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Production of microbial chitinases – A revisit

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Abstract Production of microbial chitinases have received increased attention in recent years due to its potential application in various fields. There has been an all around effort to increase the production of chitinases by using genetically engineered organisms and by incorporating modern fermentation techniques. This review presents a brief outline on the production of microbial chitinases which includes information on random screening and selection of chitinolytic organisms and the rationale behind the use of genetically engineered organisms. The problems and perspectives involved in large scale production of chitinases is discussed with special reference to bioreactor studies and new fermentation methods. The present limitations in the understanding of the chitinase fermentation process is also discussed and the scope for future investigation is outlined in this communication.

1 Introduction

Chitinases are enzymes capable of hydrolysing chitin to its monomer *N*-acetyl glucosamine (GlcNAc). The definition of chitinase runs as "hydrolysis of *N*-acetyl glucoseamide (1–4)-β-linkages in chitin and chitodextrins". Chitin is the (1–4)-β-linked homopolymer of *N*-acetyl *D*-glucosamine [1]. Gooday [2] defines chitinolytic organisms as "those organisms that are capable of degrading chitin solely by hydrolysis of glycosidic bonds". It is available in large amounts in the biosphere and an estimate in 1990 have shown that chitin is produced to the order of 10^{10} to 10^{11} tonnes per annum [2]. Chitin occurs in three different forms, viz., α-chitin with antiparallel monomer chains, β-chitin with parallel monomer chains and γ-chitin with a three chain unit cell [3]. Of these α-chitin is most widely found and forms the structural component of cell walls of various fungi and exoskeleton of many invertebrates.

The classification of chitinolytic enzymes is not yet very clear. Chitinases are broadly classified as endochitinases and exochitinases. The exochitinase activity is defined as "the progressive action starting at the non-reducing ends of chitin with the release of successive diacetyl chitobiose units". The endochitinase activity is defined as "the random cleavage at internal points in the chitin chain" [4]. Chitinase was first observed by Bernard in 1911 when he isolated a thermostable and diffusable chitinolytic fraction from orchid pulp which is next supported by Karrer and Hoffmann for the presence of chitinolytic enzymes in snail [5].

Due to the important applications of chitinase, which will be discussed later, there has been a lot of research in the recent years for enhanced production of chitinases from microbial sources. A better understanding of the genetics of chitinase expression and the advent of recombinant DNA technology has led to a large increase in chitinase production. Along with recombinant organisms the introduction of novel fermentation technologies have also contributed to the improved production of chitinases by microorganisms.

2 Screening and selection of microorganisms producing chitinase

Screening and isolation of organisms capable of producing chitinase is usually done on a medium containing chitin. The organisms are isolated from soils, chitinious wastes, and marine environments. Gupta et al. [6] screened and isolated a variety of *Streptomyces* species for chitinase production and their subsequent use for fungal cell wall lysis. *Streptomyces viridificans*, *Streptomyces aureofaciens*, *Streptomyces coelicolor*, *Streptomyces glaucescens*, *Streptomyces kanamycitis*, *Streptomyces lividans*, *Streptomyces parvies*, and *Streptomyces venezuelae* were studied for their ability to produce chitinase. Of these, *Streptomyces viridificans* was found to be the best producer with the maximum levels of chitinase obtained at 1.5% colloidal chitin after six days of fermentation at 30 °C and 200 rev/min. Among the various pentoses and hexoses studied, arabinose doubled enzyme production while glucose repressed enzyme synthesis.

Similarly, Sabry (1992) isolated forty bacterial strains and tested them for their ability to produce chitinase from shrimp shell waste. The waste had a composition of 21.4% chitin, 40% calcium carbonate, 27.9% protein, 20% moisture and 6% ash. The fermentation medium containing 0.5 kg/m^3 yeast extract and 1% shrimp shell waste was inoculated with the selected microorganisms and incubated on a rotary shaker for 7 days. The most active

