Enzymatic Depolymerization of Nano Chitin Particles into N-Acetylglucosamine

G. N. Rameshaiah, Y. K. Suneetha

Abstract- Chitin is a crystalline polysaccharide widely spread in nature with three structures: alpha, beta and gamma chitins. Chitin is gaining importance for their biotechnological applications. Enzymatic depolymerisation of chitin to produce oligomers was carried out using the filamentous fungi Trichoderma harzianum (MTCC 3928). The bioprocess offers many advantages and helps to overcome the limitations of conventional chemical treatment which is presently used in industries. Chitin is treated with hydrochloric acid for chitin demineralization and to obtain colloidal nano size particles. Production of N-acetyl glucosamine was studied as a function of acid washed chitin in the particle size range of 74-125µm, pH of the broth media, and concentration of chitin and trace nutrients. N-acetylglucosamine yield was highest with particles of 125 µm size at solution pH5 and when incubated at 34℃ for 120 h in an orbital shaker with 160 revolutions per minute. Higher yield was obtained with initial chitin concentration of 10 g/L and lowered yield may be due to diffusion resistances and substrate inhibition at other concentrations. Trace nutrient concentration has an impact on both enzyme activity and product yield.

Keywords: Trichoderma harzianum, Chitin, N-acetylglucosamine

I. INTRODUCTION

Chitin, a water insoluble linear β-1,4-linked homopolymer of N- acetylglucosamine, is one of the most abundant natural nitrogen containing renewable biopolymer. Chitin comprises 22 to 44% of cell walls of fungi[1-2]. It is a major cell wall component of higher fungi belonging to chitidomycets, ascomycets and as a byproduct of seafood processing industry. More than 80,000 metric tons of chitin isobtained per year from the marine waste[2-3].The current interest in chitin and its derivatives is related to their biological and biotechnological characteristics. Chitin and its derivatives have numerous applications. Treatment of natural chitin using a mineral acid is a pre-requisite for most of these applications.Enzymatic methods have been studied as an alternative to the conventional chemical processes. Biotechnology offers many advantages [4] on chitin processing. Mild conditions of reaction biocatalysts can replace the aggressive conditions that are used in the chemical processing which results in the deterioration of the products , erosion of equipment surfaces and unhealthy working conditions [5] By-products (like shrimp protein and shrimp pigment) that are denatured by chemical methods remain applicable for high value application for human nutrition [6].

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High protein alkaline waste produced during conventional chemical procedure is not generated instead all materials of the end up in one of the useful fractions produced in bioprocessing. The solubility of chitin is enhanced by partial deacetylation under mild conditions that do not degrade the polymer, thus increasing the polarity and electrostatic repulsion of the amino groups. Besides the loss of crystalline structure is a consequence of reduction of the hydrogen bonds caused by the elimination of acetyl groups.
It has been reported that chitins with a degree of acetylation (fraction of the chitin sample with acetyl group) of 0.45-0.55 display a good solubility in aqueous media [7]. Degree of deacetylation (deacetylated fraction of chitin) of chitin is an important factor of the activity of enzymes. Even though α-chitin has lower solubility and swelling compared to β-chitin, the interest in studying hydrolysis of α-chitin is in its abundance in nature.Enzymes involved are of two types, viz. endochitinases and exochitinases [8]. Chitinase, chitosanase, N-acetyl glucosaminase (GlcNAc) enzymes produced at different stages from fungi can be utilized for chitin depolymerisation which includes deacetylation, breaking down of the polymer to give monomer GlcNAc and then followed by glucosamine formation. The chitinolytic enzyme is produced by many organisms for their basic physiological needs. The bio catalytic conversion of natural chitin has been hampered by the low yield of the process. This has been attributed to the non-accessibility of acetyl group in the interior of the crystalline chitin particle for deacetylation. Using a new method to produce a super fine chitin powder in combination with a pretreatment in a mineral acid, can increase the conversion to more than 90%. The cell wall of fungi has widely distributed chitin. This microorganism is expected to sustain high growth rates, yield, and productivity on the substrate. Suggested growth conditions are the absence of stringent requirements for growth factors, ability to grow at high temperatures (above 35°C). The studies of chitnolytic enzymes from plants, insects, and microorganisms with respect to their role and applications have been extensively reviewed[9–14]. The production of chitinase by *Trichoderma* species is of interest due to their wide applications in bio control and as a source of mycolytic enzymes. *Trichoderma harzianum* 39.1 was the best strain for chitinase which produced nearly 3-17 times more enzyme than any other strain and significant chitinase activity was present when the fungus was incubated in chitin medium in absence of other sugars (Cirano J). N-acetyl D-glucosamine is the acetylated derivative of the amino sugar glucosamine, which is a constituent of cartilage proteoglycans. It is derived from marine exoskeletons or produced synthetically. GlcNAchas been proposed as a treatment for autoimmune diseases, and recent tests have claimed success in some of the cases. N-acetyl D-glucosamine is also used for osteoarthritis and inflammatory

