The purified enzyme had the optimal temperature and pH as 50°C and 6.0 respectively. It retained more than 90% and 50% of its activity at NaCl concentrations of 1.25 % and 4.5% respectively. The activity was enhanced in the presence of divalent cations like Ca^{2+} and Fe^{2+}. Most of the solvents had a notable negative impact on chitinase activity. Ionic surfactants reduced the enzyme activity considerably while the non-ionic surfactants like Tween and Triton had either a neutral or positive effect on enzyme activity. Results of the activity staining experiment showed that only a single protein (enzyme) molecule, which had chitinase activity, was produced. The pI and molecular weight of the chitinase enzyme were determined to be 6.9 and 40 kDa respectively. The ability to function even at extremely low pH conditions combined with a reasonably high optimum temperature, and its stability makes the *Streptomyces* sp. chitinase a lucrative enzyme to be used for industrial processes.

Chapter 4

Isolation of Microbial Culture and Optimisation of Bioprocess Conditions for Chitin Deacetylase Production

4.1. Introduction

Microbial enzymes which act as biocatalysts for a large number of reactions have widespread industrial, pharmaceutical and environmental applications. They are also more active and stable than plant and animal enzymes. In addition, microorganisms represent an alternative source of enzymes because of their ability to be cultured in large quantities in a short time by fermentation. With a view of fulfilling the current enzyme requirements, industries are always in search for new microbial strains. A host of challenges are associated with microbial production of enzymes at the industrial scale. The enzyme production process which can be divided into multiple phases has been previously mentioned in Chapter 2.

The enzyme chitin deacetylase (CDA) hydrolyzes the acetamido group of the NAG units in chitin and chitosan, thus generating glucosamine units and acetic acid. From application point of view, CDA is a low value- high volume based enzyme product, hence it is very important to have an organism that can produce large amounts of enzyme using a cost-effective bioprocess. In order to reduce the currently popular but environment-damaging chemical process of chitin deacetylation involving strong basic conditions at high temperatures, chitin deacetylases are an environmentally friendly substitute for deacetylation of chitin. CDAs in microorganisms have been produced primarily by different fungi but also have been reported in bacteria to a limited extent. Usually the CDA producing microorganisms are isolated from the soils, especially those contaminated by chitinous wastes and those from marine environments.

Microbial fermentation may be carried out either by SmF or SSF. In SmF, the process could be batch fermentation, continuous fermentation or fed-batch fermentation. In SSF, the substrate characteristics play an important role, especially its usability, cost and availability. Also, the solid substrate should not only supply nutrients to the growing microbial culture, but also serve as an efficient anchorage for the cells. The popular substrates are, hence, agro-

industrial residues. The general objectives of the fermentation medium optimization are to maximize the productivity, minimize the by-products and costs, along with maintenance of the product quality.

Microbial CDA production has been predominantly carried out by SmF (Cai et al., 2013; Pareek et al., 2011; Sun et al., 2014; Zhang et al., 2014). SmF has advantages in process control and easy recovery of extracellular enzymes, but the products are dilute as compared to those obtained by SSF. In order to make the enzyme applications more cost-effective at industrial level, their production using low cost substrates under different fermentation systems has been recommended. Use of cheap raw material and the relative easiness of process operations are advantages of SSF, although problems are encountered with substrate sterilization, temperature and pH control, maintenance of culture purity and length of the process. CDA production by SSF is relatively a very less explored area at present and only a few attempts of SSF based production have also been done (Aye et al., 2006; Pareek et al., 2011). In the current study production of CDA using a fungal isolate has been carried out using both SmF and SSF methods. The optimization of the nutritional media requirements as well as other physical factors including aeration, pH, and incubation temperature of the culture has been done using one-factor-at-a-time approach as well as statistical approach.

4.2. Materials and methods

4.2.1. Preparation of colloidal chitin

The colloidal chitin was prepared by the method of Hsu and Lockwood (1975) with minor modifications. Forty grams of flaked chitin was dissolved in 400 ml of concentrated HCl by stirring for 1 h. The chitin was precipitated as a colloidal suspension by adding it slowly to 4-L of water at 4°C. The suspension was repeatedly washed by centrifugation at 11,000 ×g until

the pH of the suspension was about 4.0. The colloidal chitin thus prepared was stored at 4°C for subsequent use.

4.2.2. CDA assay

Extracellular CDA activity was determined using the protocol suggested by Jayamurthy et al. (2014) with some modifications; 0.5 mL of enzyme sample was incubated with 1.0 mL 0.1 M citrate-phosphate buffer, pH 6.5 and 0.5 mL of 1% (w/v) colloidal chitin prepared in the same buffer. The mixture was kept in a water bath at 50°C for 1 h along with appropriate substrate and enzyme blanks. After this, the reaction mixture was heated in a boiling water bath to inactivate the enzyme and centrifuged at 11,000 ×g for 5 min, and the aqueous supernatant was removed into a new tube. The supernatant was then analysed for its acetate content using HPLC (Shimadzu). The conditions maintained in the HPLC were as follows; ROA-Organic acid H+ (Rezex, 300×7.8 mm, Phenomenex®) column, refractive index detector (Shimadzu), 0.01N H_2SO_4 as mobile phase, flow rate at 0.6 mL min^{-1} and temperature maintained at 55°C. A standard graph using different concentrations of acetate was generated using the HPLC method and the equation obtained was used for calculating the unknown concentration of acetate after the chitinase assay. The equation obtained was:

$y = 13690x - 857.6$ ($R^2 = 0.999$)

where y is the DNS assay reading at 540nm and x is the unknown NAG concentration. One unit of the enzyme activity was defined as the amount of enzyme which catalysed the release of 1 µmole mL^{-1} min^{-1} of acetate under the above mentioned assay conditions and was denoted as **U**. In case of SSF, the enzyme activity was defined as **U gds^{-1}** (gram dry substrate).

Enzyme activity in $U = (X*10^6*4)/(60.05*60)$

Where X= concentration of acetate in mg mL^{-1}; 10^6=conversion of moles to micromoles; 4=dilution factor; 60.05= molecular weight of acetic acid; 60= time in minutes

4.2.3. Isolation of cultures

Twenty-six soil samples were collected from different regions along the coast near Kochi, Cherthala, Alappuzha, Kollam and Trivandrum in Kerala state, India (Fig. 4.1). One gram of the each sample was suspended in 100 ml sterile saline individually and incubated on a rotary shaker at 30^{o}C, 200 rpm for 2 h. The samples were then serially diluted and plated on CDA selective agar medium containing (g L^{-1}): $NaNO_3$, 2.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgCl_2.6H_2O$, 0.5; 4-nitroacetanilide, 0.5; agar, 20 and colloidal chitin 1% (w/v), pH 7.2 and incubated at 30°C for four days (Zhou et al., 2010). The microbial colonies that produced CDA were screened by colour reactions of 4-nitroacetanilide. The highest-producing strain, developed from a single colony, which was a fungus, was obtained from the secondary screening using liquid medium of the above mentioned composition at 30°C, 200 rpm for four days, and showing the highest CDA activity. This fungal isolate was named as I8 and was maintained on potato dextrose agar (PDA) slants with periodic sub-culturing for further use.

Fig. 4.1. Places of collection of environmental samples in Kerala for isolation of CDA producing microorganisms (Map not to scale).

4.2.4. Microscopical studies

The vegetative (mycelial) and reproductive (conidial) features of the fungal strain was observed under a clinical light microscope (Leica DM2000, Wetzlar, Germany) and scanning electron microscope (SEM). For SEM analysis, the sample was directly picked from agar medium plate containing mature culture for conidial structures and was fixed in a mixture of 2.5% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde in freshly prepared 0.1 M phosphate buffer for 2 h on clean glass coverslips. After being gently washed with distilled water, fixed samples were dehydrated through a graded ethanol wash (a series of 20-40-60-80 and 100% ethanol for 5 min each), and then the sample was mounted on a stub using double sided carbon tape and dried overnight in a desiccator. The dried samples were sputter coated with gold using SC7620 sputtercoater device and analysed under SEM at an operating voltage of 30 kV (Zeiss Evo-18, Carl Zeiss, Germany).

4.2.5. Isolation of genomic DNA from the fungal culture

Fungal spores (10^6 spores) were inoculated in the above mentioned liquid medium in 250 mL Erlenmeyer flasks and were incubated at 30°C, 200 rpm for 48 h. One gram wet weight of the mycelium was frozen in liquid nitrogen and was ground to a fine powder using mortar and pestle. It was suspended in 5.0 mL of extraction buffer (250 mM NaCl, 50 mM EDTA, 2% (w/v) SDS and 100 mM Tris-HCl, pH 8.0). The suspension was incubated at 60°C for 30 min with intermittent gentle mixing. After centrifugation at 11000 ×g at 4°C for 10 min, the supernatant was transferred to a fresh tube and polysaccharides and proteins were precipitated by adding equal volume of Phenol:Chloroform:Isoamyl alcohol mixture (25:24:1; v/v). The clear aqueous fraction was transferred with a wide bore tipped pipette into a fresh tube. The DNA was precipitated by adding two-thirds the volume of ice-cold iso-propanol to the aqueous fraction. The solution was gently mixed by inversion, placed at -20°C for 1h and the

DNA was recovered by centrifugation at 15,000 ×g for 10 min at 4°C. The DNA precipitate was washed twice with 70% ethanol and was allowed to air dry after which it was suspended in 50 µL 10 mM Tris-EDTA buffer (pH 8.0). RNAse was added to a final concentration of 10 µg mL^{-1} and incubated at 37°C for 30 min. The above mentioned purification procedure was repeated. The purified DNA was stored at -20°C until further use.

4.2.6. **Identification of isolate by ITS sequencing and phylogenetic analysis**

For the identification of fungal isolate I8, the Internal Transcribed Spacer (ITS) region in the genomic DNA was amplified using universal eukaryotic forward (ITS1F- 5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and reverse (ITS4R- 5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') primers (White et al., 1990). The PCR conditions were maintained as an initial 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min; and a final extention step at 72°C for 10 min. This ITS amplicon was further analysed for its sequence data by dye terminator sequencing method (3500 Genetic Analyser, Hitachi, Tokyo, Japan). The identification of the phylogenetic neighbours was initially carried out by the BLAST (Altschul et al., 1997) and megaBLAST (Zhang et al., 2000) programs against the database of type strains with validly published prokaryotic names (Chun et al., 2007). The 20 sequences with the highest scores were then selected for the calculation of pairwise similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org) (Chun et al., 2007). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Evolutionary analyses were conducted using MEGA 5 software (Tamura et al., 2011).

4.2.7. **Optimisation of CDA production (SmF)-Single Parameter Studies**

The initial production medium consisted of (g L^{-1}): glucose, 10.0; yeast extract, 3.0; peptone, 5.0; KH_2PO_4, 3.0; K_2HPO_4, 1.0 and NaCl, 0.5 (pH 6.0) (Pareek et al., 2011). 0.5 ml spore suspension (10^7 spores mL^{-1}) of the fungal strain was inoculated in 50 mL CDA production medium in 250 mL Erlenmeyer flask. It was then incubated at 30°C and 200 rpm shaking with appropriate modifications as demanded by the specific conditions of the experiment. For every 24 h intervals up to 96 h, culture sample was aseptically collected, centrifuged at 11,000 ×g for 10 min at 4°C and the supernatant was used for the CDA assay as mentioned earlier.

4.2.7.1. Effect of inoculum type and incubation time

The culture was inoculated in the production medium and incubated at 30°C up to 96 h in a rotary shaker at 200 rpm. Samples were withdrawn at periodic intervals of 12 h. The experiment was carried out with both types of inoculum namely mycelial (1.0 g wet weight) and spore {0.5 mL spore suspension (10^7 spores mL^{-1})}.

4.2.7.2. Effect of inoculum size

The spore suspension was made from a week old mature PDA slant using saline supplemented with 0.1% (v/v) Tween 80. The production medium was inoculated with 1.0 mL of 10^3, 10^4, 10^5, ... 10^9 spores mL^{-1}. After optimising the spore concentration, the fermentation medium was inoculated with various volumes (0.5, 1.0, 1.5, ... 5 mL) of the optimised spore concentration inoculum.

4.2.7.3. Effect of incubation temperature and initial pH

The effect of temperature on enzyme production was determined by incubating the inoculated medium at different temperatures (26, 28, 30, 32, 34, 37 and 40°C) at 200 rpm. The effect of the initial pH value on the CDA production was investigated by varying the initial pH of the culture medium (using 1N HCl or 1N NaOH) from 3 to 12 with a difference of 1 pH unit.

Flasks were inoculated, incubated and then the contents were centrifuged as specified earlier. CDA assay was performed using the culture supernatants as per the earlier mentioned protocol.

4.2.7.4. Effect of glucose concentration

Glucose was the only carbon source provided for CDA production. To determine the effect of glucose concentration on the induction and production of CDA enzyme, it was added at increasing concentrations of 0, 2, 4, 6, 8, 10, 12 and 14 g L^{-1} to the CDA production medium. Flasks were inoculated, incubated and then contents were centrifuged as above. CDA assay was performed using the culture supernatants as per above mentioned protocol.

4.2.7.5. **Effect of variation of nitrogen source**

The effect of different sources of inorganic and organic nitrogen on CDA production was determined by individually supplementing various nitrogen sources such as urea, ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium citrate, sodium nitrite, sodium nitrate, potassium nitrate, diammonium hydrogen phosphate, dihydrogen ammonium phosphate, peptone, yeast extract, beef extract, casein enzyme hydrolysate and corn steep liquor to the production medium. The nitrogen sources were supplemented such that the final nitrogen content in the medium would be at 2.0 g L^{-1}. A control flask containing no added source of nitrogen was also maintained. Flasks were inoculated, incubated and then contents were centrifuged as above. CDA assay was performed using the culture supernatants as per above mentioned protocol.

4.2.7.6. Effect of addition of metal ions

Influence of various metal ions on CDA production was determined by individually supplementing the medium with different divalent metal cations such as Co^{2+}, Ca^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Sn^{2+} and Zn^{2+} in their chloride form; Fe^{2+}, Ni^{2+} and Cu^{2+} in its sulphate form and Ag^{2+} in its nitrate form. The metal ions were added at final concentrations of 5mM and 10

mM to the production medium. A control flask containing no added metal ions was also maintained. Flasks were inoculated, incubated and then contents were centrifuged as above. CDA assay was performed using the culture supernatants as per above mentioned protocol.

4.2.7.7. Effect of addition of surfactants

For studying the effect of addition of surfactants on the production of CDA, the production medium was supplemented with 0.2% and 0.5% (w/v) of SDS, CTAB; 0.2% and 0.5% (v/v) of Triton X-100, Tween 20, Tween 80, Tween 60 and Tween 80. A control flask containing no added surfactants was also maintained. Flasks were inoculated, incubated and then contents were centrifuged as above. CDA assay was performed using the culture supernatants as per above mentioned protocol.

4.2.8. Optimisation of CDA production (SmF)-Statistical Optimisation

The statistical software package Minitab 17.1.0, Minitab Inc., Pennsylvania, USA was used to create suitable experimental designs and analyze the results of the experiments.

4.2.8.1. Screening of parameters affecting CDA production by Plackett-Burman statistical design

Identification of essential medium constituents to improve CDA production by the *Streptomyces* sp. was carried out using Plackett–Burman design (Plackett and Burman, 1946). The important nutritional parameters affecting the enzyme production were screened in a design with six variables at two levels (Table 4.1) in a total of 28 experimental runs as shown in Table 4.2. The parameters tested were: concentrations of glucose, yeast extract, peptone, $MgCl_2$, $CuSO_4$ and $ZnCl_2$ in the production medium. The variables were represented at two levels: a higher level designated as +1 and a lower level designated as -1. The number of positive and negative signs per trial was $(k + 1)/2$ and $(k - 2)/2$, respectively.

Table 4.1. Range of experimental values for the Plackett-Burman design for CDA production.

Code	Parameter Name	Low level (-1)	High level (+1)
X_1	**Glucose**	20 g L^{-1}	30 g L^{-1}
X_2	**Yeast Extract**	10 g L^{-1}	20 g L^{-1}
X3	**Peptone**	10 g L^{-1}	20 g L^{-1}
X4	**$MgCl_2$**	1.0 g L^{-1}	2.0 g L^{-1}
X5	**$CuSO_4$**	1.25 g L^{-1}	2.5 g L^{-1}
X6	**$ZnCl_2$**	0.68 g L^{-1}	1.36 g L^{-1}

Table 4.2. Plackett Burman experimental design matrix for CDA production.

RunOrder	X_1	X_2	X_3	X_4	X_5	X_6	CDA (U)
1	1	1	-1	-1	-1	1	43.74
2	-1	1	1	1	-1	1	41.94
3	1	1	1	-1	-1	-1	37.91
4	1	-1	1	1	-1	1	36.60
5	-1	-1	-1	-1	-1	-1	21.40
6	1	1	1	1	1	1	44.11
7	-1	1	1	-1	1	1	31.28
8	1	1	1	-1	1	-1	36.77
9	-1	-1	1	-1	1	-1	36.33
10	-1	-1	-1	-1	1	1	21.74
11	-1	1	1	1	1	1	39.77
12	1	1	-1	1	-1	-1	42.28
13	-1	1	-1	-1	1	-1	35.35
14	-1	1	-1	1	-1	-1	34.61
15	-1	-1	-1	1	-1	-1	24.58
16	1	-1	1	1	1	1	44.73
17	1	1	-1	1	1	1	45.22
18	-1	-1	1	-1	-1	1	24.01
19	-1	-1	1	1	-1	-1	36.37
20	1	-1	-1	-1	1	-1	40.03
21	-1	-1	-1	1	1	-1	22.77
22	-1	-1	-1	1	-1	1	28.12
23	1	-1	1	1	1	-1	36.76
24	1	-1	-1	-1	-1	1	21.04
25	-1	1	-1	-1	1	1	22.74
26	1	1	-1	1	-1	1	44.11
27	1	-1	1	-1	1	-1	34.69
28	1	1	1	-1	-1	-1	45.31

Each row represented a trial, and each column represented an independent (assigned) variable. The effect of each variable was determined by the following equation:

$$\mathbf{E(X_i)= [\Sigma(M_i+)-\Sigma(M_i-)]/n}$$

where, $E(X_i)$ was the concentration effect of the tested variable, M_i+ and M_i- represented CDA production from the higher and lower levels respectively, for each parameter, and n was

the total number of trials. All experiments were conducted in duplicates and the mean value of CDA activity was taken as the response. CDA assay was performed as per the protocol mentioned earlier. The analysis of variance (ANOVA) was performed on the data to determine the significance of the fitted model and to test the significance of the effect of individual parameters on the CDA production. The most significant parameters affecting the CDA production were identified.

4.2.8.2. Optimization of significant parameters affecting CDA production by Response Surface Methodology (RSM)

RSM-Central composite design (CCD) was employed in the present study to optimize the concentration of the significant parameters identified by the Plackett-Burman design for further fine tuning of CDA production, which included glucose, yeast extract, peptone and $MgCl_2$. Each factor in the design was studied at five levels (-2, -1, 0, +1, +2) (Table 4.3) in a set of 30 experiments that included 8 axial points, 16 factorial points, and 6 centre points (Table 4.4). All experiments were conducted in duplicates and the mean value of CDA activity was taken as the response. CDA assay was performed as per the protocol mentioned earlier. A second-order polynomial equation with interaction terms was then fitted to the data by multiple regression analyses. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

Table 4.3. Range of experimental values for the central composite design for CDA production.

Code	Parameter Name	Levels (Values in g L^{-1})				
		-2	**-1**	**0**	**+1**	**+2**
X_1	**Glucose**	25	30	35	40	45
X_2	**Yeast Extract**	25	30	35	40	45
X3	**Peptone**	10	15	20	25	30
X4	**$MgCl_2$**	1	2	3	4	5

Table 4.4. Experimental design matrix for CDA production using CCD.

Run No.	X_1	X_2	X_3	X_4	CDA (U)
1	0	-2	0	0	40.57
2	-2	0	0	0	42.15
3	0	0	0	0	50.12
4	0	0	0	0	49.13
5	0	0	0	-2	46.70
6	0	0	2	0	39.95
7	0	0	0	2	54.29
8	0	2	0	0	45.63
9	0	0	-2	0	52.53
10	2	0	0	0	36.87
11	1	1	1	1	45.94
12	-1	1	1	1	52.84
13	-1	1	-1	-1	47.14
14	-1	-1	-1	1	47.02
15	-1	-1	1	-1	40.05
16	1	-1	-1	-1	48.19
17	-1	-1	-1	-1	42.07
18	-1	-1	1	1	40.30
19	1	-1	1	1	37.24
20	1	1	-1	-1	42.94
21	1	-1	-1	1	38.20
22	0	0	0	0	49.80
23	1	-1	1	-1	40.72
24	0	0	0	0	52.26
25	-1	1	1	-1	41.39
26	0	0	0	0	49.48
27	-1	1	-1	1	50.69
28	1	1	-1	1	44.93
29	0	0	0	0	51.08
30	1	1	1	-1	40.41

The optimum levels of media components including glucose, yeast extract, peptone and $MgCl_2$ to achieve maximum CDA production were obtained by solving the regression equation and also by analyzing the response surface contour plots. Validation of the model was performed under the conditions predicted by the model in triplicates.

4.2.9. Production of CDA by Solid State Fermentation (SSF)

Wheat bran (WB) was used as substrate for SSF. It was purchased from a local market in Thiruvananthapuram, washed twice using hot water, and the water was removed by

squeezing through a muslin cloth. Then it was dried in a hot air oven maintained at 60°C for 24 h. This was stored in air-tight boxes for further use. Five gram dry weight of this WB was weighed into 250 mL Erlenmeyer flasks, and autoclaved at 121°C, 15 psi for 15 min; and dried again overnight in a hot air oven maintained at 60°C to constant moisture content. It was then moistened aseptically with 7 mL (to attain 60% initial moisture content) of sterile liquid medium containing 15 g L^{-1} glucose, 20 g L^{-1} yeast extract, 10 g L^{-1} meat extract and 5 g L^{-1} $MgCl_2$ at initial pH adjusted to 7. Each flask was inoculated with 1.0 mL of spore inoculum containing 10^7 spores mL^{-1}. The contents of the flask were thoroughly mixed and were incubated at 32°C under conditions of controlled humidity. Incubation was carried upto 144h with periodic harvesting of flasks for sampling of CDA production. At the end of the incubation, the enzyme was recovered by extraction with 50 mL of citrate-phosphate buffer. After adding the buffer to each flask, they were kept on a rotary shaker at 200 rpm for 60 min, after which the entire slurry was recovered and filtered using a stainless steel mesh. The filtered solution was then centrifuged at 9000 ×g at 4°C for 10 min and the supernatant was used as the crude enzyme preparation.

An experiment was carried out to determine the effect of incubation time and effect of washing of the WB on CDA production. Rest of the procedure was followed as above.

4.2.10. Statistical Optimisation for CDA Production (SSF)

The statistical software package Minitab 17.1.0, Minitab Inc., Pennsylvania, USA was used to create suitable experimental designs and analyze the results of the experiments.

4.2.10.1. Screening of parameters affecting CDA production by Plackett-Burman statistical design

Identification of essential medium constituents to improve CDA production in SSF by the *Aspergillus flavus* strain was carried out using Plackett–Burman design (Plackett and

Burman, 1946). The parameters tested were: substrate mass, initial moisture content, spore inoculum size, concentrations of glucose, yeast extract, meat extract, $MgCl_2$ in the SSF production medium. They were screened in a design as seven variables at two levels: a higher level designated as +1 and a lower level designated as -1. The actual and coded values tested for each parameter are given in Table 4.5. A total of 28 experimental runs were carried out as shown in Table 4.6. The number of positive and negative signs per trial was $(k + 1)/2$ and $(k - 2)/2$, respectively. Each row represented a trial, and each column represented an independent (assigned) variable. The effect of each variable was determined by the following equation:

$$\mathbf{E(X_i)= [\Sigma(M_i+)-\Sigma(M_i-)]/n}$$

where, $E(X_i)$ was the concentration effect of the tested variable, M_i+ and M_i- represent CDA production from the higher and lower levels respectively for each parameter, and n is the total number of trials.

Table 4.5. Range of experimental values for the Plackett Burman design for CDA production by SSF.

Code	Parameter Name	Low level (-1)	High level (+1)
$\mathbf{X_1}$	**Substrate Mass**	5g	7g
$\mathbf{X_2}$	**Initial moisture content**	60 %	70 %
$\mathbf{X_3}$	**Inoculum size (spores)**	10^6	10^7
$\mathbf{X_4}$	**Glucose**	20 g L^{-1}	30 g L^{-1}
$\mathbf{X_5}$	**Yeast extract**	20 g L^{-1}	30 g L^{-1}
$\mathbf{X_6}$	**Meat extract**	20 g L^{-1}	30 g L^{-1}
$\mathbf{X_7}$	**$MgCl_2$**	4 g L^{-1}	6 g L^{-1}

Table 4.6. Plackett Burman experimental design matrix for CDA production by SSF.