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organisms were *Alcaligens denitrificans*, *Bacillius aminoliquefaciens*, *Bacillius megatrium* and *Bacillus subtilis* exhibiting 1.9 U, 3.9 U, 3.6 U and 1.7 U of chitinase activity respectively when grown on shrimp shell waste as compared to 2.7 U, 3.4 U, 2.2 U and 4.3 U respectively when grown on commercial chitin [7]. An exhaustive qualitative screening of 38 different genera of bacteria done by Shaw and Cody (1988) have revealed that most active chitinolytic strains are in the genera of *Aeromonas*, *Bacillus* and *Vibrio*. The chitinase activity was determined by measuring the ratio of lytic zone to growth colony on agar plates. The most active producers were qualitatively analysed for chitinase activity by reducing sugar determination using *N*-acetyl *D*-glucoseamine standard curve [8].

Tagawa and Okazaki (1991) screened 863 *Streptomyces* isolates for their ability to produce chitinase. Further studies with the most competent strain, *Streptomyces cinereoruber* revealed that colloidal chitin was the best substrate that gave an activity of 20.7 U while *Aspergillus niger* cell wall gave 16.4 U of enzyme. Cultures grown on nigerose, nigeran, pseudonigeran, amylose, dextran and glucose showed no chitinase activity. The minimal medium for the studies composed of 0.2% K_2HPO_4, 0.1% $MgSO_4$, 0.01% $FeSO_4 \cdot 7H_2O$ at pH 6.8 and 30 °C [9].

Huang et al. (1996) isolated about 100 microorganisms capable of producing chitinase (mostly bacteria and actinomycetes) from pond water and soil samples using colloidal crab shell chitin as substrate. Among those 100 strains, one strain of *Aeromonas* sp. (designated as strain no. 16) was found to be the best chitinase producer as shown by clearing zones on agar plates. Under optimal conditions a chitinase activity of 1.4 U was obtained in shake flask fermentations and an activity of 1.5 U was obtained with a 5 dm^3 jar fermentor. Addition of glucose, glucosamine, maltose, mannose, galactose and sucrose decreased chitinase synthesis (0.53 U, 0.48 U, 0.43 U, 0.46 U, 0.42 U, 0.51 U respectively compared to 0.59 U of control), but supported growth (2.7×10^9 CFU, 1.7×10^9 CFU, 2.8×10^9 CFU, 2.5×10^9 CFU, 2.2×10^9 CFU, and 3.3×10^9 CFU respectively compared to 1.4×10^9 CFU of control). The addition of *N*-acetyl *D*-glucosamine did not affect enzyme synthesis. Inclusion of additional nitrogen sources increased chitinase production and the best among the nitrogen sources tested was tryptone which gave an activity of 0.134 U of chitinase compared to 0.019 U of control. Chitinase production is pH sensitive. Colloidal chitin concentration of 1.5% has been found to be optimal for chitinase production [10].

An alkali tolerant chitinolytic organism *Pseudomonas aerogunisa* K-187 was isolated by Sang-Lang et al. (1995) on a minimal medium containing treated shrimp and crab shell waste as sole carbon source. The medium composition was 3% shrimp and crab shell waste, 0.1% carboxymethyl chitin, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% $ZnSO_4$. Culture pH of 9.0 and a temperature of 45 °C was found to be best for production of chitinase and maximum enzyme activity was obtained after 3 days [11].

Many strains of genus *Asperlligus*, *Pencillium* and *Trichodrema* have been isolated from soil and examined for their chitinase activity. The optimal media composition for the best producer, *Aspergillus carneus* was (in kg/m^3): chitin, 10; yeast extract, 3; $MgSO_4 \cdot 7H_2O$, 1.5; KCl, 0.5. Optimum temperature and initial pH were 28 °C and 5.0 respectively. A maximum chitinase activity of 47.3 U was obtained after 7 days of fermentation under optimal conditions [12].