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bowel disease (IBD), including ulcerative colitis and Crohn's disease. Glucosamine is required for the synthesis of glycoproteins, glycolipids, and glycosaminoglycans (mucopolysaccharides).These carbohydrate based compounds are found in tendons, ligaments, cartilage, synovial fluid, mucous membranes, structures in the eye, blood vessels and heart valves. Deficiency in N-acetylation of glucosamine in inflammatory bowel disease (IBD) is possibly reducing the synthesis of the gastric and intestinal mucosa's protective glycoprotein cover.

II. MATERIALS AND METHODS

Maintenance of Culture: *Trichoderma harzianum* MTCC 3928 obtained from the Institute of Microbial Technology, Chandigarh, India was used for chitin hydrolysis. The organism was maintained on MRBA (Medium Rose Bengal Agar) plates were prepared with the composition of 31.55 g/L. The organism was sub-cultured every three months.

Preparation of pour plates: Rose Bengal Agar Base in distilled water was prepared with the concentration of 31.55 g/L. Liquid media was poured onto sterile petri dishes in a laminar flow chamber. Pour plates were left in the laminar flow chamber for three to four days to confirm for aseptic environment of pour plate prior culture inoculation. Pour plate was inoculated with culture above the flame of the spirit lamp in a laminar flow chamber. Culture was incubated at 32^{o}C ±2^{o}C for 105 hours (four days and nine hours).

Preparation of seed inoculum of *T. harzianum* MTCC 3928and its cell count: Seed inoculum in liquid media was prepared using 105 hour pour plate culture. Medium for the seed growth of ***T. harzianum*** was prepared with following constituents: Glucose 10g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2 g/L, NaH_2PO_4 .2H_2O 6.9 g/L, $MgSO_4$ .7H_2O 0.3 g/L, Peptone 1 g/L, Citric acid monohydrate 10.5 g/L and Urea 0.3 g/L. The pH was adjusted to 5 using 2M NaOH. The media was sterilized by autoclaving at 15 psi pressure (121^{o}C) for 20 minutes and was cooled to room temperature. 10 mL of the media was transferred into a sterile 20 mL test tube. Media containing test tube was inoculated from a fresh 105 hour working pour plate above the flame in the laminar flow chamber. Cell suspension was mixed well using vortex mixer. Cell suspension was measured for cell concentration (conidia/mL). 100mL of seed growth medium was inoculated with one mL of cell suspension containing ***T. harzianum***. Inoculated media was incubated on orbital shaker maintained at 32^{o}C ±2^{o}C for 43 hour to obtain pellets of the mold which was used as inoculum for chitin depolymerization. Media pH was uncontrolled during the seed growth. Cell counting of 43hour pellet media was carried out using haemocytometer.

Measurement of inoculum (seed media) cell concentration: Cell count analysis was made to know the inoculum concentration of seed media using haemocytometer. 5mL of 43h seed medium containing pellets was transferred into a 50mL centrifuge tube. Suspension was centrifuged at 4000 rpm for 20 minutes and supernatant was discarded after autoclaving. 5 mL of phosphate buffer (50 mM, pH7.0) was added to each of the pellet samples taken in 50 mL centrifuge tubes. Pellets were mixed well. Suspension was centrifuged at 4000 rpm for 20 minutes and supernatant was discarded. Washing with buffer was repeated once again using 5 mL of phosphate buffer. Pellets were homogenized using 5 mL of 8% NaCl. 1 mL of homogenized suspension of pellet was diluted several times since the cell concentration exceeded 200-250 for four corner large squares and the middle square. Viable cell count was made in the area of 16 squares of the haemocytometer.

Demineralization and preparation of colloidal chitin: 12.5g of chitin obtained from Everest Biotech Private Limited, India was treated in 100 mL of concentrated HCl. The suspension was mixed well for about 5 hours. Gelatinous paste obtained was stored at 4°C for about 24 hours. The mixture was washed with water repeatedly for neutralization. The suspension was centrifuged to remove dissolved proteins at 10000 rpm for 10 minutes and the bottom portion of particle suspension was dried in hot air oven at 60°C. Dried sample was crushed to powder and screened to obtain different size fractions in the range 74 µm-125 µm.