RunOrder	X_1	X_2	X_3	X_4	X_5	X_6	X_7	CDA (U)
1	1	1	1	-1	-1	-1	1	60.07
2	-1	-1	-1	1	1	-1	1	89.69
3	1	1	-1	1	1	-1	-1	59.92
4	-1	1	1	1	1	-1	1	78.37
5	-1	-1	-1	-1	-1	-1	-1	68.44
6	-1	-1	1	-1	-1	1	-1	87.73
7	-1	-1	-1	-1	1	1	1	90.30
8	-1	-1	-1	1	-1	1	1	101.90
9	1	1	-1	-1	1	1	1	67.78
10	1	-1	1	1	1	-1	1	80.65
11	-1	1	1	-1	1	1	1	96.96
12	-1	1	-1	1	-1	-1	-1	85.55
13	-1	-1	1	1	-1	-1	1	92.17
14	1	-1	-1	-1	-1	1	1	83.40
15	1	-1	1	1	-1	1	1	97.07
16	1	1	1	-1	-1	-1	-1	53.85
17	1	-1	1	-1	1	1	-1	80.11
18	1	1	-1	1	1	1	-1	85.75
19	-1	1	-1	-1	-1	1	-1	90.52
20	-1	1	1	1	-1	1	-1	90.21
21	1	-1	-1	1	-1	-1	-1	74.25
22	-1	1	1	1	1	1	-1	105.04
23	-1	1	-1	-1	1	-1	1	73.26
24	1	-1	-1	-1	1	-1	-1	70.32
25	1	-1	1	1	1	1	-1	91.97
26	1	1	1	-1	-1	-1	1	52.04
27	1	1	-1	1	-1	1	1	82.40
28	-1	-1	1	-1	1	-1	-1	87.25

All experiments were conducted in duplicates, and the mean value of CDA activity was taken as the response. CDA assay was performed as per the protocol mentioned earlier. The analysis of variance (ANOVA) was performed on the data to determine the significance of the fitted model and to test the significance of the effect of individual parameters on the CDA production. The most significant parameters affecting the CDA production were identified.

4.2.10.2. Optimization of significant parameters affecting CDA production in SSF by Response Surface Methodology (RSM)

RSM-Central composite design (CCD) was employed in the present investigation to optimize the concentration of the significant parameters identified by the Plackett-Burman design for

further fine tuning of CDA production. These included substrate mass, initial moisture content, glucose and meat extract for enhanced CDA production. Each factor in the design was studied at five levels (-2, -1, 0, +1, +2) (Table 4.7) in a set of 30 experiments that including 8 axial points, 16 factorial points and 6 centre points (Table 4.8).

Table 4.7. Range of experimental values for the central composite design for SSF.

Code	Parameter Name	Levels (Values in g L^{-1})				
		-2	**-1**	**0**	**+1**	**+2**
X_1	**Substrate Mass**	4.0	4.5	5.0	5.5	6.0
X_2	**Initial Moisture Content (%)**	45	50	55	60	65
X_3	**Glucose**	25	30	35	40	45
X_4	**Meat Extract**	25	30	35	40	45

Table 4.8. Experimental design matrix for CDA production by SSF using CCD.

Run No.	X_1	X_2	X_3	X_4	CDA (U)
1	0	0	0	0	99.49
2	0	0	-2	0	71.96
3	-2	0	0	0	91.30
4	0	0	2	0	112.41
5	0	0	0	-2	85.66
6	2	0	0	0	71.15
7	0	0	0	0	101.07
8	0	2	0	0	81.46
9	0	-2	0	0	84.06
10	0	0	0	2	129.08
11	1	1	-1	1	80.91
12	-1	1	1	-1	65.05
13	1	-1	-1	1	79.62
14	-1	-1	-1	1	81.74
15	1	1	1	-1	60.58
16	-1	1	1	1	64.37
17	1	-1	-1	-1	45.62
18	0	0	0	0	98.01
19	1	1	1	1	72.44
20	-1	-1	-1	-1	45.49
21	1	-1	1	1	110.93
22	1	-1	1	-1	56.89
23	-1	1	-1	-1	53.69
24	0	0	0	0	95.72
25	-1	1	-1	1	63.85
26	-1	-1	1	1	108.81
27	0	0	0	0	96.33
28	-1	-1	1	-1	73.90
29	0	0	0	0	99.63
30	1	1	-1	-1	49.34

All experiments were conducted in duplicates, and the mean value of CDA activity was taken as the response. CDA assay was performed as per the protocol mentioned earlier. A second-order polynomial equation with interaction terms was then fitted to the data by multiple regression analyses. This resulted in an empirical model that related the response measured to the independent variables of the experiment. The optimum levels of media components including substrate mass, initial moisture content, glucose and meat extract to achieve maximum CDA production were obtained by solving the regression equation and also by analyzing the response surface contour plots. Validation of the model was performed under the conditions predicted by the model in triplicates.

4.3. Results and discussion

4.3.1. Location, sampling and isolation of cultures

A total of 10 fungal strains (I1, I2, I3,... I9, I10) were isolated from the soil samples collected from different regions along the coast in Kerala state, India based on the yellow zone developed in the 4-nitroacetanilide containing agar medium (Fig. 4.2). 4-nitroacetanilide was deacetylated to give rise to yellow coloured product 4-nitroaniline.

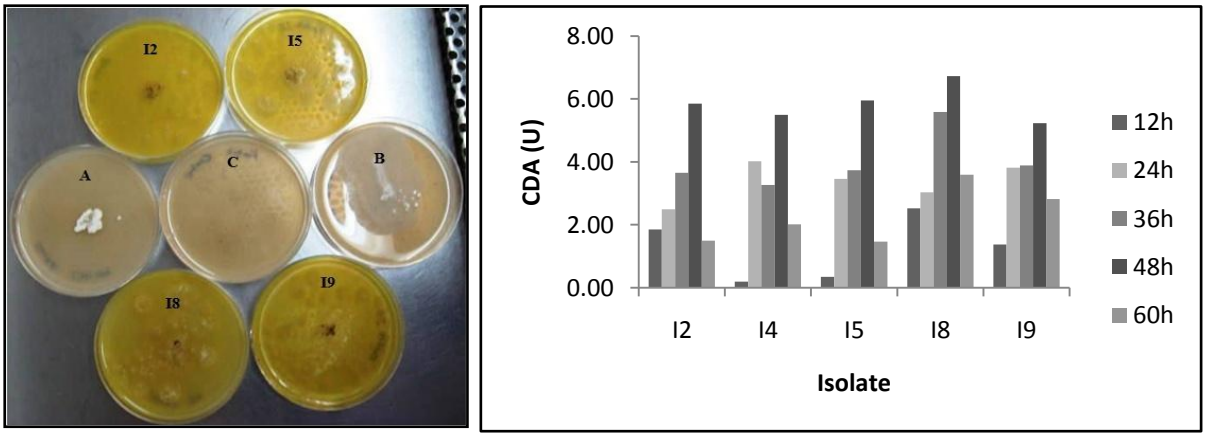

Fig. 4.2. Screening of isolates on 4-nitroacetanilide containing agar medium; A, B: Negative controls; C: control plate; I2, I5, I8, I9: isolates positive for CDA production. Bar-graph showing CDA production by positive isolates in liquid production medium.

The highest-producing strain was identified as a fungal isolate named I8. Phenotypically the strain produced velvet textured, floccose, olive coloured colonies with a white border with a creamy coloured underside on PDA and Sabouraud dextrose agar plates (Fig. 4.3).

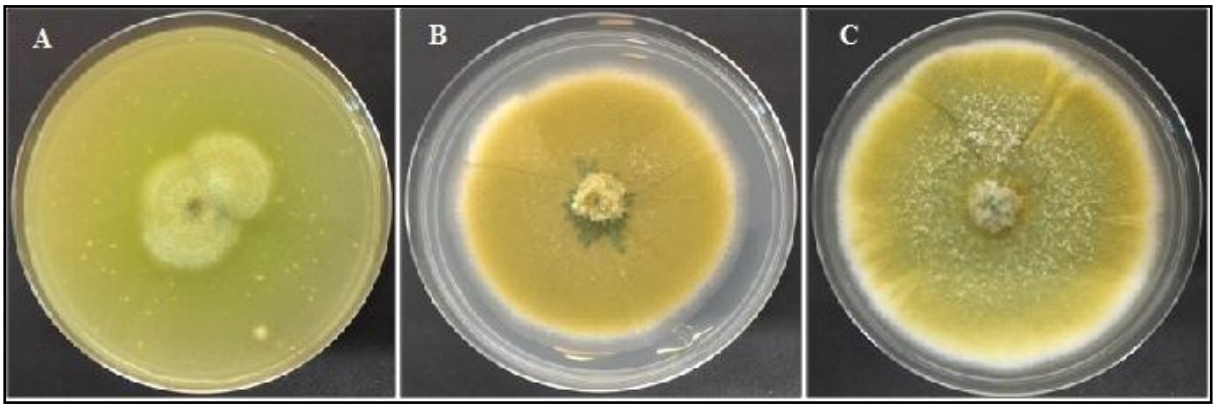

Fig. 4.3. Growth of fungal isolate on **A.** 4-nitroacetanilide containing agar medium; **B.** Potato dextrose agar; **C.** Sabouraud dextrose agar.

4.3.2. Microscopical studies

Mycelial and conidial structures were observed under a light microscope using cotton blue staining technique and also by SEM imaging (Fig. 4.4).

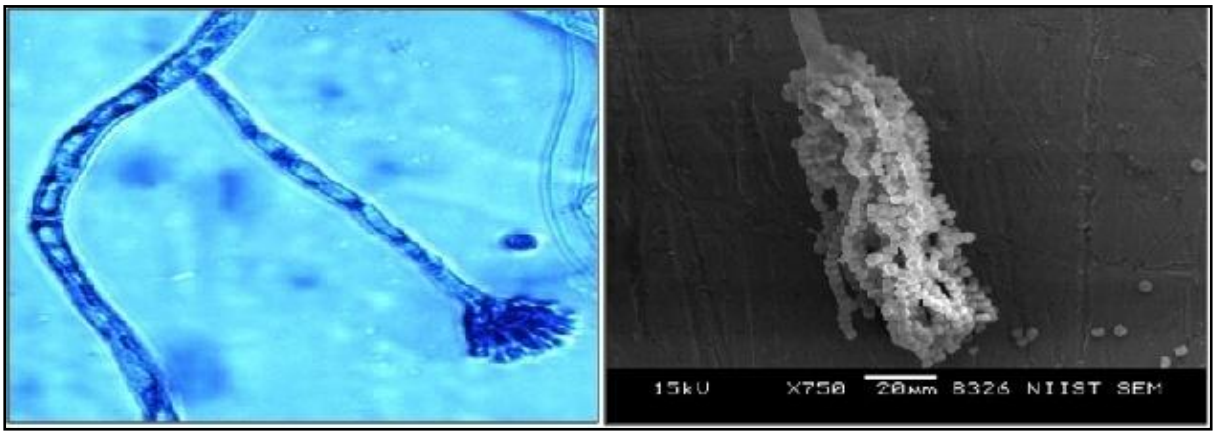

Fig. 4.4. Microscopical studies of *Aspergillus flavus*: Lactophenol cotton blue stained microscopy mount (40X) (left) and SEM image of conidiophore bearing conidia (right).

4.3.3. Identification of isolate by phylogenetic analysis

The isolate I8 was identified as an *Aspergillus flavus* by ITS DNA analysis using NCBI-BLAST and the MEGA6 software. Figure 4.5 shows the gel picture showing PCR amplified ITS product run on an agarose gel. Figure 4.6 displays the phylogenetic tree generated based on ITS sequences of the fungal strains, which were similar to the present isolate as obtained from the BLAST results. The *Penicillium chrysogenum* strain (ATCC10106) was used as the out-group.

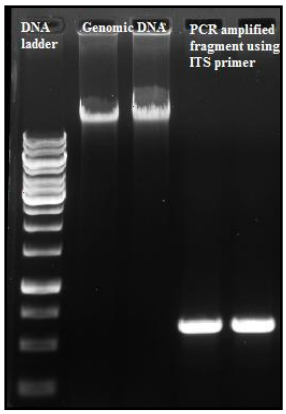

Fig 4.5. Gel picture showing PCR amplified ITS product of ~500 bp.

Sequencing Result for ITS region

TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCA
CCCGTGTTTACTGTACCTTAGTTGCTTGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCC
CCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTA
TCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA
ATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCC
CCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTT
GGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCT
CGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATC
TTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAA

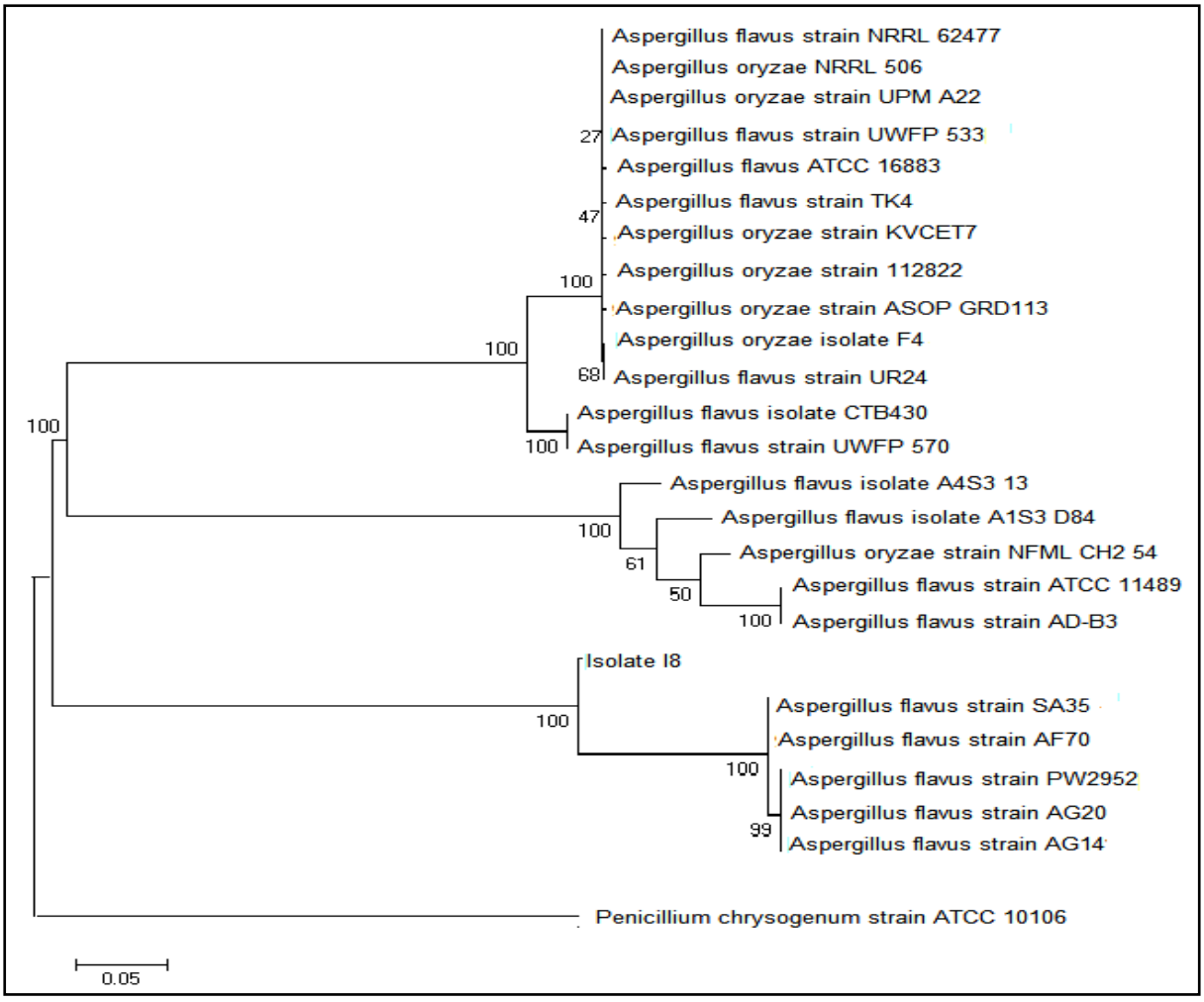

Fig. 4.6. Phylogenetic tree with species level identification of *Aspergillus flavus* isolate.

4.3.4. Optimisation of CDA production (SmF)-Single Parameter Studies

4.3.4.1. Effect of incubation time

The study of effect of incubation time on CDA production by *Aspergillus flavus* showed that the highest level of CDA was produced at 48h of incubation as observed in Figure 4.7. After this, the level of CDA in the fermentation broth decreased significantly with time. This could be either due to the depletion of nutrients in the fermentation medium or production of inhibitory products in the medium, resulting in the inactivation of secretary machinery of the enzymes; or due to the reason that the CDA enzyme plays a significant role only during the growth phase of the fungus. A significant increase in the CDA production was observed only after 24h of incubation. *Absida corymbifera* (Zhao et al., 2010), *Penicillium oxalicum* (Pareek et al., 2011), *Rhizopus oryzae* (Aye et al., 2006) and *Scopulariopsis brevicaulis* (Cai et al., 2013) were reported to produce maximum CDA after 3, 4, 5 and 6 days of incubation, respectively.

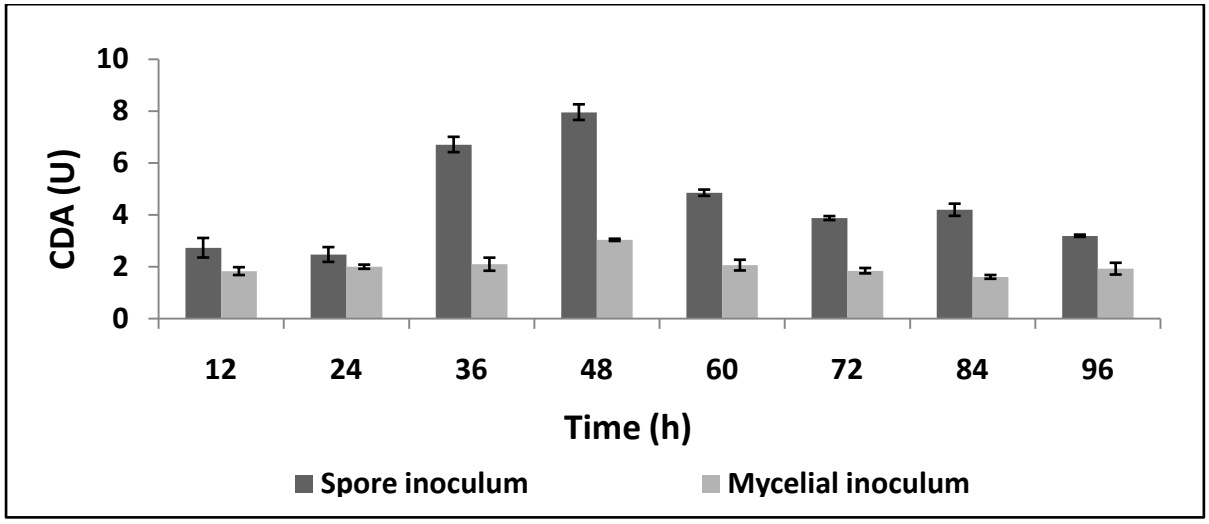

Fig. 4.7. Effect of inoculum type and incubation time on CDA production.

4.3.4.2. Effect of inoculum size

The size of the inoculum determines total biomass production during fermentation. An increase in the spore concentration in the inoculum ensures a rapid proliferation and biomass

synthesis. But after a certain limit with increased spore concentration, the competition for the nutrients results in the decreased growth and metabolic activity, which results in the decreased production of CDA. With optimum inoculum size for the enzyme production, there is a balance between proliferating biomass and availability of nutrients that supports the production of the enzyme. The inoculum size of 2.0 mL of 10^7 spores mL^{-1} resulted in the highest production of CDA by *Aspergillus flavus*. An inoculum size of 10^6 spores mL^{-1} and 3.6×10^6 spores mL^{-1} was used for production of CDA by *Mortierella* sp. (Kim et al., 2008) and *Penicillium oxalicum* (Pareek et al., 2011), respectively.

4.3.4.3. Effect of incubation temperature

To evaluate the optimum growth temperature for CDA production, the *Aspergillus flavus* strain was grown at 25–40°C. CDA production was maximum at 32°C. The enzyme production progressively decreased at temperatures above and below 32°C (Fig. 4.8). Highest CDA production was observed at incubation temperatures of 28°C for *Mortierella* sp. (Kim et al., 2008) and *Absida corymbifera* (Zhao et al., 2010), 29°C for *Scopulariopsis brevicaulis* (Cai et al., 2013), 30°C for *Rhizopus oryzae* (Aye et al., 2006) and *Penicillium oxalicum* (Pareek et al., 2011); and 37°C for *Bacillus amyloliquefaciens* (Zhou et al., 2010).

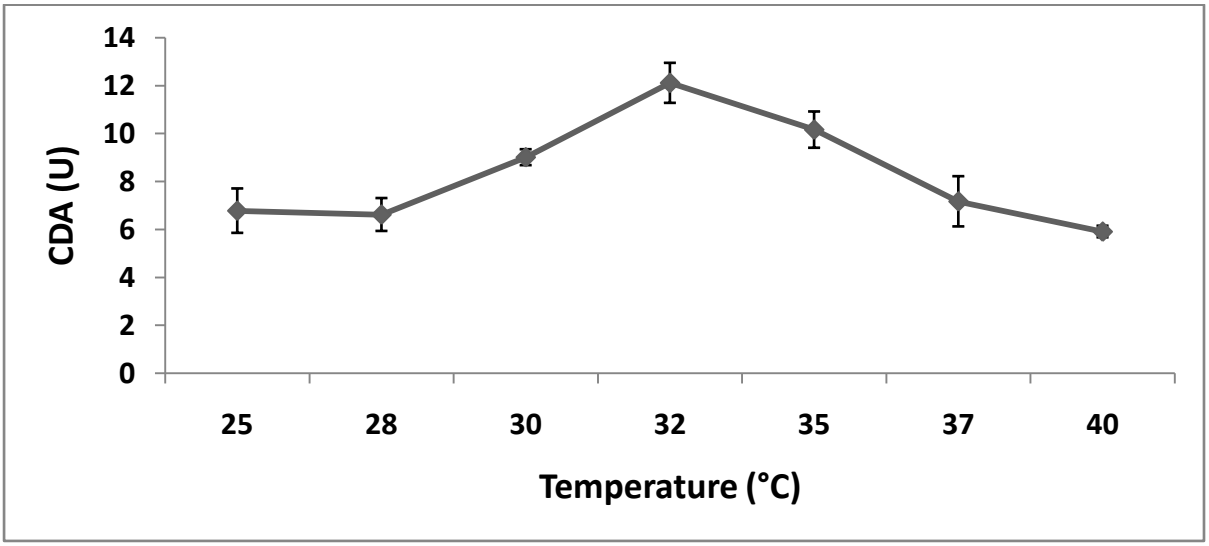

Fig. 4.8. Effect of incubation temperature on CDA production.

4.3.4.4. Effect of initial pH of production medium

Among the tested pH from 3.0 to 11.0, maximum CDA production by the *Aspergillus flavus* strain was observed at an initial pH 7.0. The level of enzyme production decreases rapidly at pH values above and below pH 7.0 (Fig. 4.9). Initial pH values of 5.0, 6.0, 7.0, 8.0 and 9.0 were optimum for CDA production by *Mortierella* sp. (Kim et al., 2008), *Bacillus amyloliquefaciens* (Zhou et al., 2010), *Scopulariopsis brevicaulis* (Cai et al., 2013), *Penicillium oxalicum* (Pareek et al., 2011) and *Absida corymbifera* (Zhao et al., 2010), respectively.

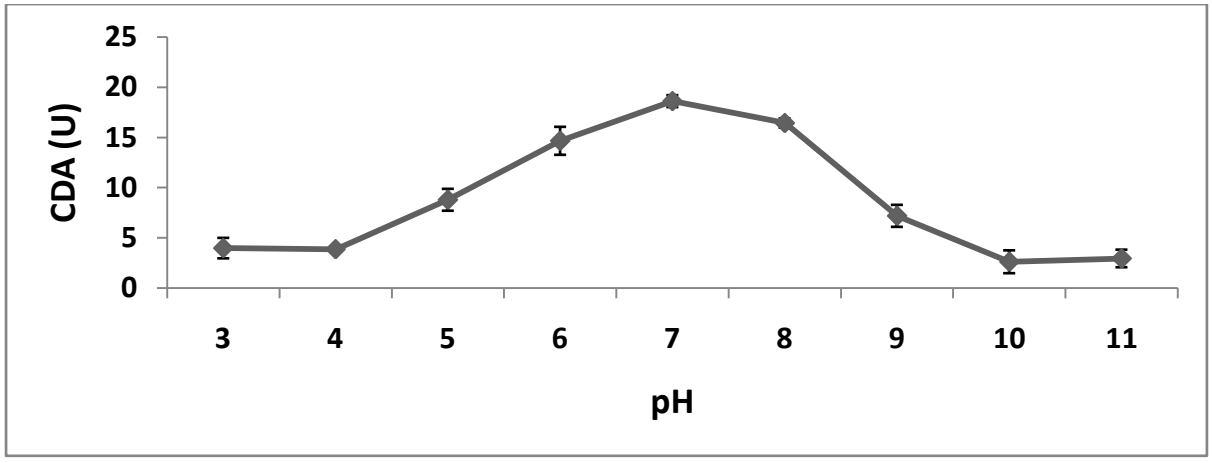

Fig. 4.9. Effect of initial pH of production medium on CDA production.

4.3.4.5. Effect of glucose concentration

Highest CDA production was observed at a glucose concentration of 10 g L^{-1}. The level of CDA production decreased gradually at glucose concentrations greater and lesser than 10 g L^{-1}.

4.3.4.6. Effect of variation of nitrogen source

Among the various nitrogen sources supplemented in the production medium individually, the organic nitrogen sources were comparatively more effective in inducing the CDA production than the inorganic nitrogen sources as observed in Figure 4.10. Yeast extract and

peptone were the most effective of the organic nitrogen sources while among the inorganic nitrogen sources sodium nitrate was the best for production of CDA by the *Aspergillus flavus* strain. However, potassium nitrate and dihydrogen ammonium phosphate entirely suppressed the CDA production. From earlier reports also, it was evident that organic nitrogen sources were more effective in inducing CDA production as compared to inorganic nitrogen sources. Beef extract, yeast extract and peptone were best for CDA production by *Bacillus amyloliquefaciens* (Zhou et al., 2010), *Paenibacillus* sp. (Dai et al., 2011) and *Scopulariopsis brevicaulis* (Cai et al., 2013). Among inorganic nitrogen sources, ammonium sulphate was best at inducing CDA production, albeit lower than organic nitrogen sources (Zhou et al., 2010; Dai et al., 2011).

