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Modification of chitin as substrates for chitinase

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Enzymes are able to bind to their substrates specifically at the active site. The proximity and orientation of the substrates strongly increase the likelihood that productive E–S complexes will arise. Treated chitin (powder or flake) is more efficient than crystalline chitin. This is because the latter is less active due to its insolubility. The structure of treated chitin is opened; this facilitates its interaction with the enzyme. The purpose of this research was to create a kind of modified chitin and study the characterization of the different types of chitin including functional groups by IR spectrophotometer, pore size, surface area and crystallinity by X-Ray diffraction. Chitin from shrimp shell was modified into colloidal, bead, amorphous and superfine chitin. The results of the IR spectra of colloidal and bead chitin showed a similar pattern with chitin powder; they peaked at 3447 and 3113 cm^{-1} (OH and NH_2 groups), 1645 cm^{-1} (amide groups N-H) and 1071 cm^{-1} (group C-O). Superfine and amorphous chitin had similar absorbance with powder chitin but appeared to peak in the fingerprint region. Characterization of physical properties based on the pore size and surface area of powder, colloidal, superfine, amorphous and bead chitin changed the pore radius of each type of chitin due to the treatment of swelling. Crystallinity showed that specific diffractogram pattern in the three main peaks 2θ was 9.5, 19.5 and 26 with varying intensity. Chitinase activity assay using modified types of chitin substrate had higher values than chitin powder. The highest activity was in amorphous chitin with values of 1.858 U/mL. This is because it has chitin chain and the rearrangement of its structure was more open, facilitating its interaction with enzyme.

Key words: Chitin modified, chitinase, substrate.

INTRODUCTION

Chitin is a polymer that is very abundant in nature and is second only to cellulose. It is widely spread in nature as in fungi, algae, nematodes, arthropods, molluscs, plants and animals (Guo et al., 2004). So far, it has been found that it has very little large-scale industrial use because of its extreme insolubility; it cannot be absorbed or digested directly in the gastrointestinal tract (Dai, 2011).

Chitin structure has three forms, namely α, β and γ. α-Chitin is a form of a dense structure isomorphous having strong hydrogen bonds. β-Chitin has a structure with weaker intramolecular bonds but slightly more stable than α-chitin. γ-Chitin is a combination of α and β chitin structure. α-Chitin structure causes the chitin not to be soluble in the solvent, while β-chitin can be swollen in water, as

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Abbreviations: SDS, Sodium dodecyl sulfate; **Ct,** chitin powder; **Cc,** colloidal chitin; **Sf,** superfine; **Cb,** bead; **Ca,** amorphous.

chitin is soluble in formic acid (Coutinõ et al., 2006). Chitin chains between each other are associated with very strong hydrogen bonds between the NH groups of one chain and the C = O groups of adjacent chains. Hydrogen bonds make chitin to be insoluble in water and to form fiber (Rostinawati, 2008). The presence of chitin in nature varies abundantly and degrades rapidly, due to the presence of some bacteria and fungi that have chitinase enzyme capable of degrading chitin. Chitin can be degraded in two lines: the first is a degradation by mechanisms that hydrolyze bonds chitinolytic 1,4-ß-glycosides, or polymers having first deacetylation and then hydrolysed by chitosanase (Herdyastuti et al., 2009).

Chitinases are extracellular inducible enzymes that catalyze the first step in chitin digestion, hydrolysis of β-1,4 linkages between the N-acetyl glucosamine (NAG) molecules. They are found in a variety of organisms including viruses, bacteria, fungi insects, higher plants and animals and play important physiological roles depending on their origin (Kuddus and Ahmad, 2013). Chitinolytic activity induces strain in the growth medium in the presence of chitin as a carbon source (Chernin et al., 1998).