Broth media for chitin depolymerisation by *T. harzianum* MTCC 3928: Chitin fermentation was carried out using required nutrients and *T. harzianum.* The modified composition [15] given below was employed for all the experiments unless mentioned. Broth media was prepared with chitin10 g/L, $(NH_4)_2$ $SO_4$4.2 g/L, KH_2PO_4 2.0 g/L, NaH_2PO_4.2H_2O 6.9 g/L, $MgSO_4$ .7H_2O 0.3 g/L, Tween80 0.2 g/L, $FeSO_4$.7H_2O 0.005 g/L, $MnSO_4$.H_2O 0.0014 g/L, $ZnSO_4$.7H_2O 0.0014 g/L and $CaCl_2$.2H_2O 0.002 g/L, 10mL of stock solutions of trace nutrients were prepared with $FeSO_4$.H_2O 0.05 g/L, $MnSO_4$.H_2O 0.016 g/L, $ZnSO_4$.7H_2O 0.014 g/L and $CaCl_2$.2H_2O 0.02 g/L separately. One millilitre of each of the above stock solutions was added to prepare one litre of broth media. Solution pH was adjusted to 5 using 2M NaOH. Solution was autoclaved at 15 psi gauge pressure (121^{o}C) for 20 minutes. Sterile media was cooled to room temperature under aseptic conditions.

Enzymatic process: Broth solution containing was inoculated with *T. harzianum* from a fresh 43 hour seed media above the flame in the laminar flow chamber. Reaction mixture was incubated at 34^{o}C±2 and 160 rpm in an orbital shaker under aseptic conditions. Broth media was uncontrolled for its pH condition during fermentation. Sampling at different intervals of incubation and followed by analysis was carried out for N-acetyl glucosamine and enzyme activity.

N-acetyl glucosamine analysis: Water insoluble chitin is a polymer composed of N-acetyl glucosamine and chitosan units which after depolymerisation reduces to water soluble oligomers and monomer as N-acetyl glucosamine. One millilitre of broth media was aseptically transferred into a 50 mL centrifuge tube and centrifuged at room temperature and 4000rpm for 20 minutes. Supernatant was preserved for GlcNAc analysis and chitinase activity. N-acetylglucosamine concentration in broth solution was found using di-nitro salicylic acid method [16]. Amber tubes were used during the estimation procedure to avoid the denaturing of dinitrosalicylic acid due to its light sensitivity. Absorbance of the reaction mixture was measured using UV-VIS spectrophotometer.

Enzyme assay: Enzyme activity was determined by the method of Monreal and Reese. This method includes two

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steps: Preparation of acid swollen chitin (ASC) and assay of enzyme activity (ASC). ASC required for chitinase activity measurement was prepared by adding one gram of colloidal chitin to 10 ml of 85% orthophosphoric acid. The mixture was stirred gently to give a gelatinous paste. Paste was re-suspended into excess of cold (2-4 mL at 15^{o}C) distilled water. It was ground using pestle and mortar and then re-suspended in 200 mL of sodium acetate buffer (50 mM, pH4.75). Suspension was stored at 10^{o}C for further use up to a maximum of one month. Toluene was added as a preservative at the concentration of 1% (volume).

Chitinase assay: The reaction mixture contained 0.55 mL of ASC suspension, in sodium 0.3 mL of acetate buffer (50mzM, pH4.75) and 0.15 mL culture filtrate. Enzymatic reaction was terminated by adding 1mL of sodium potassium tartrate reagent. It was then incubated at 47℃ for one hour in an unstirred condition [17]. One millilitre of dinitrosalicylic acid was added and mixed using vortex. Solution was kept in a boiling water bath for five minutes and then cooled to 30^{o}C. Total volume was made to 10 ml using distilled water. Mixture was centrifuged at 5000 rpm for 5 minutes and supernatant was measured for the released product in the reaction mixture which was read as absorbance at 540 nm in a spectrophotometer. Chitinase activity was determined using N-acetyl glucosamine as the standard [18] product produced. One unit (U) of chitinase activity was defined as the amount of enzyme that releases 1 µmol of N-acetyl glucosamine from the substrate in one minute per millilitre of reaction mixture under standard assay conditions [19].