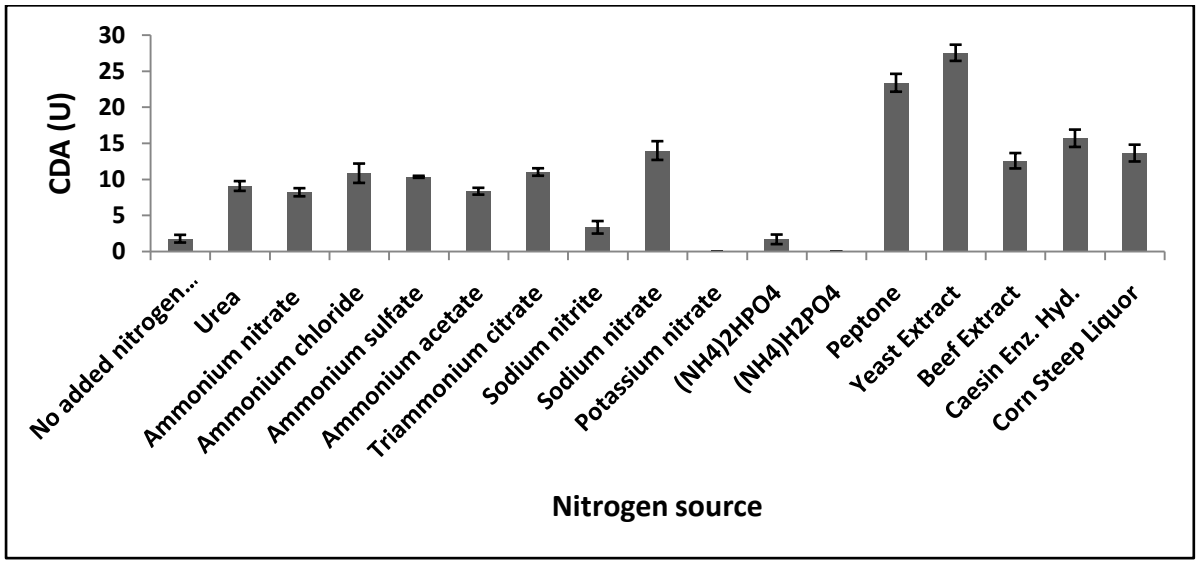

Fig. 4.10. Effect of nitrogen source variation on CDA production.

4.3.4.7. Effect of addition of metal ions

The results showed that CDA production was enhanced by the addition of Mg^{2+} in the production medium of *Aspergillus flavus* strain as given in Figure 4.11. When the concentration of Mg^{2+} was increased from 5.0 mM to 10.0 mM, there was increased production of CDA, almost double as compared to that of the control. Total inhibition of CDA production was observed when Ag^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+} and Ni^{2+} were added to the

medium even at 5.0 mM concentration. As reported by Cai *et al.* (2013), Mn^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+} enhanced the production of CDA, while Fe^{2+} and Cu^{2+} inhibited the production of CDA by *Scopulariopsis brevicaulis*. CDA production was increased by the addition of Ca^{2+} while was decreased by the addition of Cu^{2+} by *Paenibacillus* sp. (Dai et al., 2011).

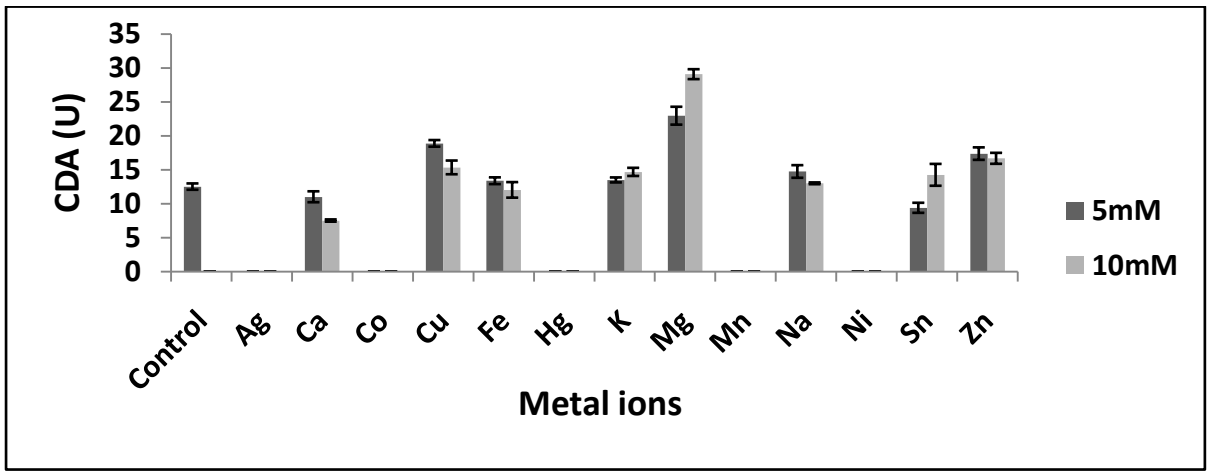

Fig. 4.11. Effect of metal ion addition on CDA production.

4.3.4.8. Effect of addition of surfactants

Non-ionic surfactants such as Tween 40, Tween 60 and Tween 80 increased CDA production while Tween 20 and Triton X-100 reduced the CDA production to one-third in comparison to that of the control as shown in Figure 4.12. The addition of ionic surfactants such as SDS and CTAB to the production medium entirely inhibited the growth of the *Aspergillus flavus* strain, and thus, the production of CDA.

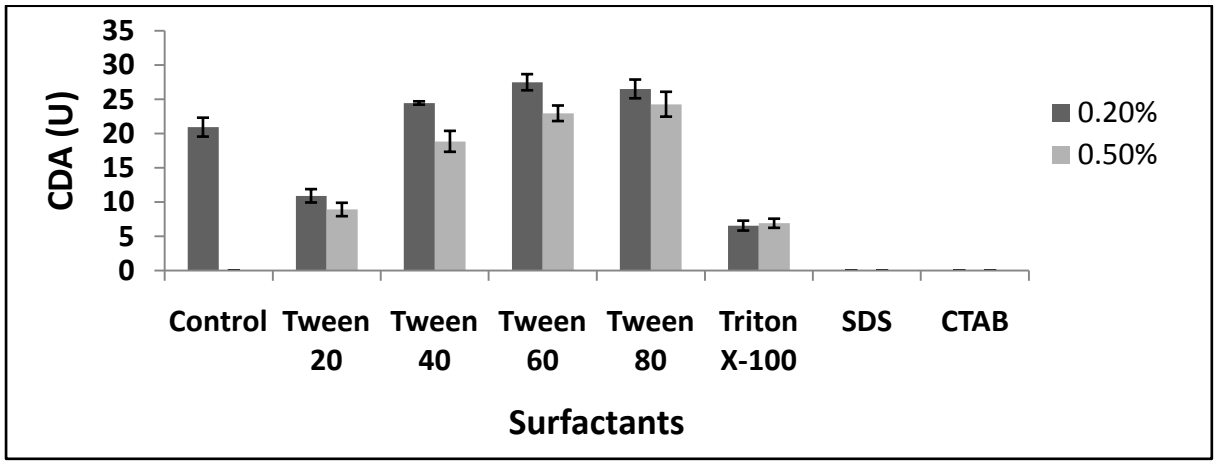

Fig. 4.12. Effect of surfactant addition on CDA production.

4.3.5. Optimisation of CDA production (SmF)-Statistical Optimisation

4.3.5.1. Screening of parameters affecting CDA production by Plackett-Burman statistical design

The concentration of the media components was optimised using statistical methods. Plackett-Burman screening experimental design was employed to determine the combined influence of media components on the production of CDA by *Aspergillus flavus*. The components were screened at the confidence level of 95% on the basis of their effects (either positive or negative). If the component shows at or above 95% confidence level and its effect is positive, it indicates that the component is effective in CDA production. But the amount required is higher than the stated high concentration in Plackett–Burman experiment. This was observed from the main effects plot (Fig. 4.13). As observed in Figure 4.14, the Pareto chart illustrated the order of significance of the variables affecting the CDA production. Here, the factors that showed significant positive effect on CDA production were glucose, yeast extract, $MgCl_2$ and peptone as in the order of significance indicated by Pareto chart. The effects of $CuSO_4$ and $ZnCl_2$ were not significant. The regression equation obtained from the PBD for CDA production was as follows.

$$CDA\ (U) = 34.797 + 3.882\ X_1 + 3.696\ X_2 + 2.200\ X_3 + 2.658\ X_4 + 0.432\ X_5 - 0.765\ X_6 \quad \text{(Eq. 1)}$$

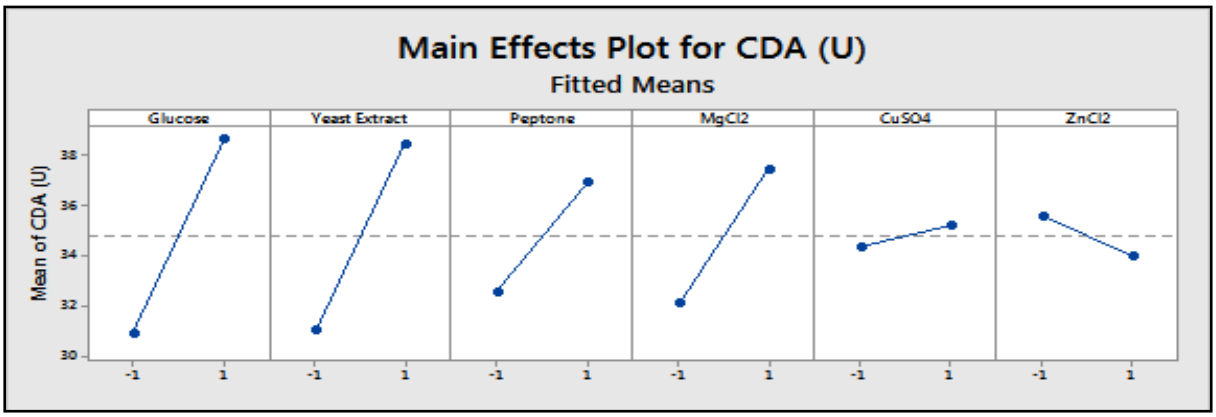

Fig. 4.13. Main effects plot showing effect of high and low level concentrations of factors influencing CDA production obtained using Packett-Burman design.

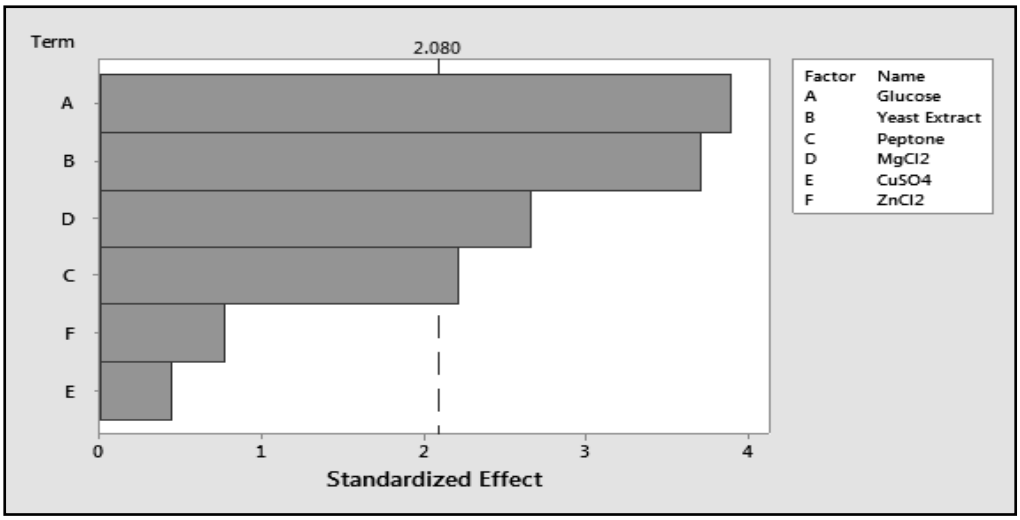

Fig. 4.14. Pareto plot showing significance of factors influencing CDA production obtained using Packett-Burman design.

4.3.5.2. Optimization of significant parameters affecting CDA production by Response Surface Methodology (RSM)

The four significant factors selected based on the results of PBD namely glucose, yeast extract, peptone and $MgCl_2$ were optimized using CCD. The results generated from the thirty experimental runs were analyzed by standard analysis of variance (ANOVA) as indicated in Table 4.9. Figure 4.15 depicts the contour plots of CDA activity showing interactions between different variables in the CCD.

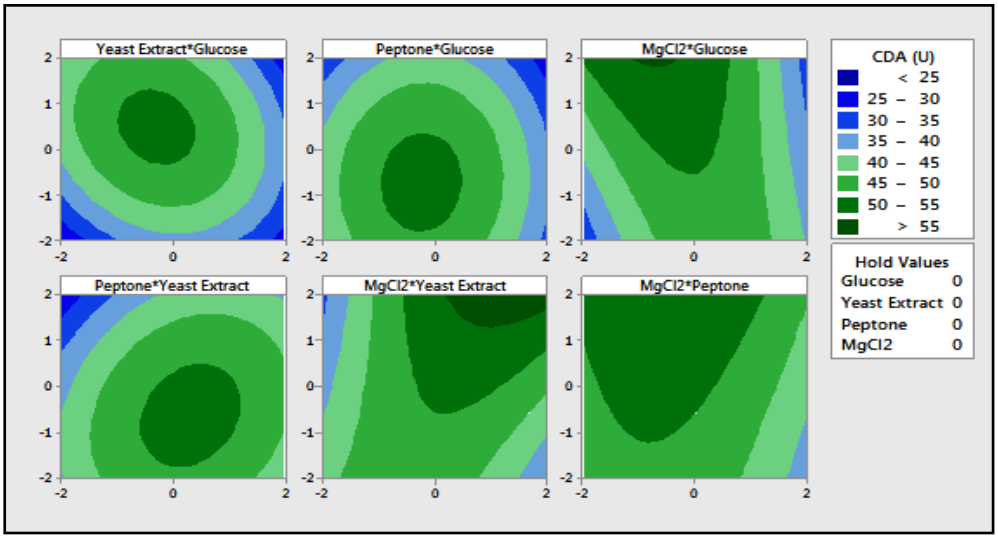

Fig. 4.15. Contour plots showing optimal concentrations and interactions between different variables in the CCD.

The quadratic regression equation obtained was as follows:

$$CDA\ (U) = 50.79 - 1.275\ X_1 + 1.896\ X_2 - 1.977\ X_3 + 1.477\ X_4 - 3.093\ X_1^2 + 2.195\ X_2^2 - 1.411\ X_3^2 + 0.029\ X_4^2 - 0.976\ X_1X_2 + 0.150\ X_1X_3 - 1.634\ X_1X_4 + 0.753\ X_2X_3 + 1.926\ X_2X_4 + 0.289\ X_3X_4 \quad (Eq.\ 2)$$

Table 4.9. Regression analysis and ANOVA for CCD for CDA production.

Source			DF	Sum of Squares	Mean Square	F-Value	P-Value
Model			16	755.173	47.198	7.950	0.000
	Blocks		2	8.861	4.431	0.750	0.493
	Linear		4	270.570	67.642	11.400	0.000
		X_1	1	34.699	34.699	5.850	0.031
		X_2	1	76.681	76.681	12.920	0.003
		X_3	1	93.810	93.810	15.810	0.002
		X_4	1	52.381	52.381	8.830	0.011
	Square		4	299.547	74.887	12.620	0.000
		X_1^2	1	214.519	214.519	36.150	0.000
		X_2^2	1	108.034	108.034	18.210	0.001
		X_3^2	1	44.642	44.642	7.520	0.017
		X_4^2	1	0.019	0.019	0.000	0.956
	2-Way Interaction		6	128.065	21.344	3.600	0.025
		$X_1\ X_2$	1	12.837	12.837	2.160	0.165
		$X_1\ X_3$	1	0.358	0.358	0.060	0.810
		$X_1\ X_4$	1	42.703	42.703	7.200	0.019
		$X_2\ X_3$	1	9.076	9.076	1.530	0.238
		$X_2\ X_4$	1	59.359	59.359	10.000	0.007
		$X_3\ X_4$	1	0.498	0.498	0.080	0.777
Error			13	77.136	5.934		
	Lack-of-Fit		9	58.510	6.501	3.340	0.129
	Pure Error		4	5.355			
Total			29	822.342			

The appropriateness of the model fitting was examined by the coefficient of determination (R^2), which for CDA production was calculated to be 0.9073, this explained up to 90.73 % variability of the response. Model terms having values of P value less than 0.05 were considered significant, whereas those greater than 0.10 were insignificant. According to the present model, CDA production was significantly affected by the linear and squared terms of glucose, yeast extract, peptone and $MgCl_2$; and interaction effect of glucose-$MgCl_2$ and yeast

extract-$MgCl_2$. However, other interactions, including glucose-yeast extract, glucose-peptone, yeast extract-peptone and peptone-$MgCl_2$, were insignificant.

Verification of the calculated maximum was done with the experiments that were performed in the culture medium representing the optimum combination found as well as in the basal medium under submerged condition. The results predicted by equation showed that combination of 30 g L^{-1} glucose, 40 g L^{-1} yeast extract, 15 g L^{-1} peptone and 7 g L^{-1} $MgCl_2$ would favour CDA production, giving 55.90 U. Under the predicted optimal conditions, CDA production experimentally reached 57.69 ± 1.68 U while the un-optimised basal medium yielded 9.64 ± 2.04 U. Thus, the CDA production increased 5.98-fold in comparison to that in the basal level medium. The predicted response for CDA production was very close to the actual response. The excellent correlation between predicted and experimental values justified the validity of the response model.

A 2.0-fold and 4.12-fold enhancement in CDA production were attained in shake flasks, when the media components were used at their optimum levels by Pareek et al. (2011) for a mutant *Penicillium oxalicum* strain and by Sun et al. (2014) for a *Rhodococcus erythropolis* strain, respectively. The optimisations were carried out using statistical methods including Plackett Burman design and RSM-Central composite design. Similarly, optimisation of the physical parameters affecting CDA production by the same *Penicillium oxalicum* strain was carried out by formulating a RSM-Central composite design (Pareek et al., 2014). Conditions for CDA production from *Rhizopus japonicus* were optimised using Plackett–Burman and Taguchi designs (Zhang et al., 2014).

4.3.6. Production of CDA by SSF

In this experiment, which was carried out to determine the effect of incubation time, it was observed that maximum CDA production was attained within 72h of incubation as seen in Figure 4.16. Also, washed WB was observed to be a better substrate than unwashed WB for CDA production.

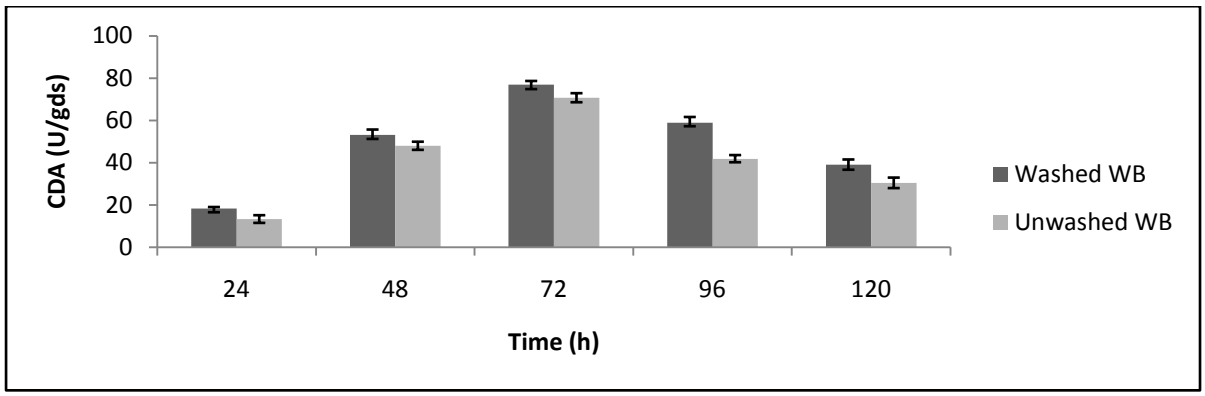

Fig. 4.16. Effect of incubation time and washing of wheat bran substrate on CDA production.

4.3.7. Statistical Optimisation for CDA Production (SSF)

4.3.7.1. Screening of parameters affecting CDA production in SSF by Plackett-Burman statistical design

Plackett-Burman screening experimental design was employed to determine the combined influence of media components on the production of CDA in SSF by *Aspergillus flavus*. The components were screened at the confidence level of 95% on the basis of their effects (either positive or negative). If the component shows at or above 95% confidence level and its effect is positive, it indicates that the component is effective in CDA production. But the amount required was higher than the stated high concentration in Plackett–Burman experiment. Similarly, if the effect was negative the amount required was lower than the stated low concentration in Plackett–Burman experiment. This was observed from the main effects plot (Fig. 4.17). As observed in Figure 4.18, the Pareto chart illustrated the order of significance of the variables affecting the CDA production. Here, the factors that showed significant positive effect on CDA production were glucose and meat extract while those that showed

significant negative effect were substrate mass and initial moisture content. The effects of inoculum size, yeast extract and $MgCl_2$ were not highly significant.

The regression equation obtained from the PBD for CDA production is as follows.

$$CDA = 81.46 - 7.21\ X_1 - 4.20\ X_2 + 0.93\ X_3 + 5.60\ X_4 + 1.21\ X_5 + 8.19\ X_6 + 0.68\ X_7$$

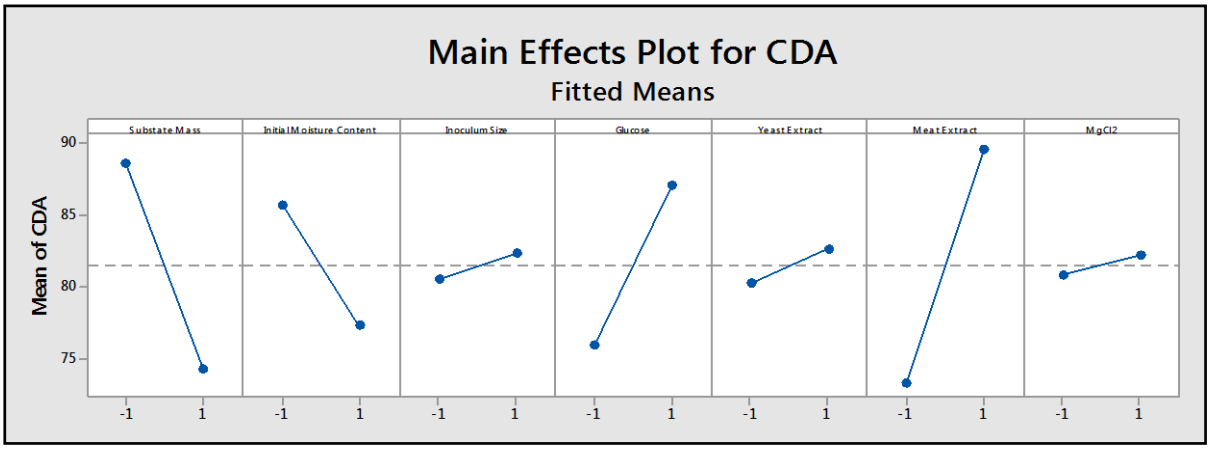

Fig. 4.17. Main effects plot showing effect of high and low level concentrations of factors influencing CDA production in SSF obtained using Packett-Burman design.

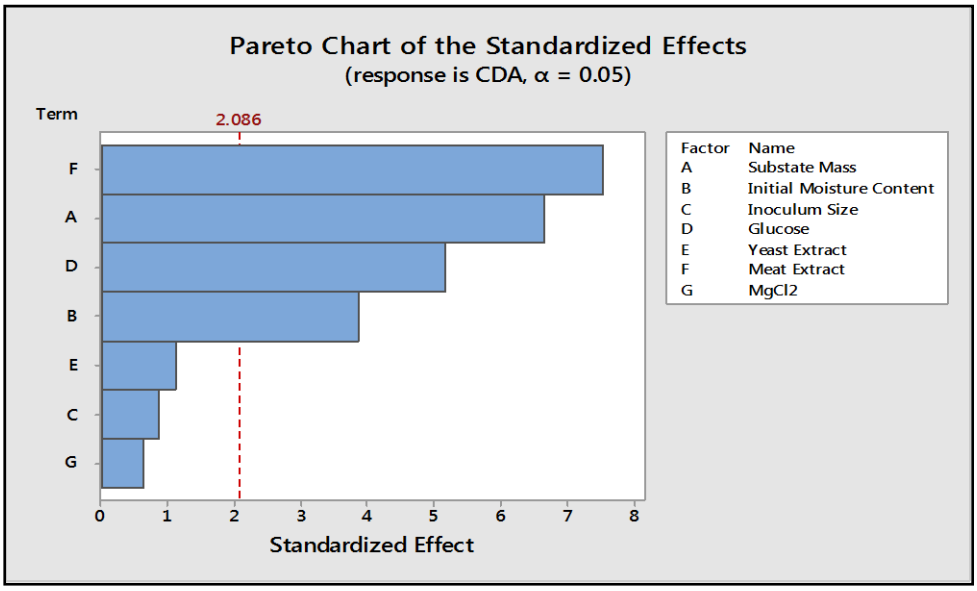

Fig. 4.18. Pareto plot showing significance of factors influencing CDA production in SSF obtained using Packett-Burman design.