Chitin can be modified by adding acid, base or detergents such as sodium dodecyl sulfate (SDS) for it to become swollen (Illankovan et al., 2007). The swelling of chitin powder process is expected to help the enzymatic reaction between chitinase and chitin. Possible modification of chitin-chitin can cause structural changes to become more open due to the restructuring of the chitin-chain. Possible rearrangement of chitin structure can cause changes in the functional group or the physical properties of each type of chitin compared with chitin powder.

MATERIALS AND METHODS

Preparation of chitin

Chitin was obtained from shrimp shells that have been dried and pulverized and the isolation was done by the method of Acosta et al. (1993). Chitin isolation process consists of two stages: deproteinisation and demineralization. Chitin is made into the form of colloids according to Hsu and Lockwood (1975). Chitin was dissolved in concentrated HCl (37%), and then precipitated as a colloidal suspension with the addition of cold water (5°C). The suspension was filtered and the residue was washed with distilled water until it got to neutral pH, and then dried with an oven. This process gives ± 85% recovery.

Chitin was then prepared into colloidal, superfine, bead and amorphous form. In the colloidal form, chitin is made by adding concentrated HCl (37%). Chitin beads were obtained by dissolving in 2% formic acid and 2 M NaOH solution. Chitin amorphous was prepared by dissolving chitin in a mixed solution of 40% NaOH and 0.2% SDS (which has been cooled to a temperature of 4°C). Solution was in-swell for 1 h at 4°C and matrix slurry was stored for 1 night at -20°C temperature, and then neutralized with HCl 6 N. Furthermore, it was filtered and washed with ethanol, water and acetone. The result was dried with a freeze dryer.

Produce of chitinase enzyme

Chitinase was produced in medium with the following composition: 0.4% chitin, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.5% $MgSO_4.5H_2O$, 0.01% $FeSO_4.7H_2O$, 0.001% $MnCl_2$ and 0.5% peptone, and incubated at room temperature for 45 h in rotary shaker at 150 rpm. The culture cells were centrifuged at 4000 rpm for 20 min (4°C). The supernatant was brought to 50% saturation with ammonium sulphate at 4°C for 30 min by stirring magnetic stirrer. The precipitate was recovered by centrifugation at 4000 rpm for 30 min (4°C) and pellet formed was solubilized in 0.1 M phosphate buffer pH 7.0. The solution was dialyzed overnight against the same buffer at 4°C.

Chitinase assay

Chitinase activity was measured by colorimetric method based on the released N-acetyl-glucosamine(Monreal and Reese, 1969). The colloidal chitin solution (2.0 mL of 1.25% (w/v)) dissolved in 200 mM potassium phosphate buffer was added to 0.5 mL enzymes solution and incubated for 2 h at room temperature. The suspensions were centrifuged at 4000 rpm for 10 min and then supernatant (1.0 mL) was added to 2.0 mL deionized water and 1.5 mL color reagent (5.3 M sodium potassium tartrate and 3,5-dinitrosalicyclic acid 96 mM). The mixed solution was placed in boiling water for 5 min and cooled at room temperature, and then the absorbance was measured at 540 nm. One unit (U) of chitinase activity was defined as the amount of enzyme required to release 1.0 mg N-acetyl D-glucosamine from chitin per hour.

Characterization of substrates

The structure of substrates was determined by FT-IR spectro-photometer (Perkin Elmer); the analysis of pore size and surface area was done with high speed surface area (NOVA 1200e). X-ray diffractograms were recorded by a Bruker type D 8 advance.

RESULTS AND DISCUSSION

Characteristic of chitin

Chitin that has been isolated from waste shrimp shells chitin powder (Ct) and has been modified into a kind of colloidal chitin (Cc), Superfine (Sf), bead (Cb) and amorphous (Ca) is as shown in Figure 1. Chitin was modified to have almost the same colour, smoother texture and lighter but more types of colloidal tawny color and form larger granules. The yield of the average obtained was 50 to 60%. The results of the analysis of the functional group on IR- spectrophotometer modified chitin (Figure 2) show the absorption at 3446 and 3113 cm^{-1} (OH and NH_2 groups).