Effect of pH on chitin hydrolysis: Chitin particles of approximately 125 µm were processed at various pH (pH4 to pH6) of the broth solution. Chitin concentration of 15 g/L and nutrients concentration:$(NH_4)_2 SO_4$4.2 g/L, KH_2PO_4 2.0 g/L, $NaH_2PO_4.2H_2O$ 6.9 g/L, $MgSO_4.7H_2O$ 0.3 g/L, Tween 80 0.2 g/L, $FeSO_4.7H_2O$ 0.005 g/L,$MnSO_4.H_2O$ 0.0014 g/L, $ZnSO_4.7H_2O$ 0.0014 g/L and $CaCl_2.2H_2O$ 0.002 g/L were used in broth solution with incubation temperature of 34℃. Experiments were carried out in triplicate. Culture filtrates were analyzed for both N-acetyl glucosamine and enzyme activity

III. RESULTS AND DISCUSSIONS

Preparation of pour plates: Initially the colour of them in the plate being white on first day, changes slowly into green from the centre portion on the second day. Fully grown filamentous fungi were obtained on the fourth day. Culture plate was stored at 4^{o}C±2^{0}C to arrest the growth of culture and for further sub culturing. Sub culturing was carried out five times before its use for fermentation. Haemocytometer was utilized for cell counting and structure of organism was observed through trinocular microscope with 100X magnification(Figure 1 and Figure 2)

Figure 1: Morphological features of filamentous mold in the pellet of growth media with 43 h incubation under 40X magnification under trinocular microscope

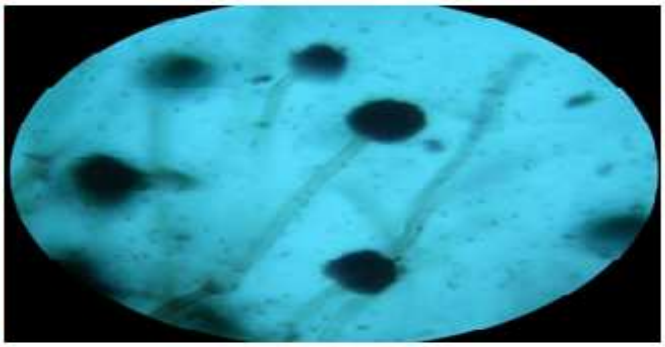

Figure 2: Morphological features of filamentous mold in the pellet growth media with 43 h incubation under 100X magnification under trinocular microscope

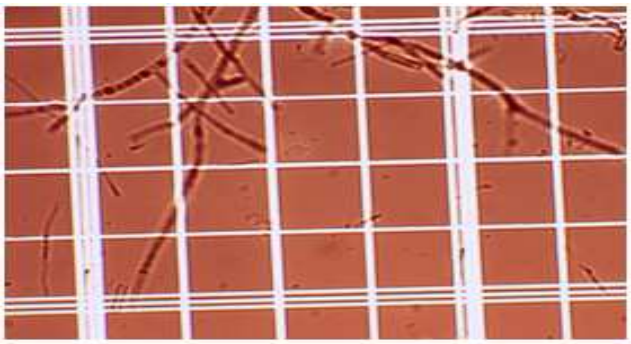

Figure 3: Pellet after 43 h of incubation under 40X magnification observed through binocular microscope during cell count with haemocytometer

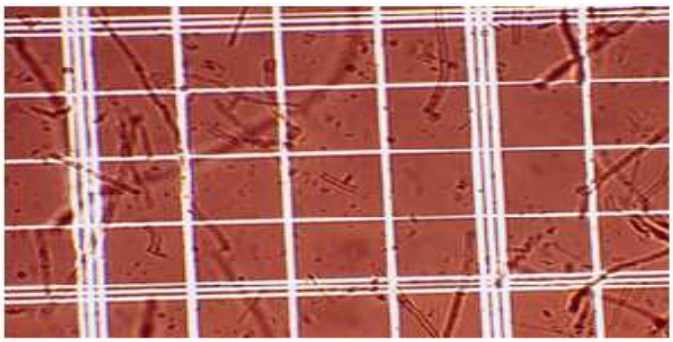

Figure 4: Pellet after 92 hours of incubation under 40X magnification observed through binocular microscope during cell count with haemocytometer

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Rose Bengal agar base was utilized as the pour plate maintenance media for the mold. 105hour old slant culture (36×10^4 $viable\ cells/mL$) was used to inoculate the seed media. Mycelium was grown from the spore inoculum in the medium containing glucose (2%, w/v) as the carbon source. Inoculum of pour plate culture and seed media were observed for the morphological structure of the filamentous fungi through the microscope. Cell density and cell count against growth of pellets were carried to understand the growth kinetics of filamentous mold. Microbial population increases with biomass in a typical batch culture. Exception to this is in molds and other filamentous organisms like where the mass and the morphology of a mold pellet or pulp varies as growth proceeds. Several experimental studies of batch submerged culture have indicated that the biomass increases at a slower rate. The rate of increase in the colony length is constant while the radius of the mold colony increases at a constant rate. Presence of spores inside the hyphae complicates the cell count analysis in the filamentous fungi which can be understood from the Figure.3& Figure4 shown.