4.3.7.2. Optimisation of significant parameters affecting CDA production in SSF by CCD

The four significant factors selected based on the results of PBD namely substrate mass, initial moisture content, meat extract and glucose were optimized using CCD. The results generated from the thirty experimental runs were analyzed by standard analysis of variance (ANOVA) as indicated in Table 4.10. Figure 4.19 depicts the contour plots of CDA activity showing interactions between different variables in the CCD. The quadratic regression equation obtained was as follows:

$$CDA\ (U) = 98.80 - 1.70\ X_1 - 4.08\ X_2 + 8.07\ X_3 + 12.46\ X_4 - 7.17\ X_1 - 6.79\ X_2^2 - 4.43\ X_3^2 - 0.64\ X_4^2 + 2.07\ X_1X_2 - 1.38\ X_1X_3 + 3.18\ X_1X_4 - 5.21\ X_2X_3 - 6.64\ X_2X_4 - 0.74\ X_3X_4$$

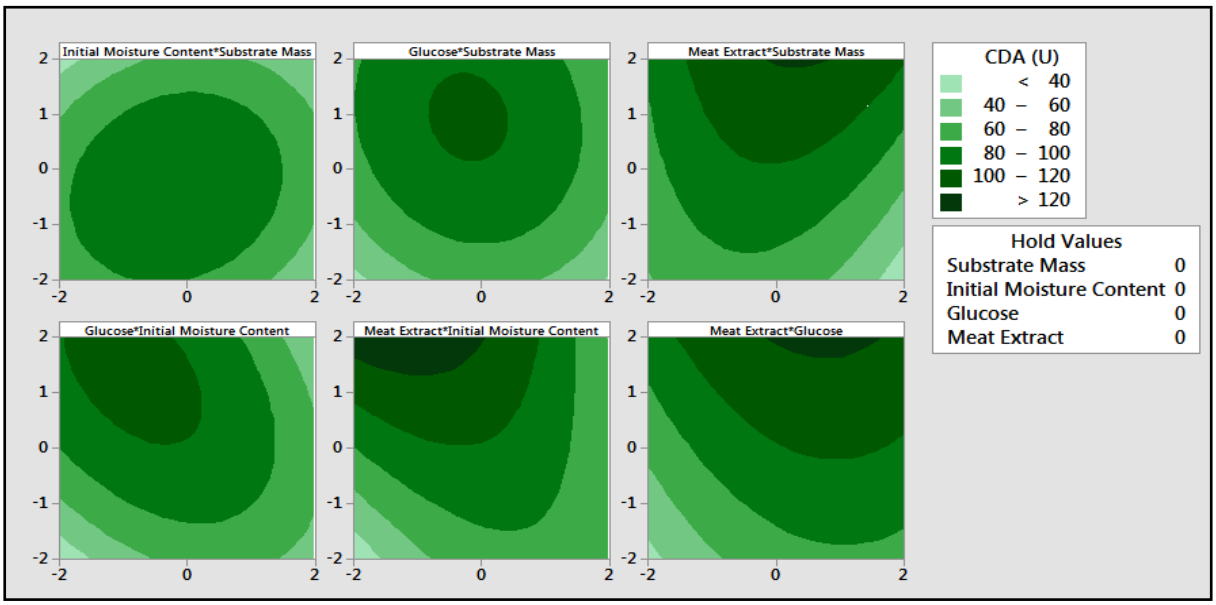

Fig. 4.19. Contour plots showing optimal concentrations and interactions between different variables in the CCD experiment for SSF production of CDA.

The appropriateness of the model fitting was examined by the coefficient of determination (R^2) which was determined for CDA production was calculated to be 0.9316, this, explained up to 93.16 % variability of the response. Model terms having values of P value less than 0.05 were considered significant, whereas those greater than 0.10 were insignificant.

According to the present model, CDA production is significantly affected by the linear and squared terms of substrate mass, moisture content, glucose and meat extract; and interaction effect of moisture content-glucose and moisture content-meat extract. However, other interactions including moisture content-substrate mass, substrate mass-glucose, substrate mass-meat extract, glucose-meat extract were observed to be insignificant.

Table 4.10. Regression analysis and ANOVA for central composite design for CDA production by SSF.

Source			**DF**	**Sum of Squares**	**Mean Square**	**F-Value**	**P-Value**
Model			15	12067.8	804.52	12.71	0.000
	Blocks		1	2298.8	2298.85	36.32	0.000
	Linear		4	5755.1	1438.78	22.73	0.000
		X_1	1	69.5	69.54	1.10	0.032
		X_2	1	400.1	400.08	6.32	0.025
		X_3	1	1562.1	1562.11	24.68	0.000
		X_4	1	3723.4	3723.40	58.83	0.000
	Square		4	2603.6	650.90	10.28	0.000
		X_1^2	1	1410.9	1410.90	22.29	0.000
		X_2^2	1	1264.1	1264.11	19.97	0.001
		X_3^2	1	538.7	538.72	8.51	0.011
		X_4^2	1	86.8	86.87	1.44	0.042
	2-Way Interaction		6	1410.2	235.03	3.71	0.020
		$X_1 X_2$	1	68.8	68.82	1.09	0.315
		$X_1 X_3$	1	30.3	30.30	0.48	0.500
		$X_1 X_4$	1	161.5	161.45	2.55	0.133
		$X_2 X_3$	1	434.8	434.79	6.87	0.020
		$X_2 X_4$	1	706.0	706.03	11.16	0.005
		$X_3 X_4$	1	8.8	8.78	0.14	0.715
Error			14	886.0	63.29		
	Lack-of-Fit		10	793.6	79.36	3.43	0.123
	Pure Error		4	92.5	23.12		
Total			29	12953.8			

Verification of the calculated maximum was done with the experiments that were performed in the culture medium representing the optimum combination found as well as in the basal medium under SSF condition. The results predicted by the equation showed that combination

of 4.5 g substrate mass, 45 % initial moisture content, 35 g L^{-1} glucose and 45 g L^{-1} meat extract would favour CDA production, giving 145 U gds^{-1}. Under the predicted optimal conditions, CDA production experimentally reached 141.89 ± 1.74 U gds^{-1} while the un-optimised basal medium yielded 78.64 ± 2.04 U gds^{-1}. Thus, the CDA production increased 1.79-fold in comparison to that in the basal level medium. The predicted response for CDA production was observed to be very close to the actual response. The excellent correlation between predicted and experimental values justified the validity of the response model.

The CDA production by *Penicillium oxalicum* SAEM-51 under SSF conditions using mustard oil cake (1162.03 ± 7.2 U gds^{-1}) was increased significantly by 1.3-fold, as compared to the production under the un-optimized condition (877.56 ± 8.9 U gds^{-1}). The optimisation was carried out using RSM-Central composite design (Pareek et al., 2014).

4.4. Summary and Conclusions

From the 10 CDA producing fungal strains isolated by enrichment technique from the natural environment, an *Aspergillus flavus* strain was selected and identified using ITS sequence identification method. The single parameter method followed by the statistical approach showed significant results for optimizing the process parameters for the CDA production in SmF using the *Aspergillus flavus* isolate and allowed rapid screening of a large number of variables. The identification of important process variables by the PBD experiments and the optimization of their levels by the RSM-CCD helped to improve the CDA yield from 8 U to 58 U. Similarly, SSF was carried out for production of CDA using wheat bran as the solid substrate. Similar identification of important process variables by the Placket and Burman design experiments and the optimization of their levels by the RSM-Central composite design helped to achieve a CDA yield of 142 U gds^{-1} as compared to un-optimised basal yield of 79 U gds^{-1}.

Chapter 5

Purification and Characterisation of Chitin Deacetylase

5.1. Introduction

During fermentation, a variety of proteins including enzymes are produced intracellularly as well as secreted into the growth medium by the microorganisms. These proteins vary from each other with respect to size, shape, charge, hydrophobicity, solubility and biological activity. For better understanding of the structure and functions of the protein of interest, it has to be separated from the rest of the proteins. This led to the development in techniques for the isolation, purification, and characterization of enzymes, so as to effectively study their mechanisms of action as well as other physicochemical properties, which may be useful in their industrial application. The classical enzyme purification procedures employed include centrifugation, ultrafiltration, selective precipitation and chromatographic procedures. The potential biotechnological significance of CDA lies in its role in the deacetylation of chitin. For this purpose the CDA should preferably be stable under standard conditions of pH, temperature, etc. that may therefore lead to more efficient yields of the end product. A variety of techniques have been used in the purification of CDAs, viz. ammonium sulphate precipitation, ion exchange chromatography (IEC), gel permeation chromatography (GPC), etc. Microorganisms, especially fungi produce CDAs having different molecular weights (Chapter 1, Table 1.6). Many purified microbial CDAs have been characterized for different parameters such as molecular weight, optimal pH, pH stability, optimal temperature, thermostability and effect of different metal ions and inhibitors. Some of them are found to exhibit appreciable activity at extremes of temperature and pH as well as in the presence of compounds usually inhibitory to enzyme activity. Such CDAs may have valuable industrial or environmental applications. In this chapter, the studies related to the purification and characterisation of the extracellular CDA from an isolated *Aspergillus flavus* strain (Chapter 4) has been presented. Kinetic parameters of the enzyme were also evaluated using various chitinous substrates. The present study would hence help decipher the characteristic features

of the CDA that appears to be compatible for industrial exploitation of the enzyme for bioconversion reactions.

5.2. Materials and Methods

5.2.1. Preparation of colloidal chitin and CDA assay

The colloidal chitin was prepared as mentioned in Chapter 4, section 4.2.1 and the CDA activity was determined as mentioned in section 4.2.2. The assay procedure was suitably modified according to the requirements of the experiments.

5.2.2. Microbial culture and CDA production conditions

An *Aspergillus flavus* strain was isolated and identified, and the bioprocess parameters for maximum CDA production by the same were optimised as mentioned in Chapter 4. The culture was inoculated in the optimised medium, and after 48 h of incubation, the culture broth was harvested, centrifugation was done at 9500 ×g at 4°C for 10 min and the supernatant that consisted of the crude CDA was further used for enzyme purification and characterisation studies.

5.2.3. Enzyme purification and determination of molecular weight

Crude enzyme obtained as above was first purified by ammonium sulphate precipitation fractionation method, followed by ion-exchange chromatography and further by gel permeation chromatography. Two hundred millilitres cell-free supernatant was precipitated with ammonium sulphate (60%). The pellets obtained after centrifugation at 11000 ×g at 4°C for 15 min were dissolved in citrate-phosphate buffer (0.1 M, pH 6.5) and dialyzed overnight in the same buffer. IEC was done using a Q-sepharose (Sigma Aldrich, USA) column (30 cm × 1 cm). The column was packed with overnight-swollen resin in 0.1 M citrate-phosphate

buffer (pH 6.5) and the above dialysed protein solution was applied to it. It was then eluted stepwise with 0.1 to 0.8 M NaCl at a flow rate of 1.0 mL min^{-1}. The protein fractions (1.0 mL) were analysed for CDA activity and those with positive activity were pooled and concentrated. This concentrated fraction was then further applied to a GPC column (50 cm × 1.7 cm). The column was packed with overnight-swollen resin (Toyopearl HW50, Tosoh Bioscience, Japan) in 0.1 M citrate-phosphate buffer (pH 6.5). The same buffer was passed through the column at a flow rate of 1.0 mL min^{-1} to separate the proteins. The protein fractions (1.0 mL) were analysed for CDA activity. The total protein content was determined for all the fractions by Bradford's method using bovine serum albumin (BSA) as the standard protein (Bradford, 1976).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli (1970) using vertical slab gel electrophoresis unit. A 14% separating and 5% stacking gel were used for PAGE analysis. The molecular mass of purified enzyme was determined by using protein molecular mass-standards (Precision-Plus) obtained from Biorad, USA. After SDS-PAGE, gel was stained by silver staining method.

GPC was used for determining the molecular mass of the CDA. The separation was carried out using the GPC column as mentioned above with 50 mM Tris-HCl, pH 7.5 with 100 mM KCl; as equilibration buffer, at a flow rate of 1.0 mL min^{-1}. One mL samples were collected for analysis. The elution volume (V_e) of the standard protein markers {Cytochrome C (12.4 kDa); Carbonic anhydrase (29 kDa); Bovine serum albumin (66 kDa); Alcohol dehydrogenase (150 kDa); β amylase (200 kDa); Blue dextran (2000 kDa)} obtained from Sigma Aldrich, USA; and the void volume (V_o) were determined. A semilog plot projecting the standard curve of molecular mass vs. V_e/V_o for each respective protein standard was plotted (Gel filtration Marker Kit, Sigma Aldrich, USA).

Native polyacrylamide gel electrophoresis (NATIVE-PAGE) using 12% gel was performed for visualization of enzyme activities in situ using glycol chitin as described by Trudel & Asselin (1990). After loading the purified CDA samples, electrophoresis was carried out. Further, the gel was incubated for 1 h at 50°C in 0.1 M citrate-phosphate buffer, pH 6.5. The gel was then stained with 0.01% (v/v) Calcofluor white M2R for 30 min, and further gently washed with distilled water. The zones were photographed under the UV-transilluminator in a Chemidoc MP imaging system (Biorad, USA).

5.2.4. Effects of pH, temperature, salinity, and addition of metal ions, solvents and denaturing agents on enzyme activity

The CDA purified by Q-sepharose-anion exchange chromatography was used for enzyme characterisation studies, including enzyme physicochemical properties and kinetics. The effect of pH was determined by incubating the CDA over a pH range of 1.0–11.0 at 50°C. The buffer systems used were HCl-KCl (100 mM, pH 1.0-2.0); citrate-phosphate (100 mM, pH 3.0–8.0); Tris-HCl (100 mM, pH 8.0–9.0) and carbonate-bicarbonate (100 mM, pH 9.0–11.0). The pH stability was tested by pre-incubation of the purified CDA in buffers with different pH from 1.0 to pH 11.0 at standard assay temperature for 2 h. The residual enzyme activity was tested at optimum pH (pH 8.0). Optimum temperature for the purified CDA activity was determined over a temperature range of 20 to 80°C. Temperature stability was tested by the pre-incubation of the enzyme at temperatures ranging from 20 to 80°C for 3 h, and further assay was carried out at optimum temperature. All further characterisation studies were carried out under conditions of optimal pH and temperature. The effects of metal ions on enzyme activity were assayed after the addition of metal ions Ag^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{2+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, Sn^{2+} and Zn^{2+} in the form of either their chloride or phosphate salts to the reaction mixtures. EDTA was added as a negative control. The metal ions and EDTA were added to the reaction mixture so as to achieve different final

concentrations of 10 and 20 mM. The enzyme activity at various levels of salinity was measured over a range of 0.25 to 5.0% (w/v) NaCl concentration in the assay mixture. The effects of addition of various solvents on enzyme activity was studied by the addition of acetone, butanol, chloroform, DMSO, ethanol, hexane, isoamyl alcohol, isopropanol, methanol and toluene at final concentrations of 10 and 20% in the assay mixture. The effects of various protein denaturants on the CDA activity were also studied. Urea was added at final concentration of 200 mM; reducing agents such as β-mercaptoethanol and dithiothreitol were added at final concentrations of 10 mM; and surfactants, viz. sodium dodecyl sulphate (SDS), Tween (20, 40, 60, 80) and Triton X-100 were added at final concentrations of 1% to the assay mixture. The inhibitory effect of acetate, which is a product of chitin deacetylation, on CDA activity was determined by assaying the enzyme activity in the presence of various concentrations of acetate (sodium acetate; 10−200 mM) in the assay mixture. The residual CDA activity was measured after each characterisation experiment. The CDA activity was expressed as the percent relative activity. Appropriate controls were maintained wherever required during the above studies.

5.2.5. Substrate specificity and enzyme kinetics

The purified CDA was incubated with various substrates such as NAG, $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, glycol chitin and colloidal chitin. These were added to the assay mixture individually at a concentration of 1% (w/v) under standard assay conditions and the degree of substrate deactylation was analysed wherein the quantity of acetate released was determined by the earlier mentioned HPLC method. The effect of substrate concentration on CDA activity was studied using colloidal chitin as substrate. Colloidal chitin with final concentration ranging from 2.0 to 50.0 mg mL^{-1} was used, and the enzyme activity was then determined by the standard assay protocol. The kinetic constants K_m and V_{max} were determined from the Lineweaver–Burk plot.

5.3. Results and Discussion

5.3.1. Enzyme purification

The crude enzyme obtained after fermentation process using the *Aspergillus flavus* strain was purified using ammonium sulphate precipitation; Q-sepharose based ion exchange chromatography (Fig. 5.1) and gel permeation chromatography (Fig. 5.2) consecutively. The CDA was 3.9-fold purified with a final specific activity of 55.91 U mg^{-1}, and a recovery yield of 2.9%. CDAs produced by different microbial cultures have been purified using ammonium sulphate precipitation method followed by multiple column chromatographic steps (Cai et al., 2006; Jeraj et al., 2006; Pareek et al., 2012). The CDA purification carried out has been summarised in Table 5.1.

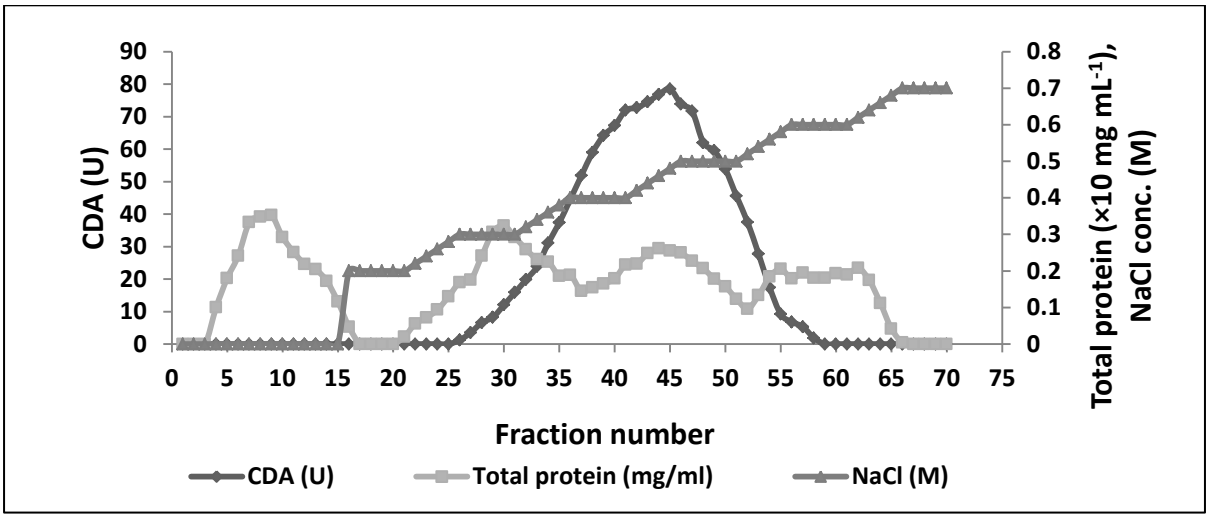

Fig. 5.1. CDA- Ion exchange chromatography elution profile.

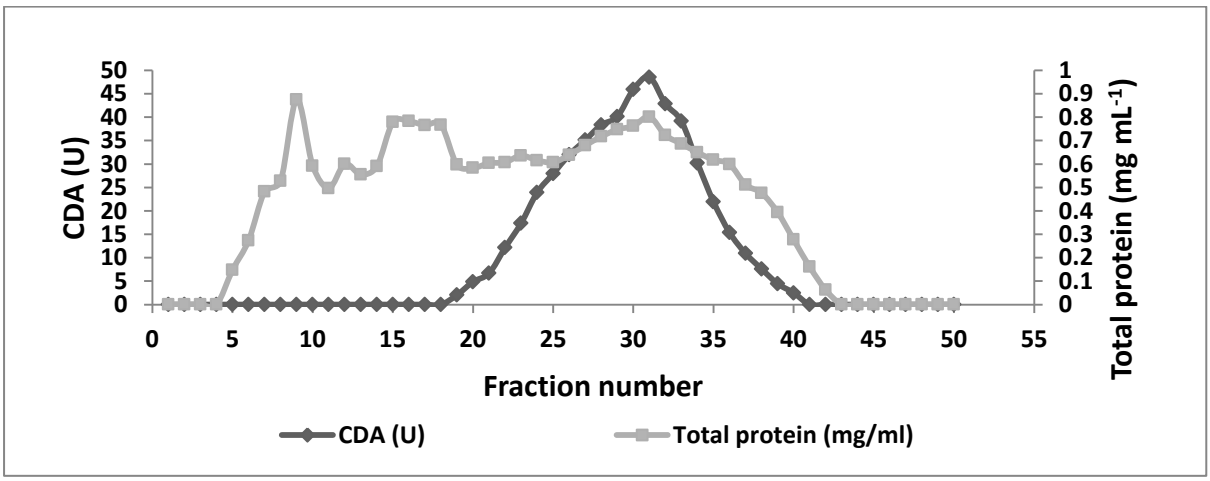

Fig. 5.2. CDA- Gel permeation chromatography elution profile.

Table 5.1. Purification of *Aspergillus flavus* CDA.

Purification Step	Volume (mL)	Total Proteins (mg)	Specific Activity (U mg^{-1})	Total Activity (U)	Purification (Fold)	Yield % (Total Activity)
Culture filtrate	100	578	14.35	8294.30	0	100
$(NH_4)_2SO_4$ precipitation (60%)	50	185.5	28.29	5247.80	1.97	63.27
Q-sepharose-ion exchange chromatography	10	20.1	38.93	782.5	2.71	9.43
Gel permeation chromatography	5	4.3	55.91	240.4	3.90	2.90

5.3.2. Gel electrophoresis and CDA molecular weight estimation

The molecular mass of the *Aspergillus flavus* CDA was estimated approximately to be 28 kDa by SDS-PAGE and was seen on the gel as a single band purified after carrying out gel permeation chromatography (Fig. 5.3). Also, the gel permeation chromatography based molecular weight determination method using protein standards (Chapter 3, Table 3.3) confirmed this observation. The standard semi-log plot used for calculations is as shown in Chapter 3, Figure 3.1. The molecular weight calculated by this method was 27.74 kDa. In accordance with the activity staining results, the purified CDA enzyme showed a single clear band by zymography, confirming that it was a CDA and only a single protein with CDA activity was produced with no isozymes. The analysis of the purified enzyme by SDS-PAGE and Native-PAGE-activity staining is shown in Figure 5.3 and Figure 5.4, respectively.

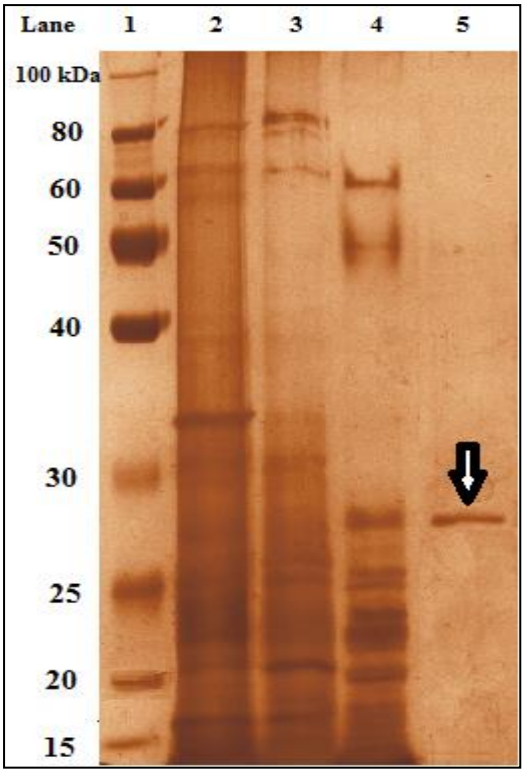

Fig. 5.3. SDS-PAGE analysis of CDA enzyme. Lane 1: Protein standard molecular weight marker, Lane 2: Crude enzyme, Lane 3: Ammonium sulphate precipitation, Lane 4: Ion exchange chromatography, Lane 5: Gel permeation chromatography. Arrow indicates the single 28 kDa band of the purified enzyme.

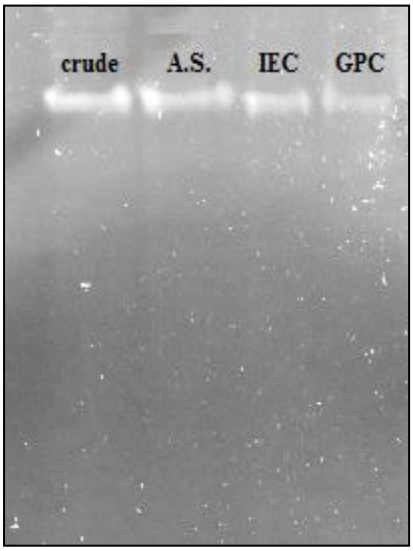

Fig. 5.4. CDA activity staining using glycol chitin stained with Calcofluor white M2R. Crude enzyme supernatant, ammonium sulphate precipitation purified fraction (A.S.), ion exchange chromatography (IEC) and gel permeation chromatography (GPC).

Molecular masses of microbial CDAs generally range from 20 to 80 kDa (Chapter 1, Table 1.9). A couple of exceptions included *Rhizopus nigricans* (Jeraj et al., 2006) and *Colletotrichum lindemuthianum* (Tsigos and Bouriotis, 1995) strains which produced CDAs of 100 and 150 kDa respectively. The molecular weight of the CDA reported in this study was similar to that reported for two *Aspergillus nidulans* strains (Alfonso et al., 1995; Wang et al., 2009). Two CDA isozymes with molecular weights of 35 kDa and 170 kDa were produced by a *Colletotrichum gloeosporioides* strain (Pacheco et al., 2013).