Sharp absorption peaked at 1645 cm^{-1} indicates the presence of amide groups (N - H) and 1071 cm^{-1} shows the group C - O. The spectra of Ca are a sharp peak in the fingerprint region below 700 cm^{-1} which is not found in other types of chitin. Spectra of superfine showed similarity with the IR spectra of chitin and colloidal chitin. The characteristic peaks of chitin are -OH group (3433 cm^{-1}), -NH (amide) at 1587.54 cm^{-1}, CH bending vibration

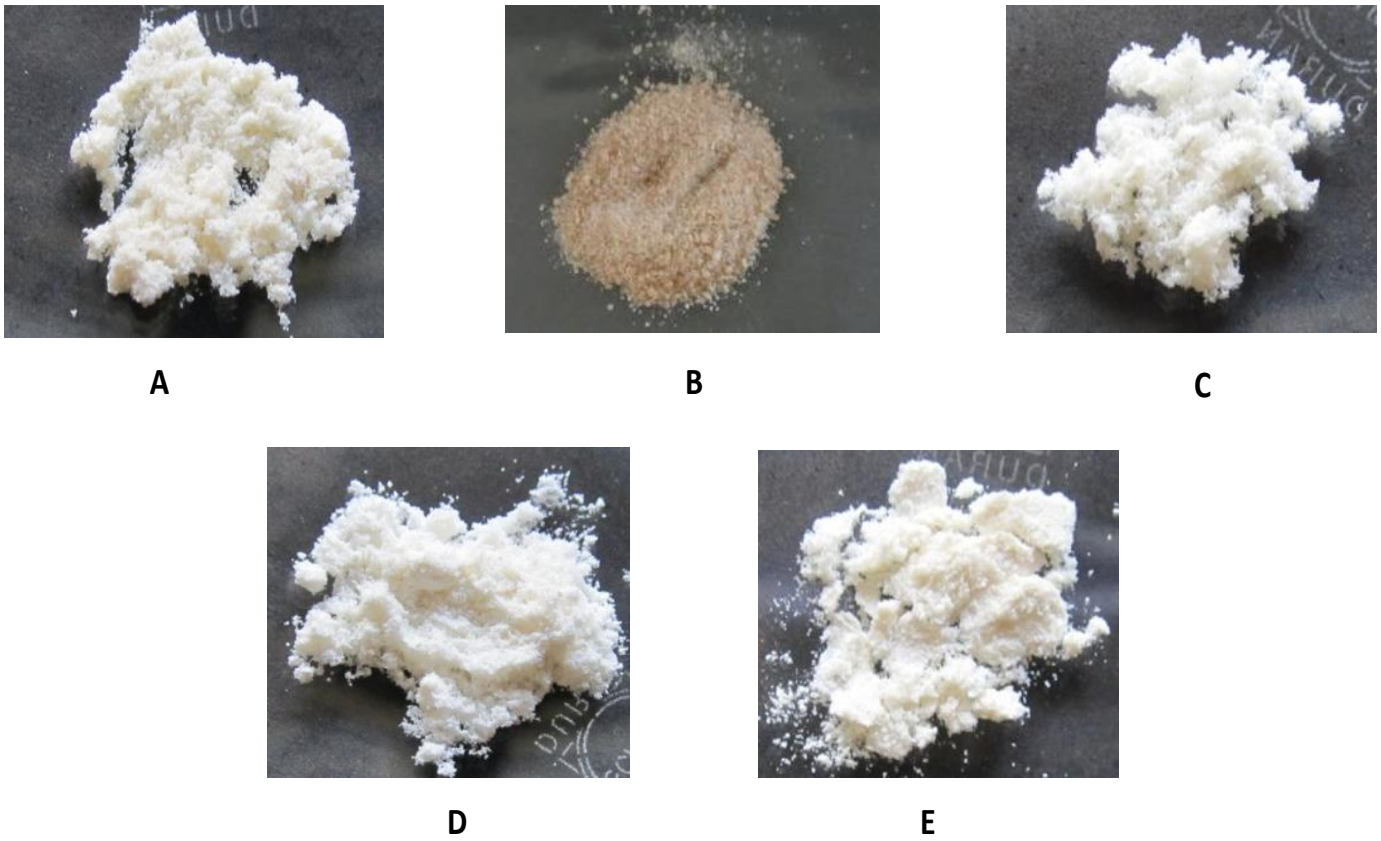

Figure 1. (A) Powder chitin. **(B)** Colloidal chitin. **(C)** Superfine chitin. **(D)** Amorphous chitin. **(E)** Bead chitin.

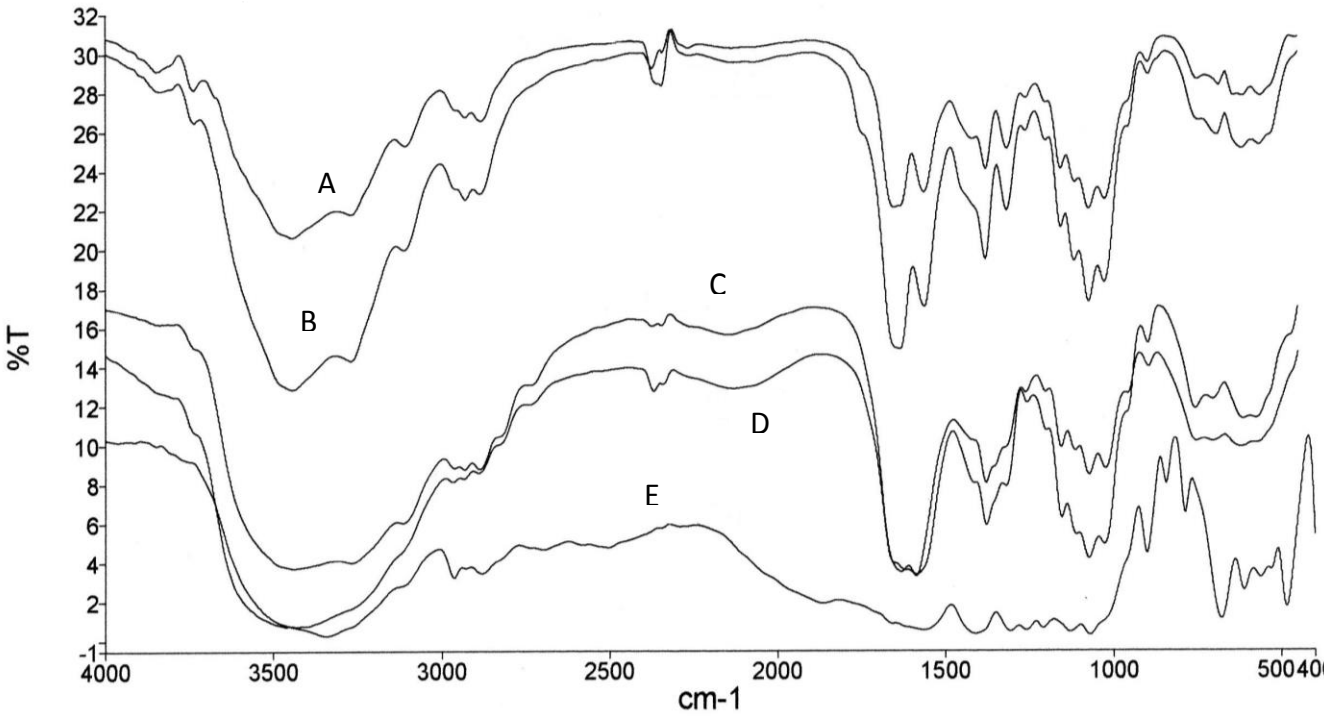

Figure 2. Spectra-IR of **(A)** Powder chitin, **(B)** Colloidal chitin, **(C)** Superfine chitin, **(D)** Bead chitin **and (E)** Amorphous chitin.