It could be inferred from the above discussion that chitinase producing organisms are predominantly isolated from soil samples and marine environments. In most cases colloidal chitin is used as substrate for isolation of chitinase producing organisms. In some cases treated crab shell and shrimp shell chitin have been used. Among bacteria, chitinase producing organisms are in the genera of *Aeromonas*, *Bacillus*, *Serratia* and sometimes in *Pseudomonas*. Among actinomycetes chitinase producing organisms occur in the genera of *Streptomyces*. In fungi chitinase producing organisms occur in the genera *Trichoderma* and *Aspergillus*. Most bacteria isolated express maximum chitinase during the third day of fermentation, while fungi and actinomycetes express maximum chitinase around the sixth day of fermentation. In all organisms addition of carbon sources other than chitin reduced chitinase production but supported growth. Chitinase is produced as an inducible enzyme with chitin or its degradation products acting as inducers. In most cases chitin concentration in the range of 1%–1.5% was found to be most suitable for chitinase production. Temperature in the range of 28 °C to 30 °C was best for chitinase production, while no such generalisation could be made for optimal pH value.

3
Production of microbial chitinases by wild type organisms

A brief summary of reports available on bioreactor studies for chitinase production is given in Table 1.

3.1
Bacterial chitinases

Pre-treated chitin from crab and shrimp shells were used as substrate for chitinase production by *Serratia marcescens*. The pre-treatment process involved size reduction and deprotenization of chitin at pH 11 and 30 °C for 60 min followed by demineralisation at the same temperature with 8% HCl for 8 h. Finally, chitin was obtained as 20–60 mesh particles by milling and sieving. The enzyme activities were observed at a temperature range of 25–35 °C and a pH range of 6.0 to 8.4 respectively. Maximum chitinase activity was predicted at 28 °C and pH 7.65 by response surface methodology. All fermentations were carried out in shake flasks [13]. A crude preparation of chitinase was obtained by growing *Serratia marcescens* QMB 1466 in a 14 dm^3 fermentor at pH 7 and temperature 30 °C in a mineral medium containing chitin. The fermentation was found to be kinetically limited by the rate of chitin hydrolysis. The kinetic data obtained were used for the development of a mathematical model to predict chitinase production. The kinetic parameters for chitin hydrolysis, cell growth and enzyme production were evaluated and were found to be in good agreement with the experimental data [14, 15].

Table 1. A brief summary of bioreactor studies on production of microbial chitinases

Organism	Medium composition (kg/m^3)	Fermentation conditions	Mode of operation	Maximum enzyme activity	Assay method	Reference
Bacillus lichiniformis	Colloidal chitin, 5.0; *N*-acetyl glucoseamine, 5.0; Yeast extract, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; Na_2HPO_4, 3.4; NH_4NO_3, 2.0; KH_2PO_4, 1.0; NaCl, 0.5	Volume: 3 l, pH = 7.0 Temperature: 50 °C Aeration: 1VVM Fermentation time: 48 h	Batch	Chitinase I: 13.7 mU Chitinase II: 3.5 mU Chitinase III: 3.1 mU Chitinase I: 2.8 mU	Reducing sugar equivalent	64
Nocardia orientalis	Colloidal chitin, 5.0; *N*-acetyl glucoseamine, 5.0; Yeast extract, 0.1; $MgSO_4 \cdot 7H_2O$ 0.5; K_2HPO_4, 0.7; KH_2PO_4; peptone, 2.0	Volume: 1 l, pH = 5.0 Temperature: 28 °C Fermentation time: 96 h	Batch	1.278 U	Reducing sugar equivalent	65
Serratia marcescens	$(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; K_2HPO_4, 1.5; Chitin, as required; glucose, as required	Volume: 750 ml for continuous culture studies and 14L for batch studies pH = 8.0 Temperature: 30 °C Fermentation time: 100 h	Batch and continuous	2×10^{-2} U	Reducing sugar equivalent	14, 15
Serratia marcescens 990E	Purified chitin, 10; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; K_2HPO_4, 1.36	Volume: 6 l, pH = 8.5 Temperature: 30 °C Aeration rate 6 l min^{-1}	Batch and fed batch	34 U	Reducing sugar equivalent	19
Talaromyces emersonii	Chitin, 10–20; plus mineral medium	Volume: 8 l, pH = 5.0 Temperature: 45 °C Fermentation time: 240 h Aeration rate: 8 l min^{-1} Agitation rate: 200 rpm	Batch	0.45 μmol · h^{-1} · ml^{-1}	Reducing sugar equivalent	21
Streptomyces cinereoruber	*A. niger* cell wall, 5.0; yeast extract, 50; $MgSO_4 \cdot 7H_2O$, 1.0; K_2HPO_4, 2.0; $FeSO_4 \cdot 7H_2O$, 0.1	Volume: 20 l, pH = 6.8 Temperature: 30 °C Fermentation time: 96 h Aeration rate: 1 VVM Agitation rate: 500 rpm	Batch	17 U	Viscometric method	9
Vibrio alginolyticus	Squid chitin, 5.0; glucose, 6; peptone, 7.5; yeast extract, 2; K_2HPO_4, 2; sea water, 75% (v/v)	Volume: 20 l, pH = 7.0 Temperature: 37 °C	Batch	Chitinase C1: 3.3 U/mg protein Chitinase C2: 5.8 U/mg protein	Reducing sugar equivalent	66
Trichoderma viride F-9	Chitin, 10; plus mineral medium	Volume: 5 l, pH = 7.0–8.0 Temperature: 40 °C	Batch	–	Reducing sugar equivalent	67
Trichoderma harzianum	Chitin 12.5; plus mineral salts	Volume: 2 l, pH = 4.9 Temperature: 30 °C Fermentation time: 120 h Aeration rate: 1.5 VVM Agitation rate: 224 rpm	Batch	0.391 U	Reducing sugar equivalent	68