5.3.3. Effect of pH and temperature on the activity and stability of the purified CDA

pH and temperature play an important role in determining the enzyme activity and stability. The CDA from the *Aspergillus flavus* strain exhibited more than 50% of the highest activity within pH range of 4.0-9.0, with maximum activity at pH 8.0, as observed in Figure 5.5. Beyond pH 9.0 there was a rapid decrease in the activity indicating that the enzyme was active more within the acidic range. Similarly, the stability was also exhibited within pH range of pH 3.0-10.0 as seen in Figure 5.6. Most of the purified microbial CDAs show moderate to high activity as well as stability within the pH range of 4.0–10.0 (Chapter 1, Table 1.9). The optimum pH for the CDA in the present study was found to be same to the CDA reported for an *Aspergillus nidulans* strain (Wang et al., 2009), showing highest activity at pH 8.0. The CDA produced by a *Colletotrichum lindemuthianum* strain was found to be active in the alkaline range of pH 7.0-12.0 (Tokuyasu et al., 1996).

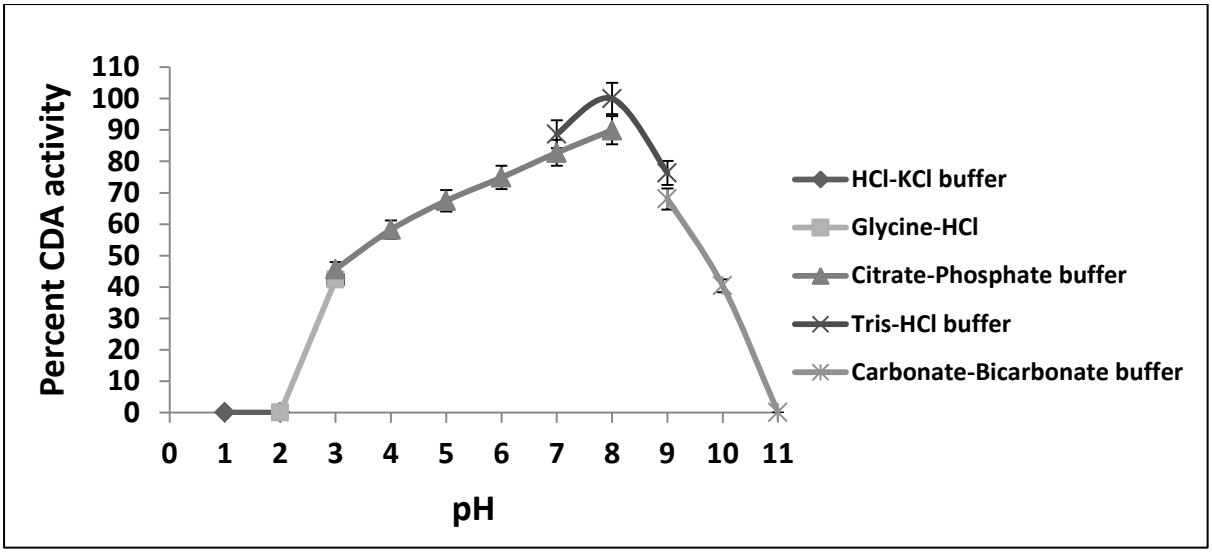

Fig. 5.5. Determination of pH optimum of CDA.

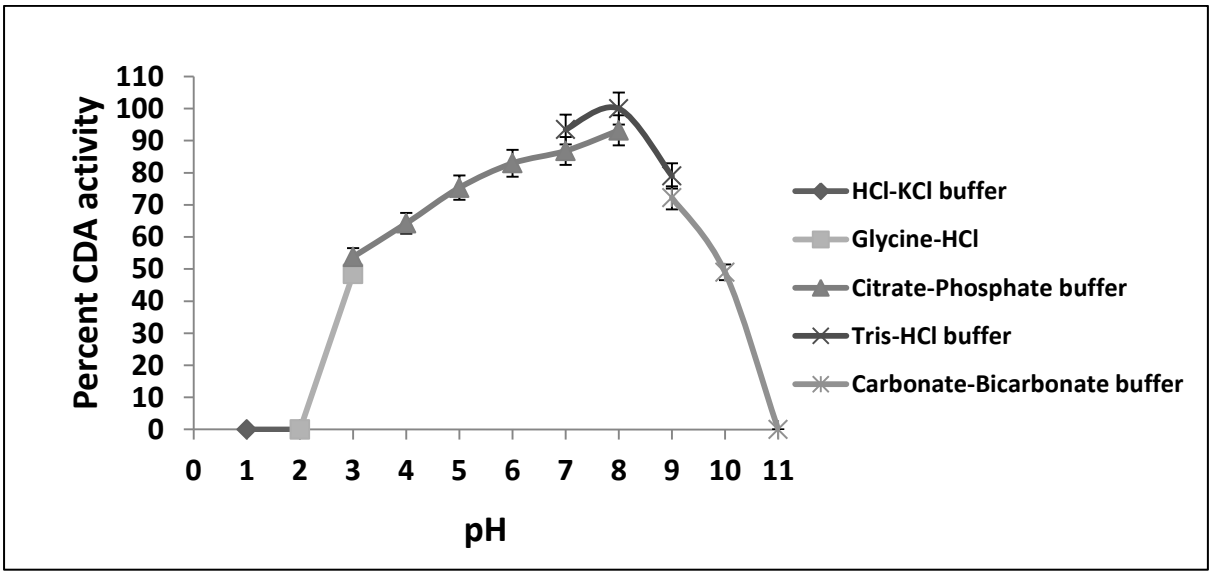

Fig. 5.6. Determination of pH stability of CDA.

CDA produced by the *Aspergillus flavus* strain exhibited a temperature optimum at 50°C (Fig. 5.7). At temperatures below and above 50°C, the enzyme activity reduced progressively and significantly. The activity dropped to below 50% of the highest activity at 30°C and 60°C. The CDA exhibited high stability up to 40°C even after three hours of incubation (Fig. 5.8). The residual enzyme activity reduced to around 40% after 3 h when incubated at 50°C showing the reduced stability of the enzyme at 50°C. At temperatures above 50°C, the

enzyme stability was significantly reduced as seen in Figure 5.8. Half-life of the enzyme at its optimum temperature was calculated as 135 min. The optimum temperature for most microbial CDAs is within 50-60°C and show moderate activity within the range of 30-70°C while the thermal stability is generally observed up to 50°C (Chapter 1, Table 1.9). A few exceptions included the CDA from a *Rhizopus circinans* strain (Gauthier et al., 2008) with an optimal temperature of 37°C and two isozymes of CDA from a *Vibrio alginolyticus* strain (Ohishi et al., 1997) having optimal temperatures of 40 and 45°C.

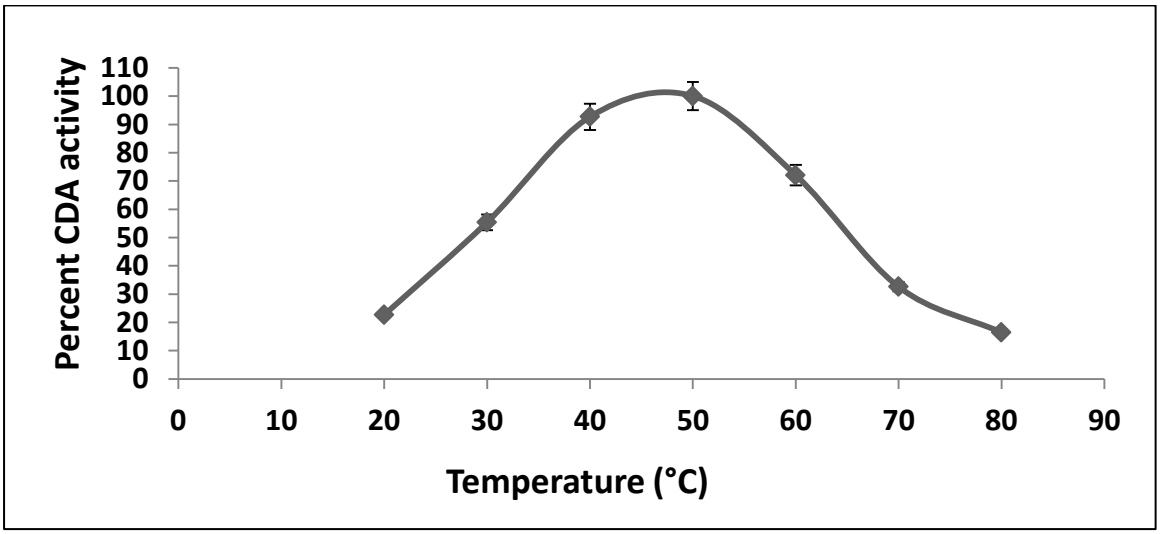

Fig. 5.7. Determination of temperature optimum of CDA.

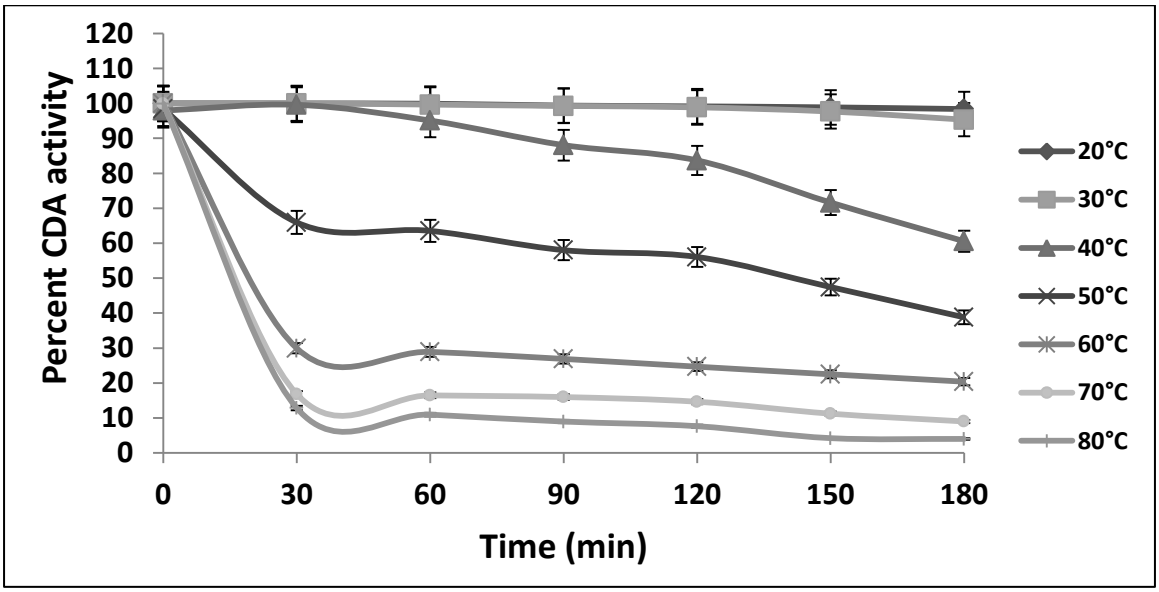

Fig. 5.8. Determination of temperature stability of CDA.

5.3.4. Effect of metal ion addition on the activity of the purified CDA

Metal ions play a significant role in biological catalysis by forming complexes with the enzymes and maintaining or disrupting the 3-dimensional structure and configuration (Andreini et al., 2008). The influence of metal ions on the activities of CDA obtained from the *Aspergillus flavus* is shown in Figure 5.9. The activity was enhanced marginally in the presence of divalent cations like Mn^{2+} and Zn^{2+} and monovalent cations like K^{+} and Na^{+}. It was inhibited moderately to strongly by Ag^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+} and Mg^{2+}. The activity was least affected by other cations. There was significant inhibition of activity in the presence of EDTA as observed in Figure 5.9.

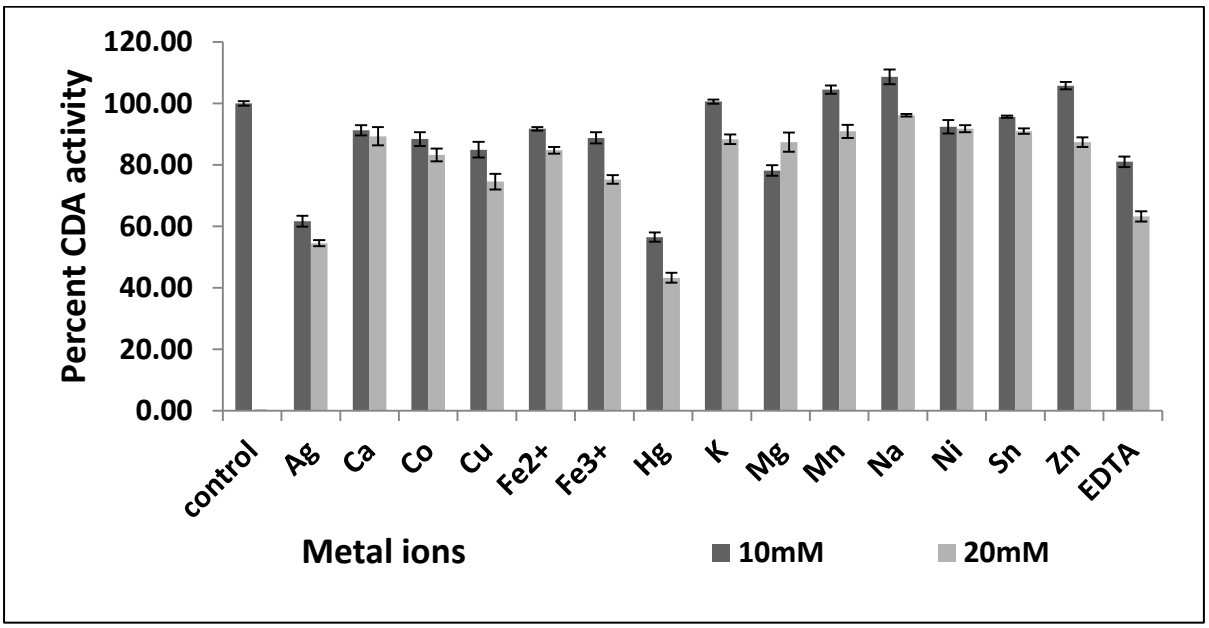

Fig. 5.9. Effect of addition of metal ions on CDA activity.

Metal ions generally shown to inhibit microbial CDA activity were Ca^{2+} and Mg^{2+} (Kafetzopouos, 1993; Martinou et al., 2002), Mn^{2+} (Tsigos and Bouritis, 1995; Tokayasu et al., 1996; Kim et al., 2008), Cu^{2+} (Martinou et al., 2002; Wang et al., 2010; Pareek et al., 2012), Fe^{3+} (Gao et al., 1995), Zn^{2+} (Martinou et al., 2002; Wang et al., 2010; Pareek et al., 2012), Co^{2+} (Tokayasu et al., 1996; Wang et al., 2010), Ag^{2+} and Hg^{2+} (Ohishi et al., 1997; Kim et al., 2008). EDTA which is a chelating agent capable of binding metal ions in solution

has been reported to reduce the activity of CDA by Araki and Ito (1975), Martinou et al., (2002) and Wang et al. (2010). Co^{2+} was found to increase the activity of CDAs from *Colletotrichum lindemuthianum* (Tsigos and Bouritis, 1995) and *Saccharomyces cerevisiae* (Martinou et al., 2002). Ca^{2+} and Mg^{2+} were reported by Wang et al. (2010) to enhance the activity of CDA produced by *Aspergillus nidulans*. Cu^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+} were found to be activators of the CDA produced by a *Penicillium oxalicum* strain (Pareek et al., 2012).

5.3.5. Effect of salinity on the activity of the purified CDA

Marine micro-organisms usually are tolerant to higher salinity levels. Salt-tolerant enzymes may play significant roles in the industrial processes that operate in high-salt conditions. Since the *Aspergillus flavus* strain was isolated from coastal environment samples (Chapter 4), it was expected that the CDA expressed would be salt tolerant. The activity of the CDA remained nearly constant up to 1% NaCl concentration. After that, it gradually reduced to give 50% residual activity at 4.5% NaCl concentration (Fig. 5.10). There are no reported studies on the effect of salinity on the activity of microbial CDA.

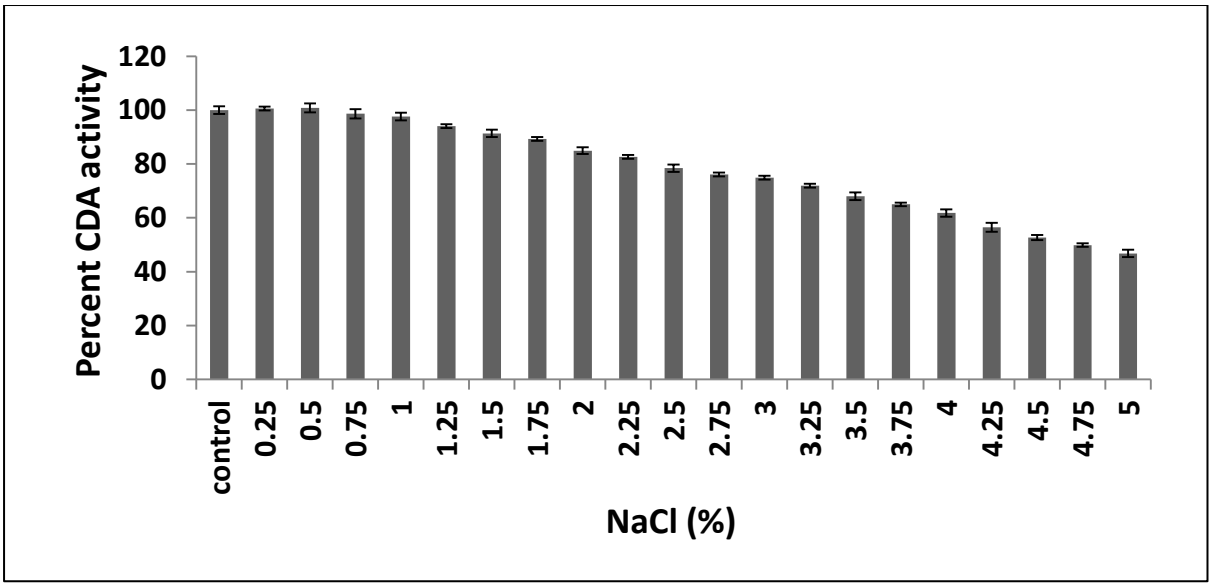

Fig. 5.10. Effect of salinity on CDA activity.

5.3.6. Effects of solvent addition on the activity of the purified CDA

The effect of solvent addition on the activity of CDA obtained from the *Aspergillus flavus* strain is shown in Figure 5.11. Most of the solvents such as butanol, chloroform, DMSO, ethanol, isoamyl alcohol, isoamyl alcohol, isopropanol and methanol significantly reduced the CDA activity. But hexane and toluene did not show any significant effect on the CDA activity. There are no reported studies on the effect of solvents on the activity of microbial CDA.

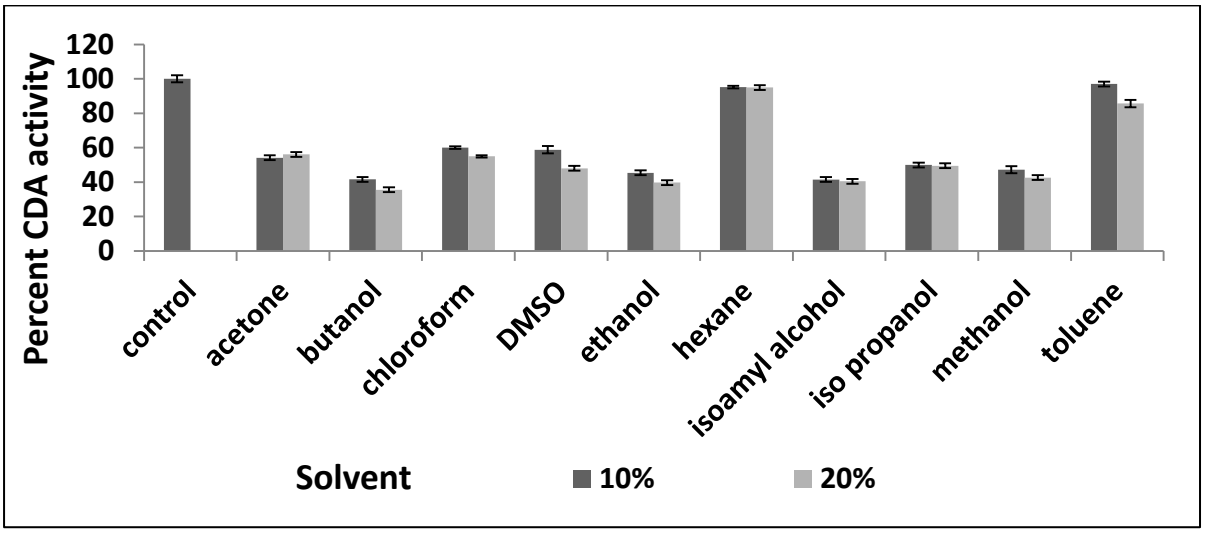

Fig. 5.11. Effect of solvent addition on CDA activity.

5.3.7. Effect of denaturing agent addition on the activity of the purified CDA

The influences of denaturing agents on the activity of CDA obtained from the *Aspergillus flavus* strain were studied. Urea, β-mercaptoethanol and dithiothreitol significantly reduced the CDA activity to 44, 18 and 17% of the original activity, respectively. In the case of surfactants, CDA activity was significantly increased in the presence of Tween (20, 40, 60, 80) and Triton X-100 as observed in Figure 5.12. The CDA activity was severely affected by SDS, causing it to reduce to 4% of the original activity. Sensitivity of the CDA enzyme towards end product inhibition was evaluated by assaying the enzyme activity in presence of acetate (Fig. 5.13). More than 80% of the enzyme activity was

retained in the presence of 0−75 mM sodium acetate and thereafter it rapidly declined with a further increase in acetate concentration. There was negligible activity beyond 100 mM concentration. Acetate was found to be the inhibitor of most microbial CDAs at various concentrations. (Araki and Ito, 1975; Kafetzopouos, 1993; Tsigos and Bouritis, 1995; Tokayasu et al., 1996; Kim et al., 2008; Wang et al., 2010; Pareek et al., 2012). In the case of CDA produced by *Colletotrichum lindemuthianum*, high residual activity of 70% was observed in the presence of acetate at concentrations as high as 800 mM (Tokuyasu et al., 1996).

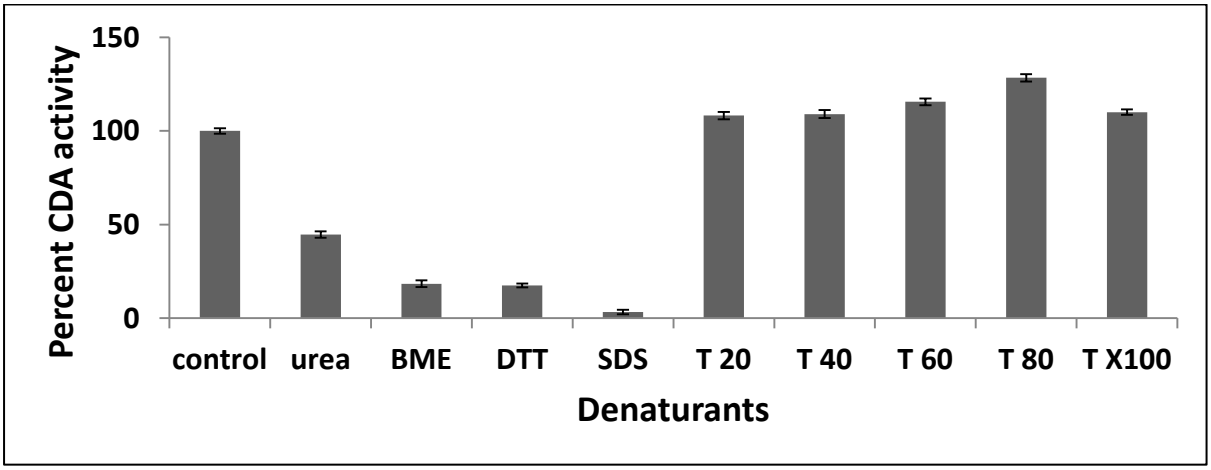

Fig. 5.12. Effect of denaturing agent addition on CDA activity. Final concentrations: Urea at 200 mM); β-mercaptoethanol (BME) and dithiothreitol (DTT) at 10 mM and surfactants SDS, Tween (20, 40, 60, 80) and Triton X-100 at 1%.

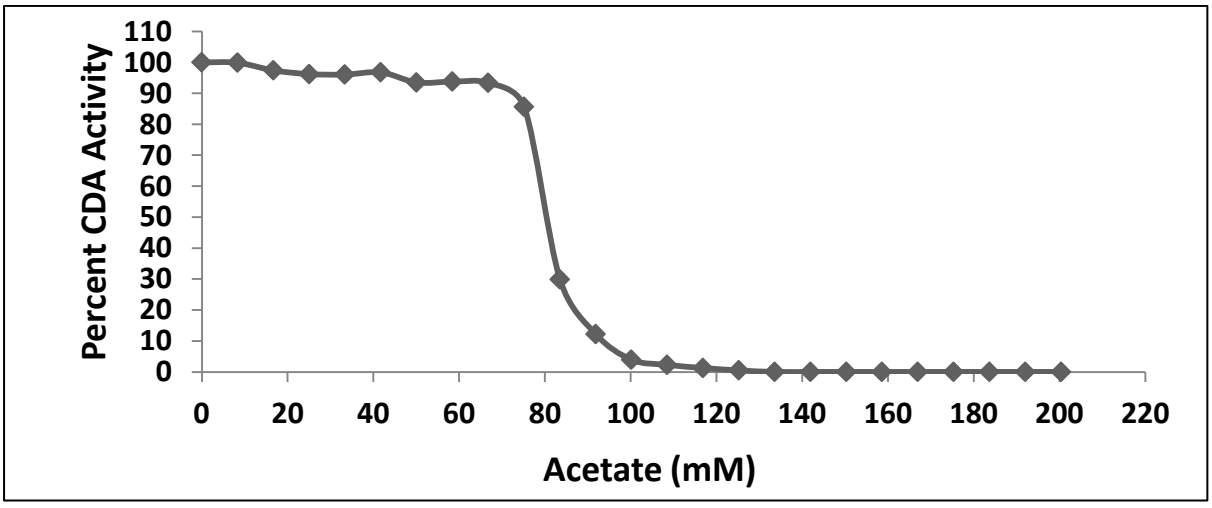

Fig. 5.13. Effect of acetate concentration on enzyme activity (product inhibition study).