Calibration plots for glucose and N-acetylglucosamine concentrations at 540 nm were prepared with the help of UV-VIS spectrophotometer (Jasco V-530). Protein content as tyrosine concentration at 700 nm and deoxyribonucleic acid of calf thymus concentration at 600 nm against absorbance were prepared with the help of UV-VIS spectrophotometer (Jasco V-530). Glucose (Fisher scientific, India) and GlcNAc (SD Fine Chemicals, India) were estimated using di nitro salicylic acid [20] method. The method of Lowry et al. [21] and the method described by Burton [22] were used for spectrophotometric estimation of tyrosine (SD Fine Chemicals, India) and Deoxyribonucleic acid (Hi media) respectively. DNA present in the spores and mycelia of pellet media was cold extracted using liquid nitrogen and its concentration was estimated using the method described by Burton [22]. Data of DNA in the seed media versus cell density was obtained.

Effect of incubation and chitin particle size: Broth media containing dried sample of acid treated chitin of 125 µm (10 g/L) size was aseptically inoculated with 43 hours old pellets of *Trichoderma harzianum*. Incubation of the media for 7 days at 34ºC±2 and 5pH with 160 rpm on an orbital shaker yielded oligomers and the monomer.Glucose of seed media and N-acetyl glucosamine of culture filtrate of broth media were analyzed using di-nitro salicylic acid method. Production of N-acetyl glucosamine was also studied with 105 µm size particles under the same standard conditions of the process. GlcNAc production was observed on the day (113.17 h) of incubation for both the sizes (Figure 5).

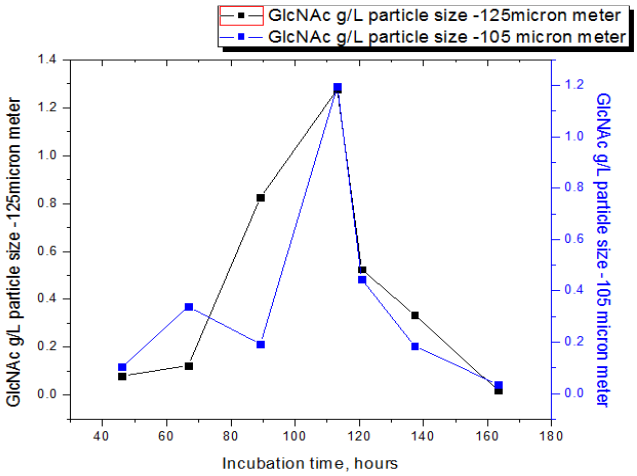

Figure 5: Effect of chitin concentration on GlcNAc production

Effect of chitin concentration: Enzymatic process was carried out with various concentrations (5 g/L to 15 g/L) of chitin particles of125 µm size under standard process conditions size for 6days. GlcNAc content of culture filtrate and chitinase activity was estimated. Highest product concentration on 5th day (Figure 6) and highest chitinase activity (Figure 7) on the 6th day respectively of incubation was observed with 10 g/L of chitin concentration. Reduction in product formation with 15 g/L of chitin may be due to substrate inhibition.

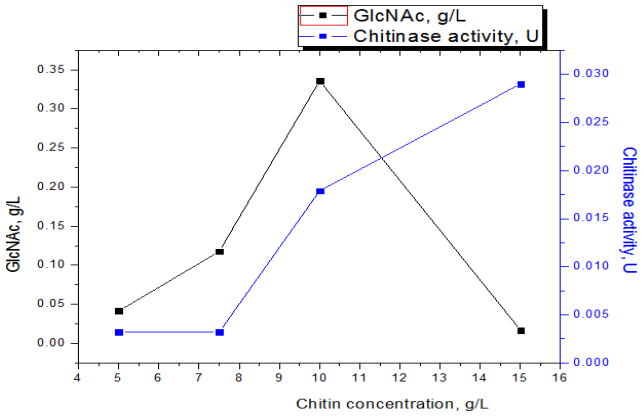

Figure 6: Effect of incubation and chitin particle size (10g/L) on GlcNAc production under standard process conditions (5th day)