Initial pH of 8.0, temperature of 30 °C , initial oxygen of 10% and water activity of more than 0.99 were found to be optimal for the production of chitinase by *Bacillus pabuli* K1. Chitinase production was increased by the addition of $(GlcNAc)_4$, $(GlcNAc)_3$ and $(GlcNAc)_2$. Best chitinase production was obtained with colloidal chitin. No chitinase was formed when the organism was grown on glucose or GlcNAc. Production of chitinase by *Bacillus pabuli* K1 grown on chitin was repressed by addition of glucose (>0.6%), starch (>0.6%), lamanarin, β-glucan and glycerol. Addition of pectin and carboxymethyl cellulose increased chitinase production [16].

Simultaneous fermentation/separation of extracellular chitinase produced by *Serratia marcescens* ATCC 990 was achieved in a PEG/dextran aqueous two phase system. The production medium contained (in kg/m^3): yeast extract,

0.5; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 1.36; swollen chitin, 15. Fermentations were carried out in a stirred tank reactor at a temperature of 30 °C with agitation rate of 300 rpm and aeration rate of 0.4 $dm^3/(dm^3)$(min). The enzyme partitioned into the bottom dextran phase and the cells into the top PEG phase. The best composition was 2% (w/v) PEG-2000 and 5% (w/v) dextran T5000 which gave highest yield of 85.2% in the bottom phase with an partition coefficient of 0.92 and a phase volume of 0.19. Higher PEG or dextran concentration decreased the partition coefficient to 0.79 and 0.76 respectively, but volume ratio increased to 0.67 to 1.0 respectively. The maximum enzyme concentration was about 1.5 times the polymer free reference system. Cell growth and production kinetics were similar to those of polymer free system [17].

Chitin at a concentration of 3% and peptone at a concentration of 3% were found to be the best carbon and nitrogen source for the production of chitinase by *Pseudomonas stulzeri* YPL-1. Optimum culture pH was 6.8 and maximum chitinase activity was obtained after 84 h of fermentation [18]. Khoury et al. (1997) have studied the effect of dissolved oxygen concentration on the production of chitinase by *Serratia marcecens* 990E. Their investigations have shown that the dissolved oxygen tension in the range of 20% to 50% saturation was best with a maximum chitinase activity of 34 U. Chitinase activity decreased rapidly at higher dissolved oxygen tension values. This observation indicates the ability of *Serratia marcescens* to produce chitinase at mild agitation and aeration conditions and will be very important for large scale process development. A 5–10 fold increase in cell growth was observed between aerated and non-aerated cultures. The experiments were conducted in a 6 dm^3 stirred tank bioreactor at 30 °C with an aeration of 6 dm^3/min. Their efforts to increase chitinase production by addition of 6 kg/m^3 chitin increased cell growth but not chitinase production. Various other substrates added were able to reactivate growth but not enzyme production [19].