5.3.8. Substrate specificity and kinetic parameters

The purified *Aspergillus flavus* CDA was assayed using different substrates and it showed highest activity towards glycol chitin. A comparatively lower activity was observed in the presence of colloidal chitin. It showed no activity towards chito-oligosaccharides including NAG, $(NAG)_2$, $(NAG)_3$, but showed deacetylase activity in presence of $(NAG)_4$ (Fig. 5.14). The efficiency of deacetylation increased with increased polymerisation from $(NAG)_2$ to $(NAG)_6$ as observed for CDAs from *Scopulariopsis brevicaulis* (Cai et al., 2006) and *Saccharomyces cerevisiae* (Martinou et al., 2002). A *Flammulina velutipes* CDA catalysed the deacetylation of N-acetyl-chito-oligomers, from dimer to pentamer, glycol chitin and colloidal chitin (Yamada et al., 2008). Among various substrates tested, water-soluble chitin, glycol chitin and crab chitosan and chito-oligomers from dimers to heptamers were deacetylated by the CDA produced by *Mortierella* sp. (Kim et al., 2008). It was observed that a *Penicillium oxalicum* CDA deacetylated glycol chitin and chitin oligomers having degree of polymerization of more than four (Pareek et al., 2012).

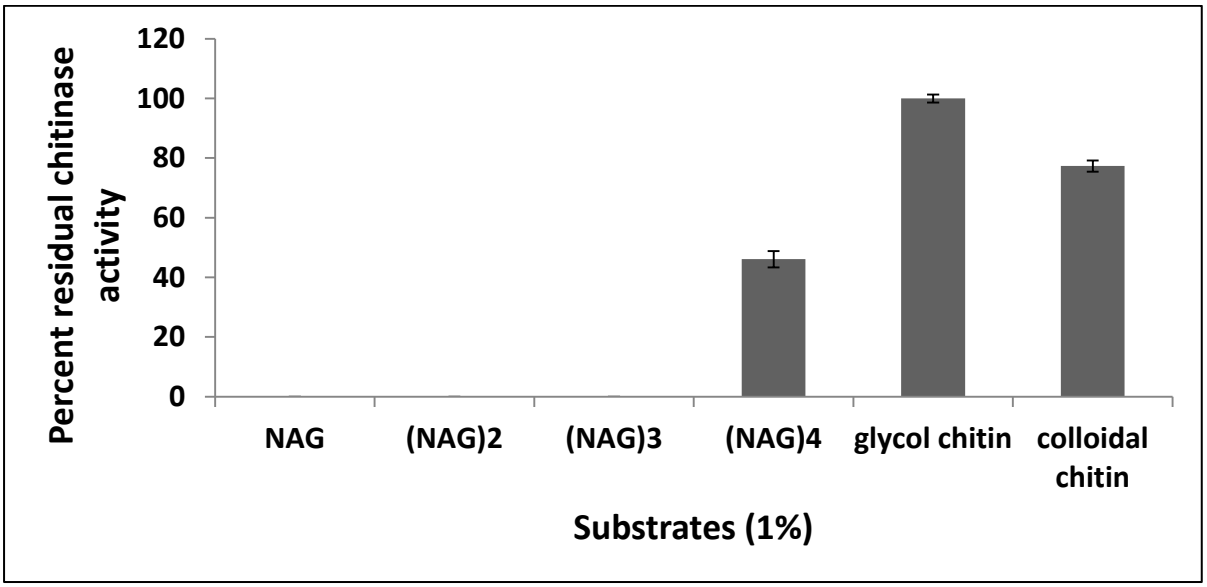

Fig. 5.14. Substrate specificity of purified CDA.

The reaction rate i.e., velocity (V) of the CDA enzyme using different concentrations of colloidal chitin (2 to 50 mg mL^{-1}) was determined as observed in Figure 5.15. The Michaelis-Menten kinetic constants K_m and V_{max} of the *Aspergillus flavus* CDA were determined using a Lineweaver–Burk Plot (Fig. 5.16) to be 9.45 mg mL^{-1} and 123.11 U mL^{-1}. A CDA enzyme produced by *Penicillium oxalicum* strain exhibited maximum affinity towards $(NAG)_5$ with the K_m being 3.07 mg mL^{-1} and V_{max} being 6.76 μmol min^{-1} (Pareek et al., 2012). The K_m value of CDA produced by *Aspergillus nidulans* was 4.92 mg mL^{-1}, and the V_{max} was 0.77 μmol min^{-1} mL^{-1} when ethylene glycol chitin was used as the substrate (Wang et al., 2010). The K_m value of chitin deacetylase produced by *Colletotrichum lindemuthianum* was 2.55 mM, 0.6 mM and 0.4 mM, and the V_{max} was 51.3 μmol min^{-1} mg^{-1}, 184 μmol min^{-1} mg^{-1} and 158 μmol min^{-1} mg^{-1} when ethylene glycol chitin, $(NAG)_4$ and $(NAG)_5$ were used as substrates respectively (Tokayasu et al., 1996).

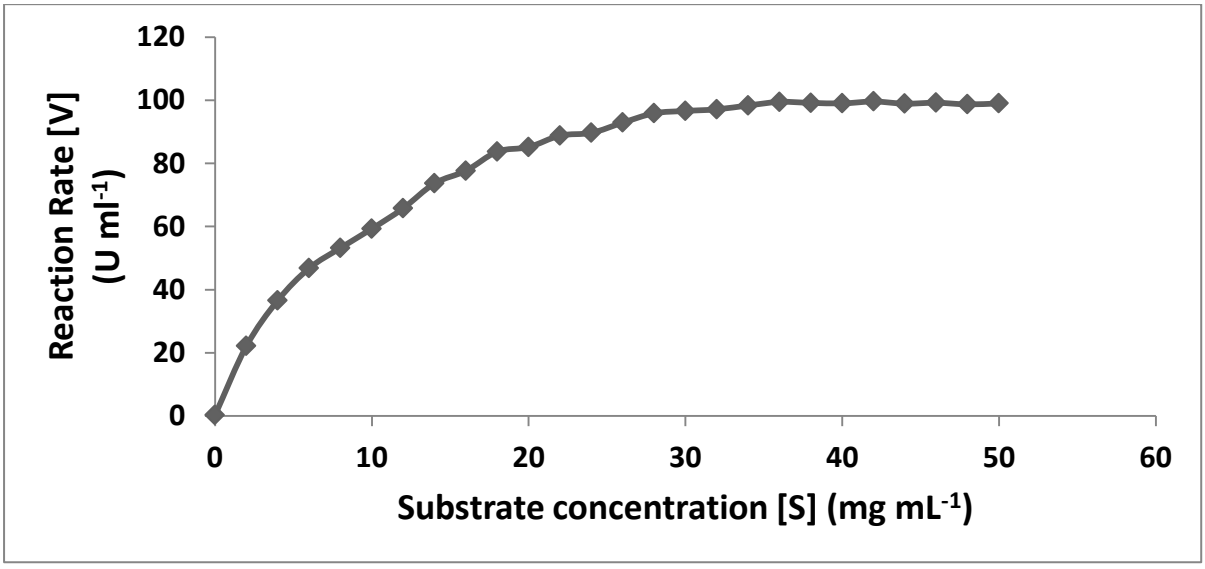

Fig. 5.15. Effect of substrate concentration on CDA enzyme.

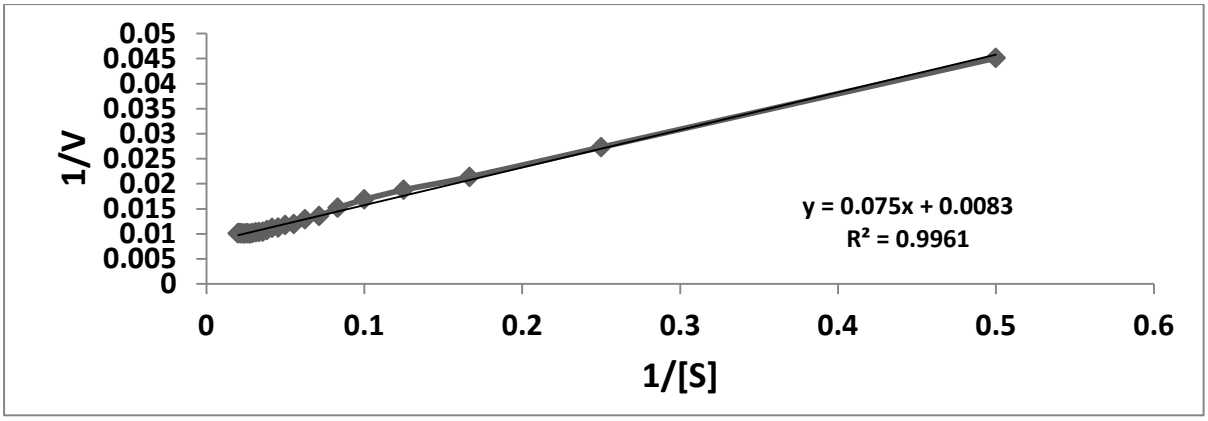

Fig 5.16. Double reciprocal - Lineweaver–Burk Plot for CDA enzyme.

5.4. Summary and Conclusions

Purification of CDA enzyme was carried out using methods like ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography. A 4-fold purification was achieved with 2.9% final yield. Characterization of CDA enzyme was done with respect to parameters such as effect of pH, temperature, salinity, addition of metal ions, solvents and denaturing agents. The purified enzyme had the optimal temperature and pH as 50°C and 8.0 respectively. It retained more than 90% and 50% of its activity at NaCl concentrations of 1.5% and 4.5% respectively. The activity was enhanced marginally in the presence of divalent cations like Mn^{2+} and Zn^{2+}. Most of the solvents had a notable negative impact on CDA activity. Ionic surfactants reduced the enzyme activity considerably while the non-ionic surfactants like Tween and Triton had either a neutral or positive effect on enzyme activity. Results of the activity staining experiment demonstrated that only a single protein (enzyme) molecule which has CDA activity is produced. The molecular weight of the CDA enzyme was determined to be 28 kDa. The ability to deacetylate water insoluble chitin (colloidal chitin) combined with a reasonably high optimum temperature of enzyme activity, and its stability makes the *Aspergillus flavus* CDA a potential candidate enzyme to be used for industrial scale deacetylation of chitin by at-least partially replacing the currently used chemical method of deacetylation.

Chapter 6

Application of the Chitinase and Chitin Deacetylase Enzymes

6.1. Introduction

Chitinases have been proven to have various applications in agricultural, industrial, biochemical and medical fields (Dahiya et al., 2006; Khoushab & Yamabhai, 2010; Nagpure et al., 2013; Stoykov et al., 2015). Chitinases from different microbial sources are used for the production of chito-oligosaccharides of specific polymeric length and NAG. These have been found to function as antibacterial agents (Dai et al., 2009), as elicitors and immunomodulators (Khor and Lim, 2003; Gupta et al., 1995) and other pharmaceutically and biomedically important applications (Khor and Lim, 2003; Khoushab & Yamabhai, 2010; Chen et al., 2010). They have also been used in single cell protein production (Dahiya et al., 2006). Table 6.1 lists out chitinases from various microorganisms mainly used in the production of chito-oligosacharides.

Table 6.1. Chitinase utilisation in various applications.

Microorganism	Application	Reference
Aspergillus flavus	Production of chito-oligosacharides	Thadathil et al., 2014
Bacillus sp.	Production of chito-oligosaccharides	Lee et al., 2007
Bacillus cereus	Production of chito-oligosaccharides	Wang et al., 2012
Bacillus licheniformis	Production of chito-oligosaccharides	Songsiriritthigul et al., 2010
Bacillus licheniformis	Chitin detecting probe	Islam et al., 2010
Chitinibacter sp.	Production of NAG	Gao et al., 2015
Enterobacter sp.	Protoplast generation in combination with cellulase	Dahiya, et al., 2005
Fusarium oxysporum	Production of chito-oligosacharides	Thadathil et al., 2014
Oerskovia xanthineolytica	Protoplast generation	Waghmare & Ghosh, 2010
Penicillium aculeatum	Production of NAG	Binod et al., 2007
Penicillium monoverticillium	Production of NAG	Suresh & Kumar, 2012
Penicillium ochrochloron	Protoplast generation	Patil et al., 2013
Streptomyces sp.	Production of chito-oligosaccharides	Prakash et al., 2013
Streptomyces violascens	Production of NAG	Gangwar et al., 2016

Application of chemical based pesticides cause long lasting adverse effects on ecosystems and human health. Hence it is necessary to search and develop non-hazardous biologically compatible alternatives. Such eco-friendly substitutes of chemical pesticides are considered as biocontrol agents. These agents could be formulations of bacteria, fungi, viruses, plant extracts or antibiotics. Inhibition or killing of harmful pests by biocontrol agents is biologically safe, target specific and does not create any environmental pollution. Diseases caused by phytopathogenic fungi not only have major economic impacts in the agriculture industry but also on human well being and biodiversity (Anderson et al., 2004; Wegulo et al., 2011). The fungal cell wall and structural membranes of mycelia, stalks and spores of most *Basidiomycetes, Ascomycetes,* and *Phycomycetes* contain chitin as the major component (George et al., 2011). Hence chitinases which are able to degrade chitin are used as an effective biocontrol remedy against many fungal pathogens especially those of plants. In last two decades, chitinases have received the attention of researchers for their anti-insects and antifungal biocontrol activities (Singh et al., 2014).

Many reports have proven the efficacy of microbial chitinases in degrading pathogenic fungal cell walls and inhibit fungal growth in vitro. Table 6.2 lists out chitinases from various microorganisms which have been reported to inhibit various fungal pathogens. Chitinases from some bacterial strains have been reported to be active against a broad range of pathogenic fungi including *Bacillus licheniformis* (Gomaa, 2012), *Bacillus thuringiensis* (Liu, et al., 2010), *Streptomyces griseus* (Itoh et al., 2003) and *Streptomyces violaceusniger* (Nagpure & Gupta, 2013). Chitinases from thermophilic fungi are also promising for biocontrol and enzymatic conversion of chitin because of their thermostability (Ma et al., 2012). Similarly chitinases have been purified from other fungi including *Tricothecium roseum* (Li et al., 2004) and *Talaromyces flavus* (Li et al., 2005) have been shown to have biocidal activity against a large number of fungal pathogens.

Table 6.2. Utilisation of microbial chitinase in pathogenic fungal inhibition.

Chitinase producing bacterial strain	Fungal strain inhibited by chitinase activity	Reference
Bacillus cereus	*Fusarium oxysporum, Fusarium solani, Pythium ultimum.*	Fleuri et al., 2009
Bacillus licheniformis	*Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Fusarium oxysporum, Fusarium* sp., *Ralstonia solanacearum, Rhizopus* sp.	Gomaa, 2012
Bacillus pumilus	*Fusarium oxysporum*	Bhattacharya et al., 2016
Bacillus subtilis	*Rhizoctonia solani*	Yang et al., 2009
Bacillus subtilis	*Fusarium oxysporum*	Chang et al., 2010
Bacillus thuringiensis	*Rhizoctonia solani ,Botrytis cinerea, Penicillium chrysogenum, Psylla piricola, Penicillium glaucum, Sclerotinia fuckelian*	Liu, et al., 2010
Bacillus thuringiensis	*Aspergillus flavus, Aspergillus niger, Aspergillus terreus*	Gomaa, 2012
Cellulosimicrobium cellulans	*Mucor miehei,Streptomyces phaerochromogenes, Trichoderma viride*	Fleuri et al., 2009
Chitiniphilus shinanonensis	*Trichoderma reesei*	Huang et al., 2012
Enterobacter sp.	*Aspergillus niger, Fusarium* sp., *Mucor* sp.	Salam et al., 2008
Gliocladium catenulatum	*Sclerotinia scleotiorum, Rhizoctonia solani, Botrytis cinerea*	Ma et al., 2012
Monascus purpureus	*Fusarium oxysporum and Fusarium solani*	Wang et al., 2002
Serratia marcescens	*Rhizoctonia solani, Bipolaris sp, Alternaria raphani, Alternaria brassicicola*	Zarei et al., 2011
Serratia marcescens	*Fusarium graminearum, Bipolaris* sp., *Alternaria raphani, Aspergillus brassicicola, Rhizoctonia solani.*	Babashpour, et al., 2012
Stenotrophomonas maltophilia	*Alternaria alternata, Rhizoctonia solani, Fusarium solani, Fusarium oxysporum.*	Jankiewicz et al., 2012
Streptomyces sp.	*Aspergillus niger, Candida albicans*	Han, et al., 2009
Streptomyces sp.	*Fusarium solani, Aspergillus brasiliensis*	Pradeep et al., 2014
Streptomyces griseus	*Aspergillus nidulans, Botrytis cinerea, Fusarium culmorum, Guignardia bidwellii, Sclerotia sclerotiorum*	Itoh et al., 2003
Streptomyces rimosus	*Alternaria alternata, Fusarium solani*	Brzezinska et al., 2013
Streptomyces roseolus	*Aspergillus sp., Rhizosporus chinensis, Mucor* sp., *Penicillium* sp.	Jiang et al., 2012
Streptomyces sporovirgulis	*Alternaria alternata, Pencillium purpurogenum, Penicillium* sp.	Brzezinska et al., 2013
Streptomyces tendae	*Aspergillus niger, Fusarium oxysporum*	Kavitha & Vijayalakshmi, 2011
Streptomyces venezuelae	*Aspergillus niger, Helminthosporum sativum, Alternaria alternata*	Mukherjee & Sen, 2006
Streptomyces violaceusniger	*Phanerochaete chrysosporium, Schizophyllum commune, Gloeophyllum trabeum, Polyporus agaricans, Coriolus versicolor, Pityriasis versicolor, Postia placenta, Polyporus friabilis*	Nagpure & Gupta, 2013
Talaromyces flavus	*Verticillium dahliae, Sclerotinia sclerotiorum* and *Rhizoctonia solani, Alternaria alternata, Fusarium moniliforme, Magnaporthe grisea*	Li et al., 2005
Trichoderma asperellum	*Fusarium oxysporum, Fusarium solani* and *Fusarium udum*	Kumar et al., 2012
Trichoderma harzianum	*Sclerotium rolfsii*	El-Katatny, et al., 2001
Tricothecium roseum	*Alternaria alternata, Fusarium moniliforme and Magnaporthe grisea*	Li et al., 2004

6.2. Materials and Methods

6.2.1. Scanning Electron Microscopy

To observe the effect of chitinase on the surface morphology of chitin, chitin flakes were treated with ammonium sulphate fractionation purified enzyme for 2, 4, 8 and 12 h duration. After the incubation time the sample was heated in boiling water bath for 5 min to inactivate the enzyme and centrifuged at 11000 ×g for 5 min. The supernatant was removed and the treated flakes were fixed in a mixture of 2.5% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde in freshly prepared 0.1 M phosphate buffer for 2 h on clean glass coverslips. After being gently washed with distilled water, fixed samples were dehydrated through a graded ethanol wash (a series of 20-40-60-80 and 100% ethanol for 5 min each), and the sample was then mounted on a stub using double sided carbon tape. The dried samples were sputter coated with gold using SC7620 sputter-coater device and analysed under SEM at an operating voltage of 30 kV (Zeiss Evo-18, Carl Zeiss, Germany).

6.2.2. Study of chitin degradation products and pattern of degradation

6.2.2.1. Thin Layer Chromatography Analysis

Determination of pattern of chitinolytic activity and analysis of of chitinase degradation products using chito-oligosaccharides of different degrees of polymerisation was done by silica gel thin layer chromatography (TLC). The following chito-oligomers were used: N-acetyl-glucosamine (NAG) (Sigma, USA), N,N'-diacetylchitobiose (NAG_2) (Sigma, USA), N,N',N''-triacetylchitotriose (NAG_3) (TCI, Tokyo, Japan), N,N',N'',N'''-tetraacetylchitotetraose (NAG_4) (TCI, Tokyo, Japan), chito-oligosaccharide mixture (TCI, Tokyo, Japan). Reaction mixtures contained 5 mM of each oligosaccharide individually, in 0.2 M citrate-phosphate buffer (pH 6.5) at a final volume of 2.0 mL, and were incubated at 50°C for the following time periods: 15, 30 and 60 min. Colloidal chitin at 1% (w/v)

concentration was used under identical conditions at a final volume of 2.0 mL but was incubated for upto 6 h. The reaction was stopped by incubating the tubes in a boiling water bath for 5 minutes and aliquots of 10 μL of the reaction mixtures were loaded and analyzed on a silica gel plate (Silica Gel 60, 0.25mm Merck, Berlin, Germany) with 5:4:3 (v/v/v) n-butanol/methanol/16% aqueous ammonia as the mobile phase (Kadokura et al., 2007). The degradation products that were released were compared with standards of chitin oligomers of known sizes. After developing the TLC plates, the compounds were visualized by spraying with an aqueous solution of 97% ethanol and 3% acetic acid containing 0.5% (w/v) ninhydrin (ninhidrin reagent) followed by heating on a hot plate until the bands were clearly visible.

6.2.2.2. HPLC Analysis

The chitinase degradation products were also determined using the HPLC method. The chitinase reaction was carried out as mentioned above and assay supernatant was analysed using gel permeation chromatography (GPC) column (Shodex OHpak SB804) with deionised water as mobile phase at a flow rate of 1 mL min^{-1} and detected using refractive index detector. Released products were compared to standards composed of chitin oligomers of known sizes. The enzyme-hydrolyzed chitin degradation products were analyzed by sampling at 30 min intervals.

6.2.3. Antifungal activity of chitinase

6.2.3.1. Agar plate diffusion method

Antifungal activity was assayed by agar diffusion method using various phytopathogenic fungal species including *Botrytis cineria*, *Colletotrichum gleosporoides*, *Penicillium expansum*, *Pythium alphanidermatum*, *Fusarium oxysporum* and *Penicillium chyrosogenum*. Yeast lytic activity of chitinase was assayed using *Candida albicans*. Table 6.3 provides

information about the phytopathogen and its corresponding host. Potato dextrose agar (PDA) plates were spread plated with 0.1 mL fungal spore and yeast cell suspension having a concentration of 10^7 spores/cells mL^{-1}. Further two wells of 10 mm diameter each were bored into the agar as shown in Figure 6.7. Two hundred microlitres of the ion exchange chromatography purified chitinase was added to the experimental well while heat inactivated purified chitinase was added to the control well. The plates were then incubated at 37°C for up to 6 days and observed for zones of clearance around the wells that indicated the cell wall lysis and thus growth inhibition.

Table 6.3. Fungal phytopathogens used in the current study and their host range.

Fungus	Pathogenic host
Colletotrichum gloeosporioides	Infects a variety of fruits and vegetable species including mangoes and apples.
Pythium expansum	Major post harvest rot in apples. Wide host range, including pears, strawberries, tomatoes, corn and rice.
Penicillium chrysogenum	Infects majorly during grain storage in moist conditions.
Pythium aphanidermatum	Soil borne pathogen. Infects beets, peppers, chrysanthemum, cucurbits and cotton.
Fusarium oxysporum	Wide host range, including potato, sugarcane, garden bean, peas, bananas and mellons.
Botrytis cineria	Grapes and strawberries.

6.2.3.2. Microscopic study of invitro lysis of phytopathogenic fungi using chitinase

The phytopathogenic fungi *F. oxysporum, P. chyrosogenum* and *P. expansum* were used for microscopic observation of cell lysis induced by the chitinase. The fungi were grown in potato dextrose agar plates for 10 days at 30°C. The spore germination was carried out in 250 mL Erlenmeyer flasks containing 50 mL of potato-dextrose broth, incubated at 30°C, 200 rpm for 48 h. The mycelial suspension was then centrifuged at 9500 ×g for 5 min at 4°C. The mycelium was washed three times with 0.1 M citrate-phosphate buffer, pH 5.5 and re-suspended in the same. The reaction mixture containing 1.0 mL of fungal suspension in 0.1 M citrate-phosphate buffer, pH 5.5 and 1.0 mL (60 U) ammonium sulphate fractionation purified chitinase, was incubated at 30°C for 30 and 60 min with gentle shaking at regular

intervals. The samples were loaded on the slides and stained with lactophenol cotton blue stain and observed for cell lysis under an optical microscope. The reaction mixture containing the fungal suspension, but without the addition of the enzyme and incubated for the same time at 30°C, was used as control.

6.2.4. Deacetylation studies of chitin and chitosan using CDA enzyme

6.2.4.1. Sodium Hydroxide Deacetylation of Chitosan

NaOH based deacetylation was carried out with slight variation in the method as suggested by Yuan et al. (2011). Two chitosans, one from Himedia Laboratories (India) and the other a low molecular weight chitosan from Sigma (USA) and colloidal chitin was used for the study. They were added to a 45% sodium hydroxide solution in a 1:10 (w/v) ratio in a screw-cap flask and the reaction was maintained under nitrogen atmosphere. It was then incubated at 90°C for 60 min. After reacting, the product was filtered and washed with distilled water until a neutral pH was obtained. The resulting products were dried in a vacuum oven for 24 h at 45°C.