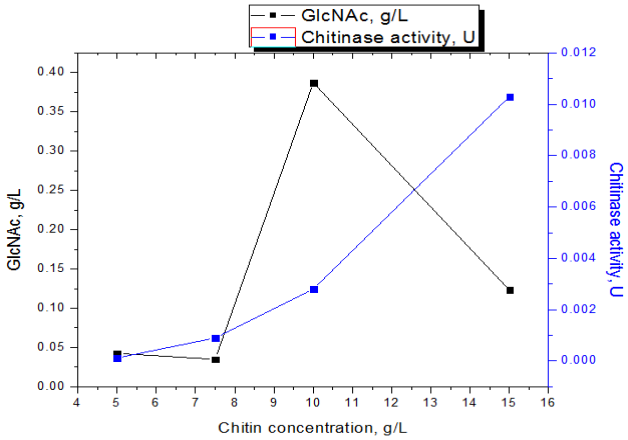

Figure 7: Effect of incubation and chitin particle size (10 g/L) on GlcNAc production under standard process conditions (6th day)

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Effect of pH on chitinase and GlcNAc production: To determine the optimum pH for chitinase production, the filamentous fungi was inoculated into the chitin medium at different pH (4-6) for 120 h at standard conditions and composition of the broth media. After 4th, 5th and 6th day of incubation, the supernatant of broth media was centrifuged and culture filtrate was used for chitinase assay and GlcNAc content. Both were observed to be very low till the 4th day with repeated experiments. Thus estimation was done only after 5th day of incubation in all experiments. Broth solution of pH5 produced maximum chitinase activity and desired product (Figure 8).

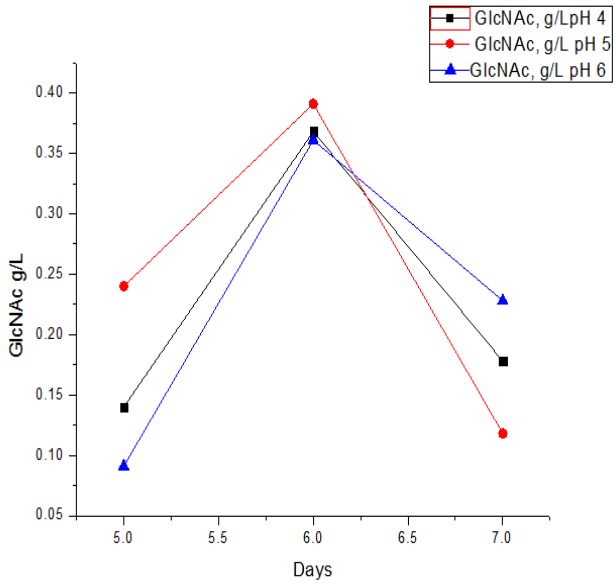

Figure 8: Effect of pH (15 g/L, 105 µm) and incubation on GlcNAc production at standard process conditions

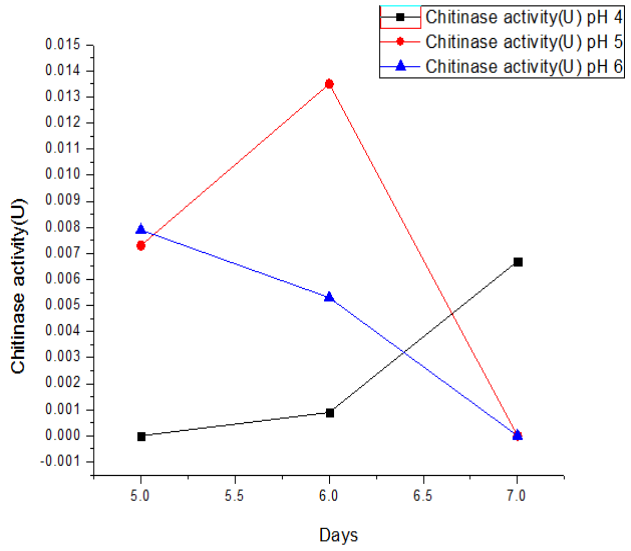

Figure 9: Effect of pH (15g/L, 105µm) and incubation on Chitinase activity at standard process conditions