3.2 Fungal chitinases

Myrothecium verucaria was grown on a medium containing (in kg/m^3): KH_2PO_4, 3; K_2HPO_4, 1; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract, 0.5; peptone, 0.5; chitin, 5.0 and incubated at pH 5.0 and 28 °C for seven days with agitation of 200 rev/min. *Myrothecium verucaria* produced maximum chitinase activity when grown on chitin as the sole carbon source. Addition of 0.03% urea increased chitinase synthesis by 4 fold. The chitinase produced was active over a temperature range of 25–55 °C and a pH range of 4.0–6.5. This enzyme was used for fungal mycelia degradation and had 5 to 6 times more chitinase activity than the commercial lytic enzymes [20].

Chitinase production by *Talaromyces emersonii* CBS81470 was studied in submerged cultures both in shake flask and in fermentors. The optimal chitin concentration was 1–2% (w/v) at a pH of 5 and 45 °C. Under optimal conditions maximum chitinase activity of 2.3 U was obtained after two days of fermentation in fermenter cultures, while maximum chitinase activity of 1.5 U was obtained after four days in shake flask cultures. The enzyme produced had maximum activity at pH 5.0–5.5 and 65 °C and had an half-life of 20 min at 70 °C and pH 5 [21]. Chitinase production by *Stachybotrys elegans* in a mineral medium containing different carbon sources showed that enzyme activity in cultures grown on chitin was higher than that of cultures grown on other carbon sources (chitinase production of 3.3 U, 1.1 U and 0.2 U were obtained when cultures were grown on chitin, cell wall fragements and lamanarin respectively). No chitinase production was observed when *Stachybotrys elegans* was grown on glucose, sucrose or *N*-acetyl glucosamine. The mineral medium contained (in kg/m^3): $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 0.9; KCl, 0.2; $FeSO_4 \cdot 7H_2O$, 0.002; $MnSO_4$, 0.002; $ZnSO_4$, 0.002 and appropriate carbon and nitrogen source. Maximum chitinase production was observed at pH 5 and 24 °C. Optimum chitin concentration was 1 kg/m^3. Chitinase production was stimulated under acidic pH and the addition of $NaNO_3$. Addition of ammonium nitrate, ammonium tartrate and aspargine as nitrogen source reduced chitinase production by three fold [22].

Statistical experimental designs were used to optimize media composition, microbiological parameters and environmental factors for the production of chitinase by *Trichoderma harzianum*. Chitin concentration of 12.5 kg/m^3 and ammonium sulphate concentration of 4.2 kg/m^3 was found to be optimum. An initial pH of 5.6 and temperature of 28 °C were best suitable for chitinase production. The optimal age of organism in the slant and the optimal age of organism in the inoculum were 105 h and 43 h respectively. Exclusion of urea and peptone from the medium increased chitinase synthesis. The chitinase activity increased from an initial value of 0.054 U before optimization to a final value of 0.197 U after optimization. Exclusion of urea from the medium increased chitinase production [23]. This observation is unlike the observation made by Ulhoa and Peberdy [24] for chitinase production by *Trichoderma harzianum* and the observation of Vyas and Deshpande [20] for *Myrothecium verrucaria* where addition of urea increased chitinase production. Mahadevan and Crawford [25] have investigated the high level chitinase production by *Streptomyces lividans* in the presence of cell walls of *Phythium* and *Aphanomyces* species. Colloidal chitin concentration in the range of 0.8–1.4 kg/m^3 gave maximum chitinase activity after five days of fermentation. The optimal temperature for chitinase production was 25–30 °C. Chitinase production was stimulated by the addition of 1% colloidal chitin as substrate. Chitinase production was induced by *N*-acetyl *D*-glucosamine and was repressed by glucose, xylose, arabinose and carboxymethyl cellulose. Addition of pectin, lamanarin, starch or β-glucan to the medium increased chitinase production. These results were similar to that obtained by Frandberg and Schnurer [16] using *Bacillus pabuli* K1. Mathivannan et al. [26] have studied the production of extracellular chitinase by *Fusarium chlamydosporum* in a minimal medium containing different carbon and nitrogen sources. They observed maximum chitinase activity after 8 days of fermentation in

a medium containing a combination of colloidal chitin (0.5%) and sucrose (10 mM) as carbon sources and 15 mM sodium nitrate as nitrogen source. All studies were conducted in 250 cm^3 Erlenmeyer flask with 50 cm^3 medium at 28 °C and 75 rev/min.