6.2.4.2. CDA Deacetylation of Chitosan

The enzymatic deacetylation was carried out using 1.0 g colloidal chitin/chitosan incubated at 45°C in a 10mL CDA enzyme (crude)-buffer (0.1M phosphate citrate buffer, pH 7) mixture which contained 90 U final enzyme activity. It was incubated for 6 h with intermittent mixing. After that the product was filtered and washed with distilled water twice. The resulting products were dried in a vacuum oven for 24 h at 45°C.

6.2.4.3. Determination of Degree of Deacetylation (DDA)

6.2.4.3.1. Acid-Base Titration

The acid-base titration method was performed as specified by Yuan et al. (2011). Chitosan (0.3–0.5 g) was dissolved in 30 mL 0.1 M HCl at 20 ± 5°C with stirring in a 250 mL flask

and then two drops of methyl orange indicator was added. NaOH at 0.1 M concentration was used to titrate the solution. At the final point of titration, the colour changes from pink to orange yellow. A pH meter was also used to make the final titration point determination more precise. To calculate water content, 0.5 g chitosan was heated in an oven at 105°C until a constant weight was reached. Each experiment was carried out in triplicates. The percentage of free -NH_2 groups in chitosan were calculated as follows:

$$NH_2\% = [(C_1V_1 - C_2V_2) \times 0.016] / [G\,(100 - W)] \times 100 \quad (1)$$

Free $NH_2\% = NH_2\%/9.94\% \times 100\%$

Chitosan theoretic NH_2 content % = (16/161) × 100% = 9.94%

C_1: Concentration of HCl (M); C_2: Concentration of NaOH (M); V_1: the volume of HCl added (mL); V_2: the volume of NaOH added by titration (mL); G: Sample weight (g); W: sample water content (%); 0.016: equal to NH_2 content (g) in 1 mL of 1 M HCl.

6.2.4.3.1. Ultraviolet-Vis Spectrophotometry

The UV-vis spectrophotometric method was performed as specified by Yuan et al. (2011). NAG was dissolved in 0.001 mol L^{-1} HCl to prepare 0.1 mg mL^{-1} standard solution. A series of 0.01, 0.02, 0.03... 0.1 mg mL^{-1} standard solutions was prepared from the 0.1 mg mL^{-1} standard solution and the absorbance of each solution at 199 nm was determined on a UV-vis spectrophotometer (Agilent 8453 instrument) using 0.001 mol L^{-1} HCl as reference. A standard curve of concentration vs. absorbance was generated. Chitosan (10–20 mg) was dissolved in 10 mL 0.01 mol L^{-1} HCl in a 100 mL volumetric flask. After the chitosan was dissolved completely, the solution was diluted to 100 mL using de-ionized water. With reference to the standard curve, the concentration of acetyl could be determined by measuring the solution absorbance at 199 nm. DDA can be calculated according to the equation:

$$DDA = 100\% - C_1/C \quad (2)$$

where C_1 is the acetyl concentration of sample and C is concentration of sample.

6.3. Results and Discussion

6.3.1. Scanning Electron Microscopy (SEM)

To observe the surface changes of chitin powder treated by chitinase, SEM was performed. In the absence of chitinase treatment (control), chitin powder showed almost a smooth surface (Fig. 6.1). However, when chitin was exposed to chitinase, it showed degradation and distortion of the chitin surface which increased significantly by increasing the time of exposure. Similar morphological changes on the chitin surface were observed for a chitinase isolated from *Serratia marcescens* (Zarei et al., 2011), *Streptomyces roseolus* (Jiang et al., 2012) and *Penicillium* sp. (Lee et al., 2009). The results implied that isolated chitinase is quite effective in degradation of chitin based on the evidence from SEM data.

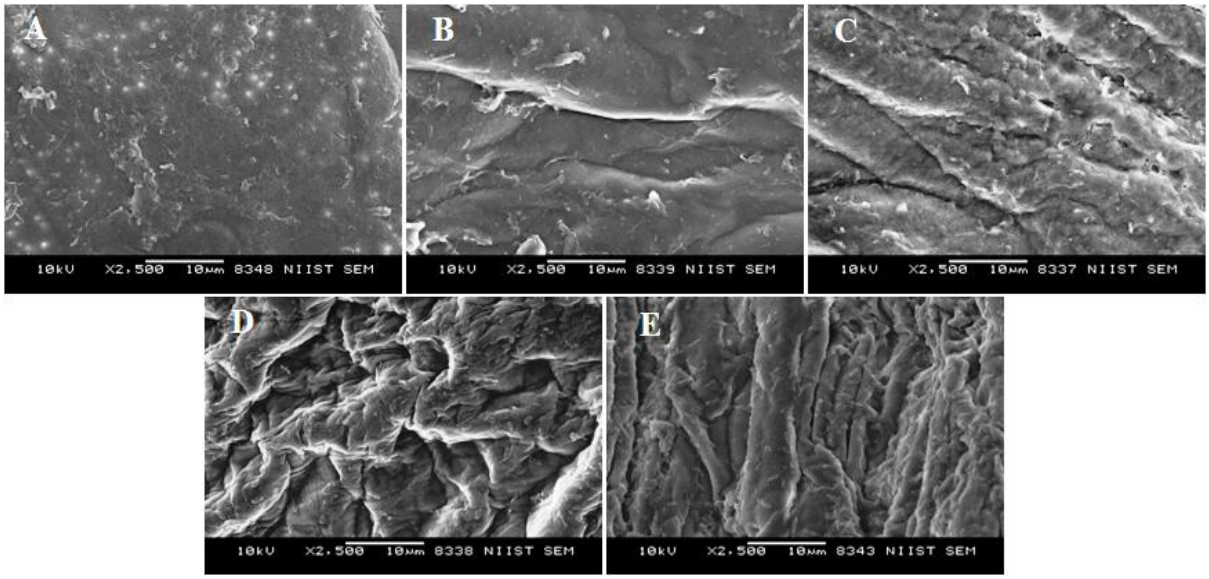

Fig. 6.1. Effect of chitinase on chitin flakes over time- SEM study; A: non-treated control; B: 2 h; C: 4 h; D: 8 h; E: 12 h.

6.3.2. Study of chitin degradation products and pattern of degradation

It was observed that NAG_2 was hydrolysed gradually but fully to NAG over a time period of 60 min (Fig. 6.2 and Fig. 6.3). In the case of chitinase hydrolysis of NAG_3, NAG_4 and chito-oligosaccharide mixture, between 15 min and 30 min of incubation time, there was an increase in the concentration of both NAG and NAG_2. But by the end of 60 min, the end product consisted of only NAG. There was some residual NAG3 even at the end of 60 min. This was verified when compared with standard NAG and its polymers using TLC (Fig. 6.2 and Fig. 6.3). When colloidal chitin was used as substrate, NAG_2 was initially generated which was further hydrolysed to NAG as observed, over a period of 6 h. But the rate of hydrolysis of colloidal chitin was comparatively slower than the chito-oligosaccharides. The degradation products of colloidal chitin after 6h are observed in Figures 6.4.

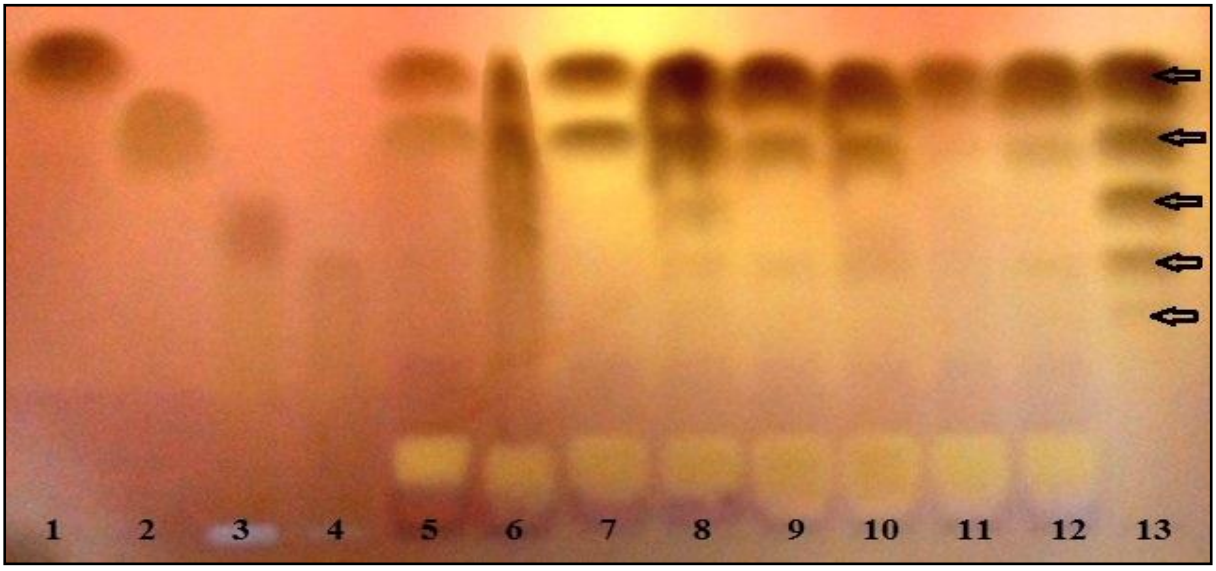

Fig. 6.2. TLC analysis of chitinase degradation products (I). **1.** NAG standard; **2.** NAG_2 standard; **3.** NAG_3 standard; **4.** NAG_4 standard; **5.** NAG_2+chitinase (15 min); **6.** NAG_3+chitinase (15 min); **7.** NAG_4+chitinase (15 min); **8.** Chito-oligosaccharide mixture+chitinase (15 min); **9.** NAG_2+chitinase (30 min); **10.** NAG_3+chitinase (30 min); **11.** NAG_4+chitinase (30 min); **12.** Chito-oligosaccharide mixture+chitinase (30 min); **13.** Oligosaccharide mixture (Arrows indicate bands corresponding to NAG and its polymers).

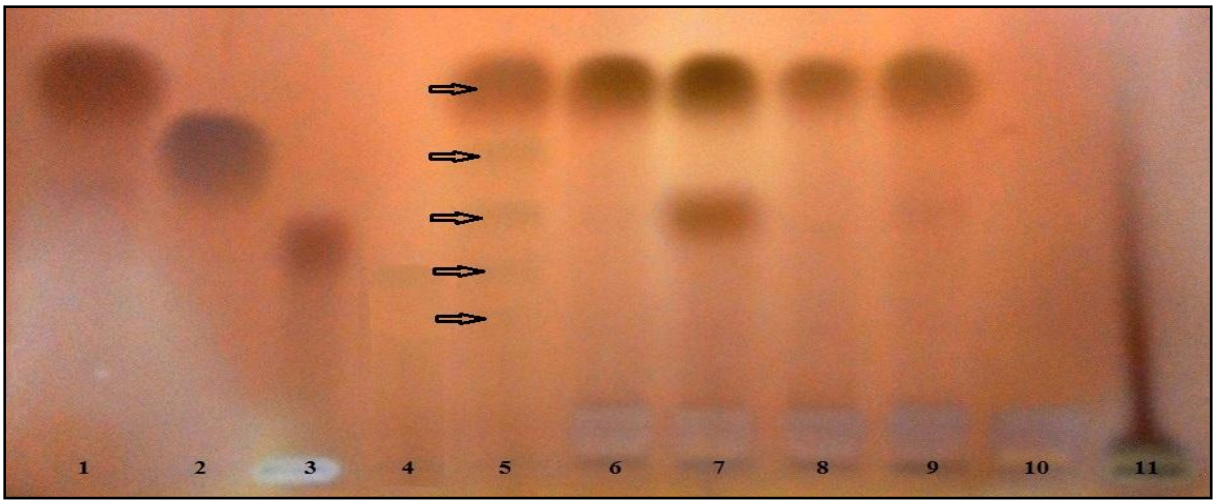

Fig. 6.3. TLC analysis of chitinase degradation products (II). **1.** NAG standard; **2.** NAG_2 standard; **3.** NAG_3 standard; **4.** NAG_4 standard; **5.** Chito-oligosaccharide mixture (Arrows indicate bands corresponding to NAG and its polymers); **6.** NAG_2+chitinase (60 min); **7.** NAG_3+chitinase (60 min); **8.** NAG_4+chitinase (60 min); **9.** Chito-oligosaccharide mixture+chitinase (60 min); **10.** Colloidal chitin+chitinase (0h); **11.** Colloidal chitin +chitinase (3 h).

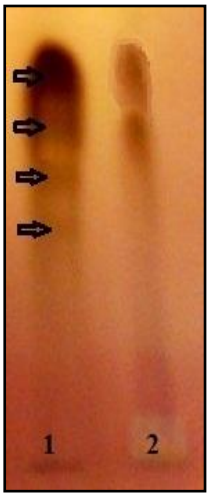

Fig. 6.4. TLC analysis of chitinase degradation products (III). **1.** Chito-oligosaccharide mixture (Arrows indicate bands corresponding to NAG and its polymers); **2.** Colloidal chitin +chitinase (6 h).

Similar results were observed using HPLC analysis. The only drawback with HPLC analysis was that NAG_2, NAG_3 and NAG_4 did not separate into individual peaks while NAG was eluted out as an individual peak as observed in Figure 6.5.

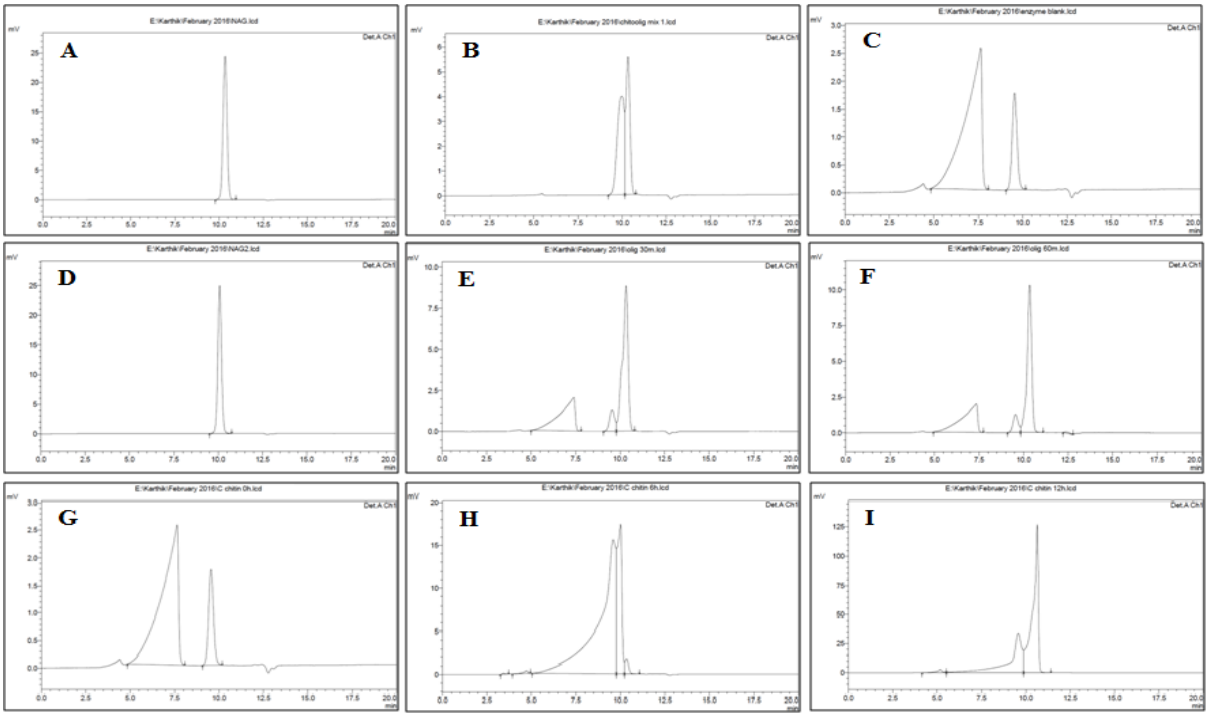

Fig. 6.5. HPLC analysis of chitinase degradation products. **A.** NAG standard; **B.** Chito-oligosaccharide mixture standard; **C.** Enzyme blank; **D.** NAG_2 standard; **E.** Chito-oligosaccharide mixture+chitinase- 30 min; **F.** 60 min; **G.** Colloidal chitin untreated; **H.** Colloidal chitin +chitinase- 6h; **F.** 12h.

This indicates that the chitinase produced by the *Streptomyces* sp. is an exochitinase showing both chitobiosidase and n-acetyl-β-glucosaminidase activities, thus producing NAG_2 and further hydrolysing it to NAG, from chito-oligosaccharides and colloidal chitin (Fig. 6.6).

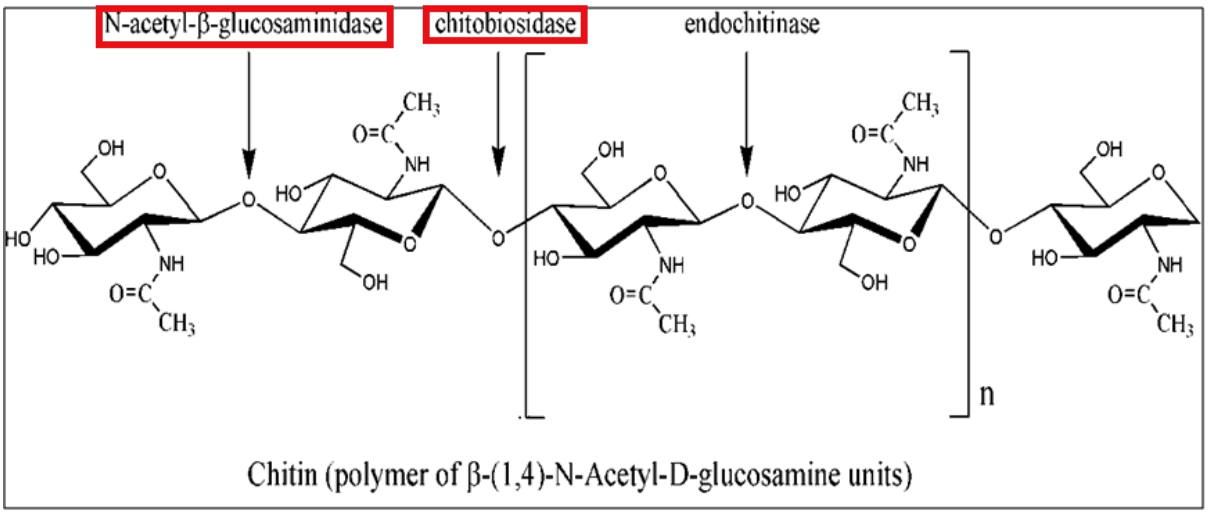

Fig. 6.6. Action of chitinase enzyme (Action of *Streptomyces* sp. exochitinase is highlighted in red).

Using the TLC method, chitinase isolated from *Rhodothermus marinus* (Hobel et al., 2005) was identified to show endochitinase, chitobiosidase and n-acetyl-β-glucosaminidase activities while chitinases produced by *Bacillus circulans* (Siwayaprahm et al., 2006) and a *Streptomyces* sp. (Pradeep et al., 2014) showed both chitobiosidase and n-acetyl-β-glucosaminidase activities. A *Paenibacillus thermoaerophilus* chitinase showed endochitinase and chitobiosidase activities only (Ueda and Kurosawa, 2015). Chitinases produced by *Serratia* sp. (Kim et al., 2007), *Vibrio parahaemolyticus* (Kadokura et al., 2007), *Streptomyces cyaneus* (Yano et al., 2008) and *Bacillus licheniformis* (Waghmare and Ghosh, 2010) were reported to show only chitobiosidae activity. Using the HPLC method two *Trichoderma* sp. chitinases were reported to show only chitobiosidase activity (Hoell et al., 2005, Ike et al., 2006) while a *Streptomyces* sp. showed only n-acetyl-β-glucosaminidase activity (Prakash et al., 2013). An *Enterobacter* sp. was reported to produce an enzyme showing both chitobiosidase and n-acetyl-β-glucosaminidase activities (Dahiya et al., 2005).

6.3.3. Antifungal activity of chitinase

6.3.3.1. Agar plate diffusion method

The antifungal activity of the *Streptomyces* sp. chitinase was determined by agar plate diffusion method. Inhibition was observed on PDA plates inoculated with various phytopathogenic fungi and wells loaded with purified chitinase. The chitinase inhibited the growth of *C. gleosporoides, P. expansum, P. alphanidermatum, F. oxysporum* and *P. chyrosogenum*. The hyphal inhibition was observed as a zone of clearance around the well containing the active purified chitinase, while abundant growth was observed around the control well (Fig. 6.7). But there was no growth inhibition observed in the case of *C. albicans* as abundant growth was seen around both the control well as well as the well containing the

active purified chitinase (Fig. 6.8). Hence it may be said that the chitinase is active only against filamentous fungi and not against yeasts.

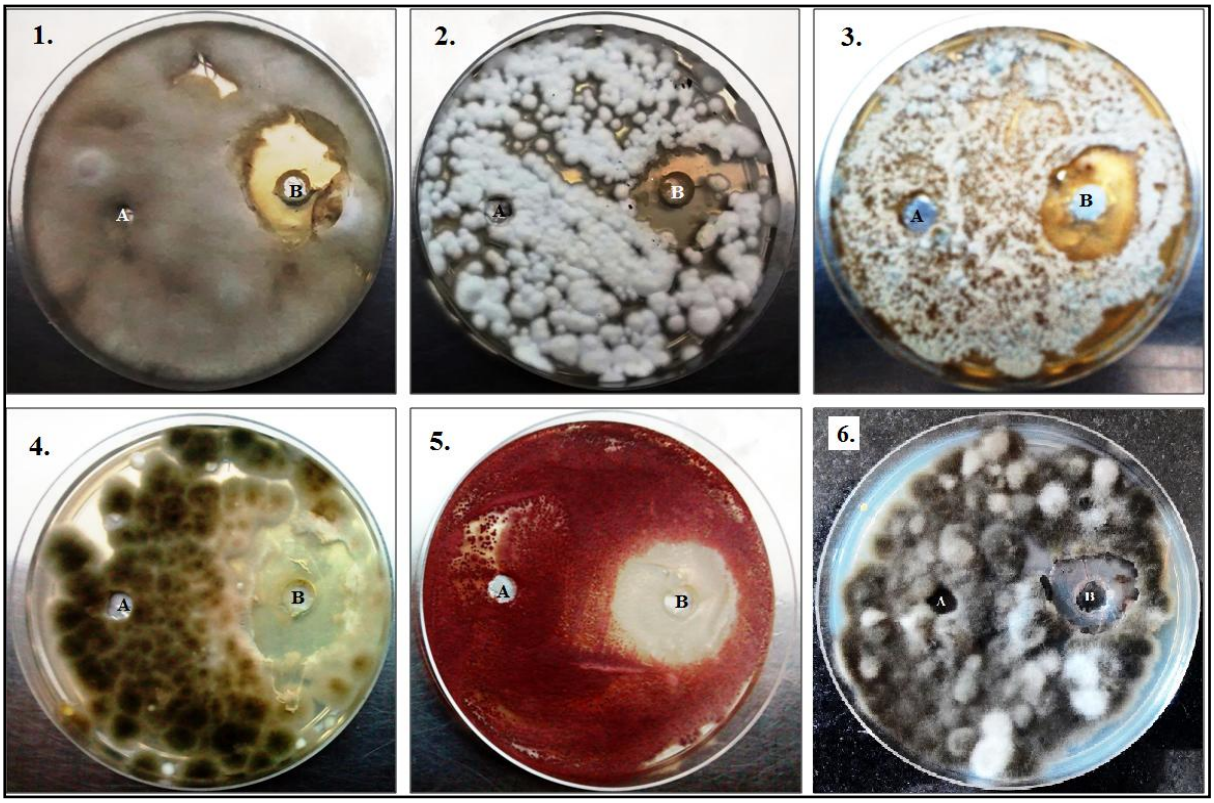

Fig. 6.7. Antifungal activity determination of chitinase- Agar plate diffusion method (**A:** Heat inactivated chitinase, **B:** Ammonium sulphate precipitation purified chitinase).
1. *Colletotrichum gloeosporioides*; **2.** *Pythium expansum*; **3.** *Penicillium chrysogenum* ;
4. *Pythium alphanidermatum*; **5.** *Fusarium oxysporum*; **6.** *Botrytis cineria*.

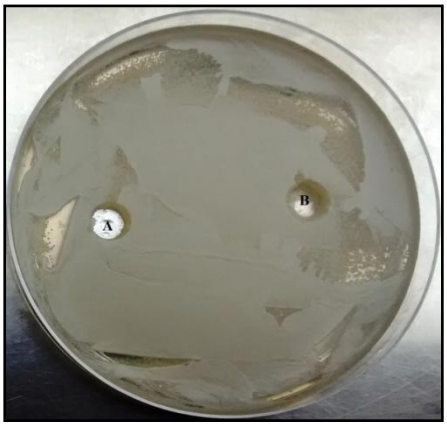

Fig. 6.8. Antifungal activity determination of chitinase- Agar plate diffusion method- *Candida albicans* (**A:** Heat inactivated chitinase, **B:** Ammonium sulphate precipitation purified chitinase).