Effect of trace nutrient concentration on GlcNAc production and Chitinase activity on chitin depolymerization: Production of N-acetyl glucosamine was studied by varying the concentration of trace nutrients at 34℃ with 160 rpm orbital speed in the incubator for all the experiments and broth media pH was adjusted to 5 using 2M NaOH before incubation. Trace nutrients $FeSO_4$. H_2O 0.0005 g/L, $MnSO_4.H_2O$ 0.00016 g/L, $ZnSO_4.7H_2O$0.00014 g/L and $CaCl_2.2H_2O$ 0.0002 g/Lwere added. This composition corresponds to $1/10^{th}$ of the standard concentration in all other experiments. This reduction in trace nutrient concentration resulted in drastic drop in GlcNAc production due to lowered chitinase activity (Figure 10**)**. Yield of GlcNAc was defined as the ratio of amount of GlcNAc produced per unit quantity of chitin fed during the process. The results of this experiment demonstrated the importance of trace nutrients during enzymatic process. Curves **(**Figure 10) corresponding to pH 4.5, 5.2 and 5.8 were obtained with this concentration. These three curves with reduced trace nutrient concentration show drop in GlcNAc production and chitinase activity (Figure 11). Experimental results with pH of 4, 5 and 6 shows considerable increase in GlcNAc content of culture filtrate.

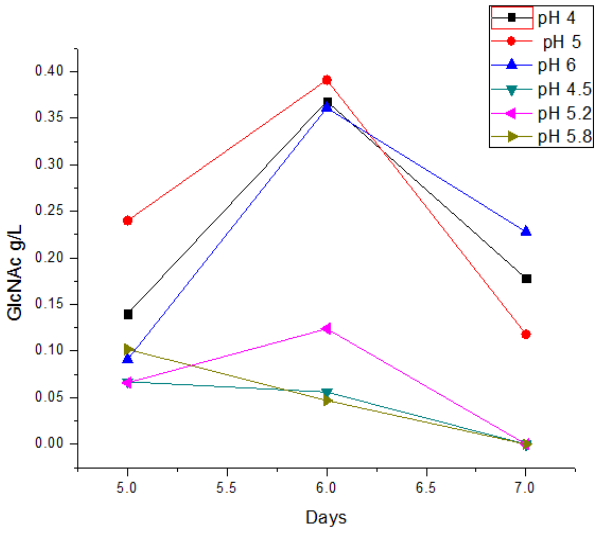

Figure 10: Effect of trace nutrients and incubation on GlcNAc production at different pH of broth media under standard process conditions with 15g/L of 105µm size chitin

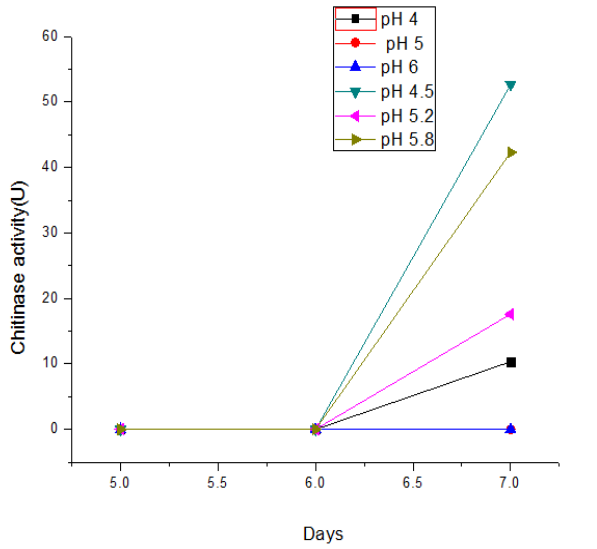

Figure 11: Effect of trace nutrients and incubation on chitinase activity at different pH of broth media under standard process conditions with 15 g/L of 105 µm size chitin

Substrate inhibition: Experimental results obtained with various substrate concentrations after incubation time of 120 h and 144 h were subjected to both Michaelis-Menten

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(Figure 12) and Lineweaver-Burk (Figure 14) methods of kinetic analysis. Both show substrate inhibition. Figure 12 and Figure 13 also depict variation of reaction rate (r, g/L h) against substrate concentration (S, g/L) and product concentration (g/L) .