The above discussion on the production of bacterial and fungal chitinase reveals that in bacterial strains, chitinase production is repressed by the addition of glucose, starch, lamanarin, β-glucan and glycerol. In fungi, addition of glucose, xylose, arabinose and carboxymethyl chitin reduces chitinase production, while addition of pectin, lamanarin, starch and β-glucan increase chitinase production. Among all the carbon sources studied for all organisms, colloidal chitin was found to be the best carbon source. Cell wall fragments was the best carbon source next to chitin for fungal chitinase production, while no reports are available on the use of cell wall fragments for bacterial chitinase production. Addition of urea increased chitinase production in most fungal chitinases, while urea addition did not have any effect on bacterial chitinase production. Fungal chitinases are active over a wide range of temperature and pH, while this range for stability of bacterial chitinase is very less. Simultaneous production/separation of chitinase using aqueous two phase systems have been reported for bacterial chitinase production, while such investigations are yet to be done for fungal chitinases. Moreover, fungal fermentation becomes complicated in terms of mixing and mass transfer limitations as the systems forms mycelia in addition to the insoluble substrate. This may be the reason for decreased productivity in fungal chitinase fermentation when compared to bacterial chitinase fermentation.

4 Enhanced production of chitinase by recombinant microorganisms

Recombinant organisms are known to increase the productivity of many fermentation processes [27–30]. In chitinase fermentation, a few examples exists for the use of recombinant organisms to increase the yield. Kole and Altosaar (1985) using a non-pigmented, stable mutant of *Serratia marcescens* designated BL40 showed about 167% increase in chitinase activity over the wild type strain under similar conditions. Chitin concentration of 8 kg/m^3 was found to be best for both the mutant and the wild type strains, but the mutant produced 184 U of chitinase while the wild type produced 76 U of chitinase. Apart from chitin, the medium contained KH_2PO_4, $MgSO_4$ and casamino acids. The culture was maintained at a pH of 7.2 and a temperature of 30 °C. Chitinase were extracellular in both the strains. Activity was observed in the cytoplasm and periplasmic space also [31]. Next, Joshi et al. (1989) used UV-irradiation and nitrosoguanidine treatment to obtain a non pigmented chitinase and chitobiase overproducing mutant of *Serratia marcescens* SJ101(named Chi^{+++} mutant). Chitinase producing strains were isolated on agar plates containing chitin. The medium composition was 1% cane molasses and 0.75% chitin at pH 7.5 and temperature 30 °C. A maximum activity of 15 U was obtained after 45 h of fermentation. Another recombinant strain was obtained by transferring a plasmid *PSJ12-6* containing cloned chitinase and chitobiase genes of *Serratis liquefaciens* into the Chi^{+++} mutant. The optimal conditions for chitinase production remained the same as that of the Chi^{+++} mutant, but the time for maximum chitinase production decreased by 40% and the maximum chitinase production increased by 80% when the plasmid *PSJ12-6* was introduced into the Chi^{+++} mutant [32].

Chen et al. (1997) used a recombinant *Escherichia coli* strain harbouring a chitinase gene from *Aeromonas hydrophilia* for enhanced production of chitinase. The expression of chitinase was induced by IPTG and the cell growth rate was controlled by varying aeration rate and the concentration of nitrogen source in the medium. Induction with 0.5 mM IPTG after 2 h of fermentation was found to be best for chitinase production. Cell growth and chitinase production showed different time profiles indicating that chitinase production is non-growth associated. A decrease in cell growth rate have resulted in increase in enzyme secretion in the periplasmic space [33].