The chitinases from *S. halstedii* (Joo et al., 2005), *B. thuringiensis* (Liu et al., 2010), *S. marcescens* (Babashpour et al., 2012), *S. griseus* (Itoh et al., 2003), *S. maltophilia* (Jankiewicz et al., 2012), *S.roseolus* (Jiang et al., 2012) and *S. violaceusniger* (Nagpure and Gupta, 2013) have been reported to inhibit the growth of *Fusarium graminearum, Bipolaris* sp., *Mucor* sp., *Aspergillus brassicicola, Rhizoctonia solani. Alternaria alternata, Rhizoctonia solani, Fusarium oxysporum, Phytophthora capsici, Colletotrichum gloeosporioides, Stemphylium lycopersici, Botrytis cinerea, Penicillium chrysogenum, Penicillium glaucum, Phanerochaete chrysosporium, Schizophyllum commune, Gloeophyllum trabeum, Coriolus versicolor, Rhizosporus chinensis and Pityriasis versicolor*. A *Streptomyces* sp. chitinase was reported to inhibit *Candida albicans* (Han et al., 2009). Reports of other microbial chitinases showing antifungal activity have been shown in Table 6.2.

6.3.3.2. Microscopic study of invitro lysis of phytopathogenic fungi using chitinase

Cell lysis was observed under an optical microscope. Figure 6.9 illustrates the lysis of the fungi *F. oxysporum, P. chyrosogenum* and *P. expansum* by the partially purified chitinase as compared to their respective controls. As observed under the microscope the fungal biomass was highly hydrolysed, and only fragmented residues were found on the slides after 60 minutes of incubation (Figure 6.9). This is because the chitinase degrades the cell wall chitin polymers and thus lyses the cell. Microscopic observations revealed that a 61 kDa chitinase obtained from *Cellulosimicrobium cellulans* was capable of lysing the fungi *Mucor miehei, Streptomyces phaerochromogenes* and *Trichoderma viride* and capable of producing protoplasts of *Rhizopus oligosporus* and *Penicillium* sp. (Fleuri et al., 2009).

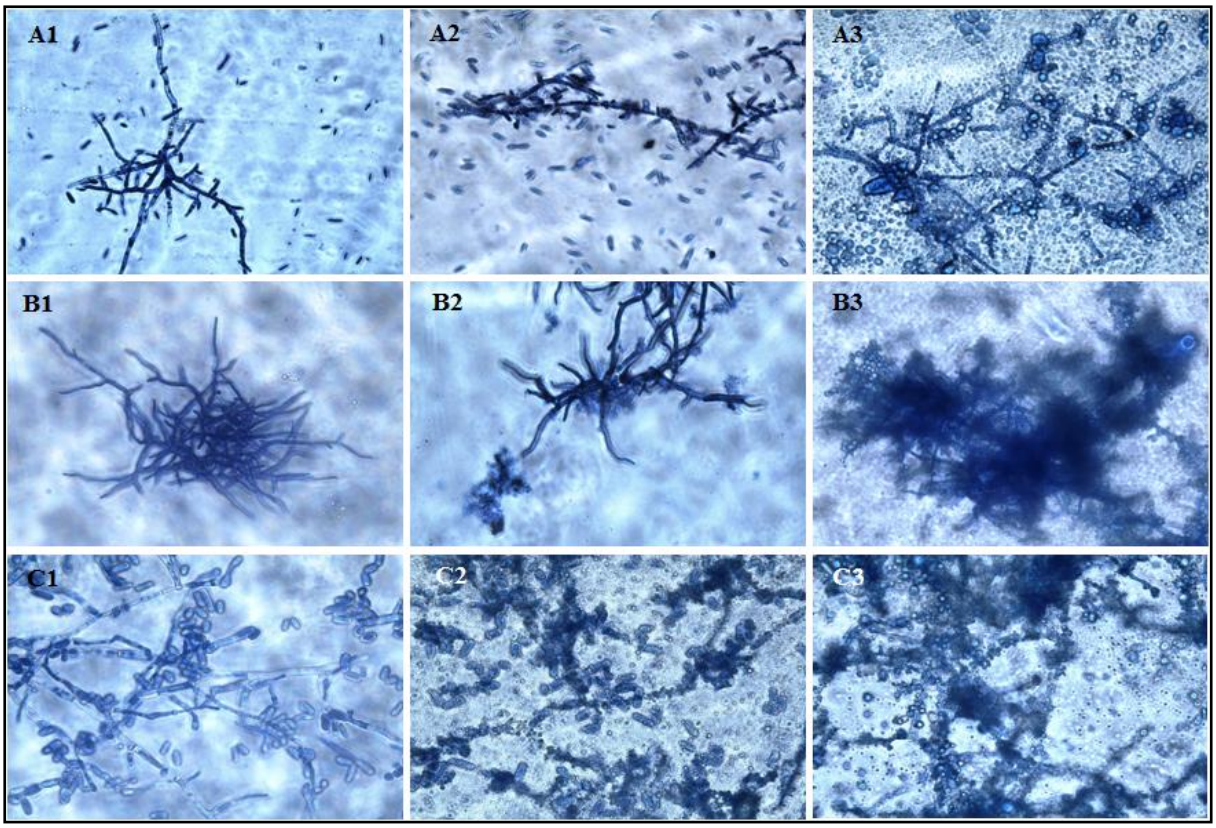

Fig. 6.9. Microscopic study of invitro lysis of phytopathogenic fungi using chitinase. **A:** *Penicillium chrysogenum*; **B:** *Fusarium oxysporum*; **C:** *Pythium expansum*; **1:** non-treated; **2:** 30 min; **3:** 60 min.

6.3.4. Deacetylation studies of chitin and chitosan using sodium hydroxide and CDA enzyme

Acid-base titration and UV-vis spectrophotometry were used to determine the degree of deacetylation (DDA) of deacetylated chitin and chitosan. The standard curve of concentration vs. absorbance of NAG dissolved in 0.001 mol L^{-1} HCl at 199 nm was generated as observed in Figure 6.10.

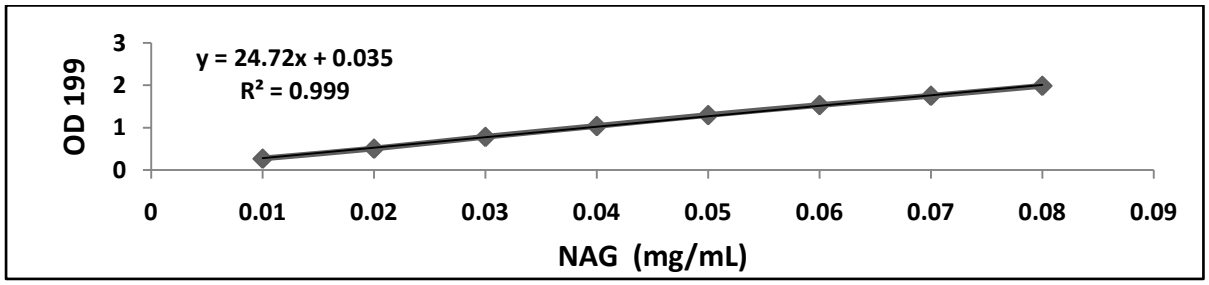

Fig. 6.10. Standard curve of concentration vs. absorbance of NAG dissolved in 0.001 mol/L HCl at 199 nm.

Table 6.4 shows that the DDA determined by the titration method was almost similar to the DDA determined by the UV-vis spectrophotometric method, though the spectrophotometric method tended to give comparatively higher DDA values than that of the titration method.

Table 6.4. Determination of DDA of different chitosan samples by different methods.

Type of chitin	Deacetylation Method	Estimation of degree of deacetylation (% DDA)	
		Titration Method	Spectrophotometric Method
Chitosan lmw	Control	75.64 ± 0.78	77.50 ± 0.96
	NaOH	86.25 ± 0.73	89.77 ± 1.23
	CDA	80.48 ± 0.85	81.90 ± 1.03
Chitosan	Control	71.54 ± 1.03	74.21 ± 1.18
	NaOH	83.46 ± 0.79	85.44 ± 0.93
	CDA	75.31 ± 0.56	77.66 ± 0.84
Colloidal chitin	Control	54.78 ± 0.78	57.88 ± 0.94
	NaOH	66.24 ± 1.27	70.14 ± 0.55
	CDA	59.10 ± 0.67	60.58 ± 0.92

Determination of the DDA of chitin and chitosan by titration method was done by Jia and Shen (2002), while the same was carried out using UV-vis spectrophotometric method by Wu and Zivanovic (2008). Past studies have also reported that titration methods yield DDA values comparable to FT-IR and ^{1}H-NMR methods suggesting that titration methods are useful and appropriate means for determining DDA (Lavertu et al., 2003; Kasaai, 2008). Among the two methods which were used here, the acid-base titration, when based on selected procedures and standard analytical solutions of well-known concentrations, is the better method, since it is convenient, easy, highly repeatable and does not require expensive equipment or reagents. The only disadvantage is the relatively long time used for sample preparation and that required for the titration itself. UV-Vis when carefully performed produced good results, with the advantage that it could be done using generally available equipment (UV-Vis spectrometer) and easy measurements. But the procedure is quite time-consuming (preparation of calibration) and is potentially sensitive to contaminants (absorbing

below 210 nm) that can influence spectra and hence the final results. An extensive comparison of different methods for determination of deacetylation of chitin-chitosan was carried out by Yuan et al., (2011) and Czechowska-Biskup et al., (2012).

6.4. Summary and Conclusions

There was observed surface degradation of chitin flakes over time on treatment with the chitinase enzyme produced by *Streptomyces* sp. Chitin oligomers as well as colloidal chitin was degraded to give rise to dimers which was further cleaved to give rise to monomeric NAG by the chitinase enzyme thus proving it to have both chitobiosidsae as well as N-acetyl-β-glucosaminidase activity. It could be hence concluded that the *Streptomyces* sp. chitinase is an exochitinase. The antifungal activity of the crude enzyme extract was evidenced by the in vitro antifungal assay. Extensive lysis and degradation was observed in 48 h old mycelium of various fungal phytopathogens including *Colletotrichum gloeosporioides*, *Pythium expansum*, *Penicillium chrysogenum, Pythium alphanidermatum*, *Fusarium oxysporum* and *Botrytis cineria*. A major concern in developing the commercial biopesticides is the speed of kill as compared to the chemical pesticides. So if it was possible to even partially replace the use of chemical pesticides with effective formulations of the chitinase spray, it might to facilitate faster killing of the pathogens in a more environmentally friendly way. It was observed that the *Aspergillus flavus* CDA enzyme was able to deacetylate chitosan and colloidal chitin, although not as much as compared to chemical deacetylation using concentrated NaOH. But its use may be explored in further efficiently deacetylating the partially deacetylated chitins coming from a mild chemical deacetylation process.

Chapter 7

Summary and Conclusions

Summary

Chitin which is one of the most abundantly produced biopolymers, is present in many organisms in different forms. It is a growing cause of environmental concern in industry (waste decomposition and processing into chitosan) and agriculture (as protective structures in pests). This has led to increased interest in chitin-hydrolyzing enzymes i.e. chitinases that break down the polymeric chitin to produce mono- and oligomers. The inducible nature of chitinases, low activity of synthesized enzymes, and inertia of the substrate are a few of the problems that can be solved by biotechnology to meet industry demands for green, energy-efficient, pollution-free, and economically profitable chitin use. Similarly the chitin-modifying enzyme viz. CDA, which deacetylates chitin to chitosan is being explored as a suitable alternative to the environmentally damaging chemical deacetylation process.

The thesis illustrates the experiments and observations related mainly to the production, purification and characterisation of the chitinase produced by a *Streptomyces* sp. under SmF, and CDA produced by an *Aspergillus flavus* strain under both SmF and SSF. These microbial strains were isolated from environmental samples collected from different places in south Kerala, India. The following could be summarized as the major results of the research carried out:

Several chitinase producing microbial strains were isolated from natural environment by enrichment technique on selective chitin agar medium of which an isolate named P6B2 was identified as the highest producer. It was identified as a *Streptomyces* sp. bearing highest homology to the type strain *Streptomyces enissocaesilis* strain NRRL B-16365 using the 16S-rDNA identification method.

Optimization of process parameters for SmF production of the chitinase enzyme in 250 mL shake-flasks was carried out by first conducting single parameter optimisation and further by multiple parameter statistical optimisation methods using Plackett-Burman design

followed by Taguchi design. The optimal conditions were identified to be a medium consisting of 2.0 g L^{-1} glucose, 10.0 g L^{-1} CC, 8.0 g L^{-1} YE / 10.0 g L^{-1} CSL, 1.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 2.0 g L^{-1} NaCl, 0.5 g L^{-1} $MgSO_4$ and 0.5 g L^{-1} $CaCl_2$ at pH 8.0 and incubated at 32ºC, 200 rpm for 60 h. A maximum chitinase production of 106 U was obtained using yeast extract and 103 U was obtained using CSL as nitrogen source after 60 h of incubation. The chitinase production in increased 2.35-fold in comparison to the unoptimised basal level medium. A bioprocess study of 1 L volume was carried out in a 2.5 L bioreactor using yeast extract as the nitrogen source and a maximum chitinase production of 79 U was obtained after 60h of fermentation when maintained at 32ºC, with the aeration being controlled between 0.5-1.5 vvm and the impeller speed at 300 rpm. When CSL was used as the nitrogen source, a maximum chitinase production of 75 U was obtained. Hence it could be concluded that use of CSL as nitrogen source gave comparable results as that of YE. Since CSL is comparatively cheaper, it may make the bioprocess economically viable.

Purification of chitinase enzyme was done using methods like ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography consecutively. A 12.44-fold purification was achieved with 26.5% final yield. Characterisation of chitinase enzyme was done with respect to parameters including effect of pH, temperature, salinity, addition of metal ions, solvents and denaturing agents. The chitinase showed highest activity at pH 2.0 and 6.0 (dual pH optima) and the temperature optimum was 50ºC. Characterisation of chitinase enzyme purity (after each step of purification) by SDS-PAGE was done. Activity staining of the chitinase was carried out by Native-PAGE method. The chitinase enzyme showed a single clear band by zymography, and confirmed that it was a chitinase and only a single protein with chitinase activity was produced with no isozymes. The pI and molecular weight of the chitinase enzyme were

identified to be 6.9 and 40 kDa, respectively. The K_m and V_{max} values of chitinase enzyme were determined to be 6.74 mg mL^{-1} and 61.3 U mg^{-1}, respectively using colloidal chitin.

Of the CDA producing microbial strains isolated from natural environment by enrichment technique on selective 4-nitroacetanilide containing agar medium, a fungal isolate named I8 was identified as the highest producer. It was identified as an *Aspergillus flavus* strain using the ITS identification method.

Optimization of process parameters for SmF production of the CDA enzyme in 250 mL shake-flasks was carried out by first conducting single parameter optimisation and further by multiple parameter statistical optimisation methods using Plackett-Burman design followed by Central-composite design. The optimal conditions were identified to be a medium consisting of 35.0 g L^{-1} glucose, 40.0 g L^{-1} YE, 15.0 g L^{-1} peptone and 5.0 g L^{-1} $MgCl_2$ at initial medium pH of 7.0 and incubated at 32ºC, 200 rpm for 48 h. The CDA production at optimal conditions was found to be 58 U. The CDA production in SmF increased 5.98-fold in comparison to the unoptimised basal level medium. Wheat bran was used as the substrate for the SSF medium. Optimization of process parameters for SSF production of the CDA enzyme was carried out by multiple parameter statistical optimisation methods using Plackett-Burman design followed by Central-composite design. The optimal conditions were identified to be a medium consisting of 4.5 g wheat bran, 45% initial moisture content, 35.0 g L^{-1} glucose, 45.0 g L^{-1} meat extract and 5.0 g L^{-1} $MgCl_2$ in 250 mL Erlenmeyer flasks when incubated at 32ºC for 72 h. The CDA production at optimal conditions was found to be 142 U. The CDA production in SmF increased 1.79-fold in comparison to the unoptimised basal level medium.

Purification of CDA enzyme was done using methods like ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography consecutively. A 3.27-fold purification was achieved with 32.03% final yield.

Characterisation of CDA enzyme was done with respect to parameters including effect of pH, temperature, salinity, addition of metal ions, solvents and denaturing agents. The pH and temperature optima of the CDA enzyme were determined to be 8.0 and 50ºC, respectively. Characterisation of CDA enzyme purity (after each step of purification) by SDS-PAGE was done. Activity staining of the CDA was carried out by Native-PAGE method. The CDA enzyme showed a single clear band by zymography, and confirmed that it was a CDA and only a single protein with CDA activity was produced with no isozymes. The molecular weight of the CDA enzyme was determined to be 28 kDa, respectively. The K_m and V_{max} values of chitinase enzyme were determined to be 60.64 mg mL^{-1} and 136.57 U mg^{-1}, respectively using colloidal chitin. The CDA enzyme activity was substantially inhibited by acetic acid and finally reduced to zero at concentrations above 75 mM of acetic acid. This suggests that the enzyme is inhibited by the product of the reaction it catalyses.

There was observed surface degradation of chitin flakes over time on treatment with the chitinase enzyme. Chitin oligomers as well as colloidal chitin were hydrolysed to give rise to dimers which was further cleaved to give rise to monomeric NAG by the chitinase enzyme thus proving it to have both chitobiosidase as well as N-acetyl-β-glucosaminidase activity. This was determined using both TLC and HPLC methods. Phytopathogenic fungal inhibition test for chitinase enzyme was done by agar plate diffusion method. Also, it was observed under the microscope, that after treating the fungal biomass with chitinase enzyme, there was degradation of the fungal mycelia. The chitinase was able to inhibit phytopathogenic filamentous fungi but not yeasts. A comparision between the degree of deacetylation of chitin and chitosan was done which was determined by both acid-base titration method and UV-vis spectrophotometric method. The spectrophotometric method tended to give comparatively higher DDA values than that of the titration method. It was

observed that the CDA enzyme was able to deacetylate chitosan and colloidal chitin, although not as much as compared to chemical deacetylation using concentrated NaOH.

Conclusions

In past two decades, a significant number of studies pertaining to chitinases were carried out due to its application in various fields. These included the discovery, purification and characterization of chitinases from numerous microbial sources. Nevertheless, chitinases are still applied infrequently. For making chitinases more accessible and to apply them at industrial levels, the development of techniques and methods for their production and purification at decreased prices is necessary. Microorganisms are the preferred source of the chitinases since they have an advantage of reduced production time and costs, coupled with higher enzyme titres. Process parameters such as carbon and nitrogen sources, pH, temperature, aeration and agitation exert significant effect on its production.

Results of the present study showed that the chitinase from the *Streptomyces* sp. possessed the characteristics suited for an industrial enzyme. The enzyme was significantly active and stable in the acidic range of pH and could tolerate significantly high salt concentrations. Temperature stability results suggest that the crude enzyme preparation can be stored under refrigerated conditions (4°C) for prolonged periods without significant activity loss. Results also showed that the chitinase could be used as efficient biocontrol agent against phytopathogenic fungi. Also the enzyme generated NAG from colloidal chitin, which has varied applications as mentioned previously.

The major prospect in the practical use of CDAs as of today is to simplify the harsh and non-environmentally friendly chemical conversion process of chitin to chitosan. The enzymatic deacetylation would provide more regularly deacetylated chitosans, since it does not proceed in a random fashion as in the case of chemical deacetylation. Even if it were not

being able to carry out efficient deacetylation using CDA, it would permit to deacetylate to a greater degree the partially deacetylated chitins, coming from a mild chemical deacetylation process. In the current research work an attempt has been made to optimise the process of CDA production from an *Aspergillus flavus* isolate as well as understand the physicochemical properties of the enzyme to validate its industrial viability. The ability to deacetylate water insoluble chitin (colloidal chitin) combined with a reasonably high optimum temperature of enzyme activity makes the *Aspergillus flavus* CDA a potential candidate enzyme to be used for large scale deacetylation of chitin, by at-least partially replacing the currently used chemical method of deacetylation. The further leads to this work would be to scale-up the process, especially in SSF, to study its viability in both production and economical aspects.

Growing interest in chitinous materials and their associated enzymes in bio-medical, agricultural and environmental field will increase market demand for chitin modifying enzymes. A complete study on the nature of such enzymes will make them more useful in a variety of processes in near future.

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Annexure I

Research Publications

[1] **Karthik, N.**, Binod, P., & Pandey, A. SSF production, purification and characterisation of chitin deacetylase from *Aspergillus flavus*. (communicated)

[2] **Karthik, N.**, Binod, P., & Pandey, A. (2015). Production of chitin deacetylase by *Aspergillus flavus* in submerged conditions. *Preparative Biochemistry and Biotechnology*, *46* (5), 501-508. [**DOI:**10.1080/10826068.2015.1084517]

[3] **Karthik, N.**, Binod, P., & Pandey, A. (2015). Purification and characterisation of an acidic and antifungal chitinase produced by a *Streptomyces* sp. *Bioresource Technology*, *188*, 195-201. [**DOI**:10.1016/j.biortech.2015.03.006]

[4] **Karthik, N.**, Binod, P., & Pandey, A. (2013). Bioprocess Optimisation for the Production of Chitinase from *Streptomyces* sp. Isolated from Coastal Environment Samples from South Kerala. *Journal of Chitin and Chitosan Science*, *1*(3), 177-185. [**DOI:**10.1166/jcc.2013.1028]

[5] **Karthik, N.**, Akanksha, K., Binod, P., & Pandey, A. (2014). Production, purification and properties of fungal chitinases-A review. *Indian Journal of Experimental Biology, 52*, 1025-1035 [**DOI**: ir.niist.res.in:8080/jspui/handle/123456789/1723]

[6] **Karthik, N.,** Binod, P., & Pandey, A. Chitinases, In: Current Developments in *Biotechnology and Bioengineering, Book 7: Production, Isolation and Purification of Industrial Products*, (Eds: A. Pandey, S. Negi and C. Soccol) Elsevier, U.K. (In Press)

Annexure II

Conference Proceedings

[1] **Karthik, N.**, Binod, P., & Pandey, A. Purification and characterization of extracellular chitin deacetylase from *Aspergillus flavus*. *Recent Trends in Biosciences*, Alagappa University, Karaikudi, Tamil Nadu. April 2016. (Poster presentation)

[2] **Karthik, N.**, Binod, P., & Pandey, A. Bioprocess optimisation for enhanced production of chitin deacetylase by an *Aspergillus flavus* isolate under submerged conditions. *BRSI-New Horizons in Biotechnology*, CSIR-NIIST, Trivandrum, Kerala. November 2015. (Poster presentation)

[3] **Karthik, N.**, Binod, P., & Pandey, A. Purification and characterization of extracellular chitinase with antifungal activity from a *Streptomyces sp*. *BRSI-International Conference on Emerging Trends in Biotechnology*, Jawaharlal Nehru University, New Delhi. November 2014. (Poster presentation)

[4] **Karthik, N.**, Binod, P., & Pandey, A. Bioprocess optimisation for the production of chitinase from a *Streptomyces sp*. isolated from coastal environment samples from south Kerala. *National Symposium on Emerging Issues in Chitin and Chitosan Research*, CSIR-IHBT, Palampur, Himachal Pradesh. June 2013. (Oral presentation)

[5] **Karthik, N.**, Binod, P., & Pandey, A. Culture Isolation from Coastal Environment Samples & Bioprocess Optimisation for Production of Chitinolytic Enzymes. *BRSI-International Conference on Industrial Biotechnology*, Punjabi University, Patiala, Punjab. November 2012. (Poster presentation)

Annexure III
AcSIR Coursework

Level 100

Course number	Title	Credits	Status
BIO-NIIST-1-001	Biostatistics	1	Completed
BIO-NIIST-1-002	Bioinformatics	1	Completed
BIO-NIIST-1-003	Basic Chemistry	1	Completed
BIO-NIIST-1-004	Research Methodology, Communication, Ethics & Safety	1	Completed

Level 200

Course number	Title	Credits	Status
BIO-NIIST-2-001	Biotechniques and Instrumentation	1	Completed
BIO-NIIST-2-256	Basic and Applied Microbiology	2	Completed
BIO-NIIST-2-257	Basic Molecular Biology	2	Completed

Level 300

Course number	Title	Credits	Status
BIO-NIIST-3-001	Seminar Course	1	Completed
BIO-NIIST-3-381	Bioprocess Technology	2	Completed
BIO-NIIST-3-382	Enzymology and Enzyme Technology	2	Completed

Level 400

Course number	Title	Credits	Status
BIO-NIIST-4-001	Review Writing	1	Completed
BIO-NIIST-4-002	Project Proposal	1	Completed

AcSIR-800		Completed

Annexure IV

Instruments used during the research period

Instrument	Brand Name
Agarose gel electrophoresis unit	Biorad
PAGE unit	Biorad
Protein purification unit	Biorad
Isoelectric focussing unit	Protean (Biorad)
Gel documentation system	Chemidoc (Biorad)
TLC sample applicator	Linomat 5 (Camag)
UV Cabinet dual wavelength, 254/366 nm	Camag
Laminar flow hood cabinet	CleanAir
Centrifugal vacuum concentrator	Eppendorf
PCR thermal cycler	Eppendorf
Temperature controlled heating block	Eppendorf
Fume-hood	Escoglobal
DNA sequencer	Hitachi
Fermentor (2.5 L total volume)	Infors
Rotary incubation shaker	Infors
Shaking water bath	Julabo
Static water bath	Julabo
Autoclave	Labline
Microscope (clinical)	Leica
Weighing balance	Mettler Toledo
Centrifuge	Remi, Kubota, Eppendorf
4°C walk-in cold storage room	Rinac
Heating oven	RRL-T NC hot air drier
-80°C deep freezer	Haier
Lyophiliser	Scanvac
Nitrogen generator	Schmidlin
HPLC	Shimadzu
UV-Vis spectrophotometer	Shimadzu
Magnetic stirrer	Tarsons
Vortex mixture	Tarsons
Microplate reader	Tecan
Temperature controlled incubator	Thermo Fisher Scientific
Bath sonicator	iUltrasonic
-20°C deep freezer	Voltas