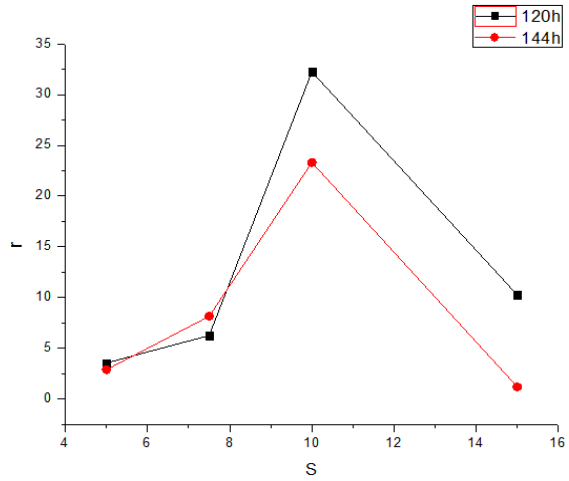

Figure 12: Substrate concentration against reaction rate

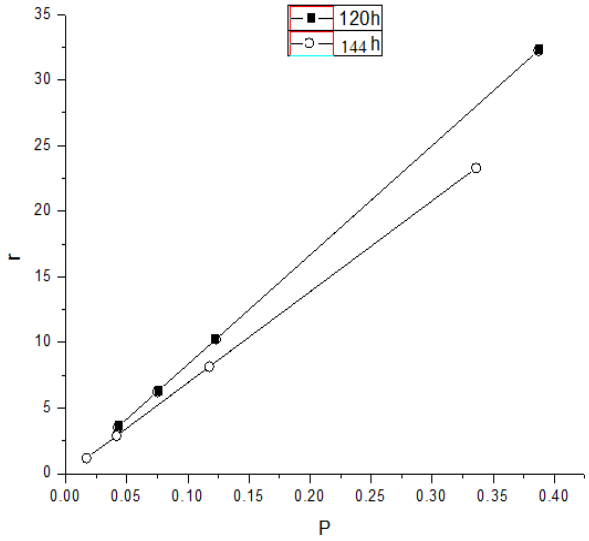

Figure 13: Product formed against reaction rate

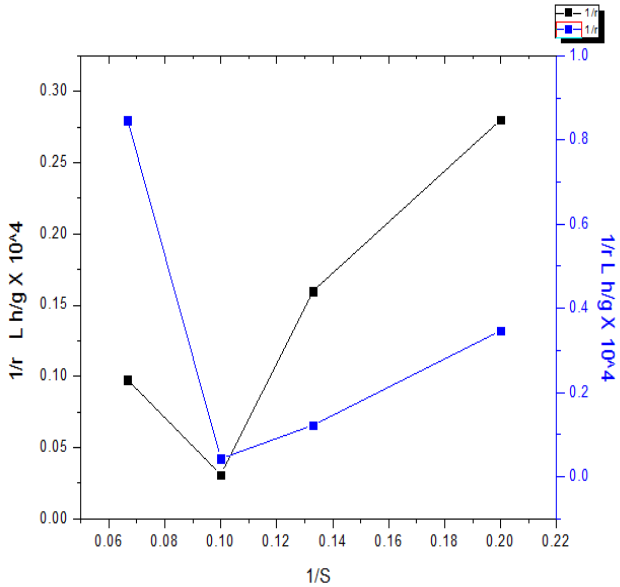

Figure 14: Lineweaver–Burk plot showing substrate inhibition during depolymerisation

IV. CONCLUSIONS

Depolymerization of chitin using chitinolytic enzymes produced by *Trichoderma harzianum* (MTCC 3928) was successful in producing oligomers analyzed using dinitrosalicylic acid method. Incubation in an orbital shaker for 6 days at 34ºC±2and 160 rpm for 6 days with 105 µm acid treated chitin at pH5 yields maximum N-acetyl glucosamine. Experiments were conducted to find the effect of particle size in the range of 74 to 125 µm. Increase in the yield of GlcNAc was observed with initial chitin concentration of 10 g/L in the broth solution compared to other concentrations. Chitinase assay and GlcNAc contents were highest after 144 h of incubation. Production of N-acetyl glucosamine was studied with 125 µm and 105 µm size particles under the same standard conditions of the process. Highest GlcNAc production (1.2765 g/L and 1.197 g/L) was observed after 113.17 h of incubation for125 µm and 105 µm of chitin sample respectively. N-acetyl glucosamine content of culture filtrate was found to increase with incubation time and as well with reduction in the chitin particle size. Cell growth kinetics and reaction kinetics during seed media preparation were studied. DNA content of seed media against incubation hours and chitinase activity in the broth solution were estimated. Effect of media pH, chitin particle size and chitin concentration on depolymerization was found out. Optimal conditions of enzyme production by *T. harzianum* were estimated to be pH 5, 34ºC±2 and 144 h of incubation hours. Substrate inhibition was observed at concentration above 10 g/L.

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