5 Immoblisation as a novel method for production of chitinases

Micromonospora chalcae, was used for the production of chitinase both in free and immoblised form. The cells were co-immoblised with chitin using calcium alginate. Both free and immoblised cells were grown in submerged cultures on a medium containing 2% chitin. Both systems showed similar enzyme production profile and maximum enzyme activity was obtained after 3 days in both cases. Free cells produced maximum of 0.6 U of chitinase while the immoblised cells produced a maximum of 0.9 U of chitinase. As fermentation continued, the immoblised cells maintained maximun activity for a long time, but the activity decreased to 0.25 U after 3 days in free cells [34].

Wasabia japonica cells immoblised on double layered gel fibres was used for the production of chitinase from chitin. Fermentations were carried out in 200 cm^3 Erlenmeyer flask, 200 cm^3 aerated flask and in a bubble column culture vessel at 25 °C in dark with an agitation of 200 rpm and an aeration of 1 $dm/(dm^3)(min)$. Product inhibition was observed in the culture during chitinase production. It was necessary to maintain the chitin concentration below 2 U to avoid product inhibition and maintain chitinase production rate above 80% of the maximum chitinase production rate. A production column containing immoblised cells was coupled to a chitin column and a continuous chitinase production was obtained by circulating the broth between these two columns. The column containing immoblised cells was used for chitinase production while a column containing chitin (shrimp chitin powder, mesh size 70) was used as an affinity adsorbant for the removal of chitinase in fermentation broth. Chitinase adsorbed on to the column was later eluted out. By this way, it was possible to obtain high and stable chitinase production for 40 days and about 3211 U (after elution from the chitinase recovery column) of chitinase was obtained [35]. Similar double layered gel fibres containing protoplasts of *Wasabia japonica* immoblised with calcium carbonate gave a chitinase activity of 2 U after 5 days as compared to 0.36 U in the free system after the same

period. Addition of 2,6-dichlorobenzonitrile inhibited cell wall regeneration and hence, active protoplasts were maintained for a long time. Chitinase activity increased significantly when the protoplasts were aerated with pure oxygen. Due to product inhibition, the chitinase concentration was kept below 1.8 U. A production column was coupled with the chitin column as above and hence continuous production and separation of chitinase was achieved. High and stable chitinase production of 26,000 U (after elution from the chitinase recovery column) was maintained for 25 days [36]. Thus chitinase production by protoplasts of *Wasabia japonica* immoblised on double layered gel fibres were more effective in producing chitinase than cells of *Wasabia japonica*.

Immoblisation of whole cells and protoplasts has proved to be very effective for chitinase production when compared to free cell systems. Immoblised systems were able to maintain high chitinase production for longer periods of time when compared to free cell systems. The major disadvantage with immoblised system is the mixing and mass transfer limitations. Chitin is an insoluble substrate and it makes mass transfer limitation even worse. Co-immoblisation of chitin along with cells was proposed as method to avoid problems associated with insoluble nature of the substrate. Co-immoblisation resulted in higher productivity in batch and batch-fed mode of operation. Similarly immoblisation was able to avoid problems associated with product inhibition. Mass transfer limitation was avoided by using two different columns for continuos production and separation of chitinase. Immoblisation techniques were useful for the enhanced production of chitinase by *Wasabia japonica* protoplasts without considerable decrease in viability. Thus, immoblisation has been very useful for increased chitinase production.

6
Application of microbial chitinases

Chitinases are well known due to their ability to degrade chitin containing cell wall of many fungi. This property makes it most valuable in the fields of pest control, pollution abatement, basic and commercial biology. The capability of chitinases to degrade chitin makes it promising enzyme in the manufacture of value added products from chitinous wastes.

6.1
Application of chitinases in biological research

As chitinases play a very critical role in the dissolution of cell walls of fungi, they are very useful for the generation of fungal protoplasts. Dissolution of cell wall by chitinase was first observed by Skujins et al., 1965 [37] where they found a *Streptomyces* chitinase to effectively lyse hyphal walls of *Aspergillus oryzae* and *Fusarium solani*. Next, Johnson et al., 1979 [38] used a chitinase containing culture filtrate of *Bacillus circulans* WL12 to generate spheroplasts of *Phaffia rhodozyme*. Similarly, a commercial preparation of chitinase and cellulase was used to release protoplast from *Caprinus pellucidus* [39] and *Caprinus macrorhizus* [40]. Chitinase from *Trichoderma harzianum* has shown most efficiency in the generation of a variety of fungal protoplasts [41, 42]. Likewise, the use of chitinase (either separately or as a combination with other enzymes) in generation of protoplasts has been extensively examined [43–48].

6.2
Use of chitinase as a biocontrol agent

The cell wall of many phytopathogens contain chitin and hence chitinases are exploited for their use as a biocontrol agent [49]. This application is considered to be the most important as cmpared to others. *Aeromonas caviae* was able to control infection by *Rhizocotonia solani* in cotton and *Sclerotium rolfsii* in beans [50] while chitinase from *Aphanocladium album* strongly inhibited growth of *Nectria heamatococca* in pea [51]. Similarly supplementing soil with chitinous waste and subsequent inoculation with chitinase producing organisms resulted in efficient biocontrol of *Verticillum dahliae* and *Fusarium oxysporum* in cotton and beans [52, 53]. Chitinase from *Trichoderma harzianum* was used to inhibit hyphal elongation in plant pathogens such as *Fusarium solani* [54], *Botrytis cinerea* [55] and *Cotricum rolfsii* [56]. Regev et al. (1996) have used a recombinant *Escherichia coli* strain producing the insecticidal protein CryIc as well as chitinase for the control of *Spodoptera littoralis* larvae. The toxicity of CryIc was greatly increased with the supplementation of chitinase [57].

6.3
Use of chitinase in degradation of fish waste

The monomer of chitin, *N*-acetyl-*D*-glucoseamine is very useful in the manufacture of chemical and pharmaceutical intermediates and food products such as sweeteners and growth factors [58]. *N*-acetyl-*D*-glucosamine is conventionally prepared by complete hydrolysis of chitin with strong mineral acids or by chemical synthesis from glucoseamine [59, 60]. These processes involve high cost and corrosion problems due to which the cost of *N*-acetyl-*D*-glucoseamine or its oligimers are still prohibitive. In the last two decades there has been a lot of focus on the production of *N*-acetyl-*D*-glucosamine through enzymatic hydrolysis of chitin. Revah-Moiseev and Carroad [61] as well as Tom and Carroad [62] have described a process for the bioconversion of shell fish waste to *N*-acetyl glucoseamine and other valuable products. Subsequently a process for production of single cell protein from chitinous waste has been described by Cosio et al. [13]. A continuous process for the enzymatic hydrolysis of chitin was described by Sakai et al. (1991) using immoblised *Nocardia orientalis* chitinase for continuous production of *N*-acetyl glucoseamine from chitin [63].

7
Future trends

In the last few decades there has been an increased attention on the production of microbial chitinases mainly due to its application in agriculture and pollution abatement. Although improved screening methods and genetic modifications have brought about a considerable increase in chitinase production, research on large scale production of chitinases is very limited. Presently, there is no clear

understanding of the kinetics of chitinase fermentation process. Kinetic studies would form the basis for any further bioreactor design considerations. Hence, the kinetics of chitinase production requires to be investigated in detail. Studies on the influence of engineering variables such as aeration, agitation etc., on the production of chitinase has largely been ignored. Without the knowledge about the effect of engineering variables, it is difficult to choose the proper parameter for scale-up of this process. Thus the influence of engineering variables needs to be studied extensively. There has been some interest in the use of new fermentation technologies for the production of chitinases and extractive fermentation using aqueous two phase systems is being studied by a few researchers. Other methods such as high cell density cultivation, cell recycle systems etc., is yet to be applied for chitinase fermentation. At present there is no research done on the use of alternate reactors (air lift, bubble column etc.) for production of chitinases. As increased shear in a conventional stirred tank reactor will lead to inactivation of the enzyme or may significantly affect the morphology, the use of bioreactors with low shear needs to investigated in detail. It is evident from the above discussion that the present knowledge on production of microbial chitinases is very limited and hence there exists a wider scope for further investigations in this area.

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