Table 1. The relevant peak FT-IR spectra of chitin substrates.

Function group	Wave number (cm^{-1})				
	Ct	Cc	Sf	Cb	Ca
C – O	1379	1380	1379	1379	1308
N – H (bending)	1562	1635	1567	1567	-
C – H (streching)	2886	2931	-	-	2963
C = O	1651	1635	1633	1633	-
O – H	3446	3446	3435	3435	3345

Ct, Powder chitin; Cc, colloidal chitin; Sf, superfine chitin; Ca, amorphous chitin; Cb, bead chitin.

Table 2. Analysis of physical characteristic in the chitin substrates.

Substrate	Pore radius (A°)	Pore area (m^2/g)	Pore volume (cc/g)
Powder chitin	19.108	1.365	4.00×10^{-2}
Colloidal chitin	19.044	2.780	5.00×10^{-2}
Superfine chitin	162.879	0.06	0,0001
Amorphous chitin	19.159	3.252	1.30×10^{-2}
Bead chitin	19.011	0.606	4.00×10^{-3}

at 1378.7 cm^{-1}, stretching vibration of C = O, amide - $NHCOCH_3$ (1633.1 cm^{-1}) and CO alcohol at 1072.9 cm^{-1} (Tamimi and Herdyastuti, 2013). Table 1 shows the amides that generate elimination of carboxyl groups (Coutin˜o et al., 2006).

The results of the analysis based on the physical properties of pore size and surface area of powder, colloidal, superfine, amorphous and bead chitin are shown in Table 2. The results of the analysis showed that treatment of swelling on each type of chitin changed pore radius and became larger. Data show pore radius of superfine chitin is 9 times greater than the powder chitin. Wide pores of colloidal chitin also increased, but the chitin beads were apparently amorphous and their volume was reduced. The changes of volume size and pore radius would affect the interaction of enzymes with the substrate.

Diffractogram of chitin powder, colloidal and bead shows the same pattern. There are 3 main peaks 2θ of 9.5, 19.5 and 26; the intensity tends to be weaker in colloidal and bead chitin than in powder chitin as shown by the studies of Illankovan et al. (2007), in which the diffractogram of powder, colloidal and amorphous chitin had main peak of 2θ of 9.4 and 20.

This shows that colloidal and bead chitin has a lower degree of crystallinity than chitin powder. Swelling process in colloidal and bead chitin causes larger pores and is easily inflated in water medium; this leads to the easy interaction of enzymes with substrates than in the form of chitin powder, which is more compacted (Figure 3).

relevant peak FT-IR spectra of chitin substrates. The other research shows that chitin has -NH peak at 3269, 1663 and 1629 cm^{-1} due to the reduction of primary

Chitinase activity

Chitin from crab shell is identified as the best carbon source and colloidal chitin is reported as the best source for producing chitinase. The results show the highest activity for the amorphous chitin after colloidal chitin types (Figure 4). Suraini et al. (2008) reported that the highest specific activity produced by colloidal chitin was 14.59 U/mg. Chitinase from *Paenibacillus* sp. D1 showed that the highest activity was 35 U/mL at 30°C after 72 h (Singh, 2010). The results of optimization of culture nutrients revealed that the amount of colloidal chitin as a sole carbon source in the growth medium of *Trichoderma viride* was 32.1 U/mL (Sharaf et al., 2012). By optimizing the above cultural conditions, the production of chitinase from *Bacillus amyloliquefaciens* SM3 increased by three fold to 33.5 U/mL at the final stage (Das et al., 2012). This form of chitin is tight due to its anti-parallel chain form and it stabilizes polymorphism shape naturally causing chitin not to dissolve in the solvent (Majtán et al., 2007). Modified chitin using SDS detergent leads to swelling of chitin structure causing changes in the physical properties of chitin powder. Amorphous type chitin has fingers longer than other types of chitin and three times larger area than the chitin powder. The data indicate that amorphous chitin is more open and more

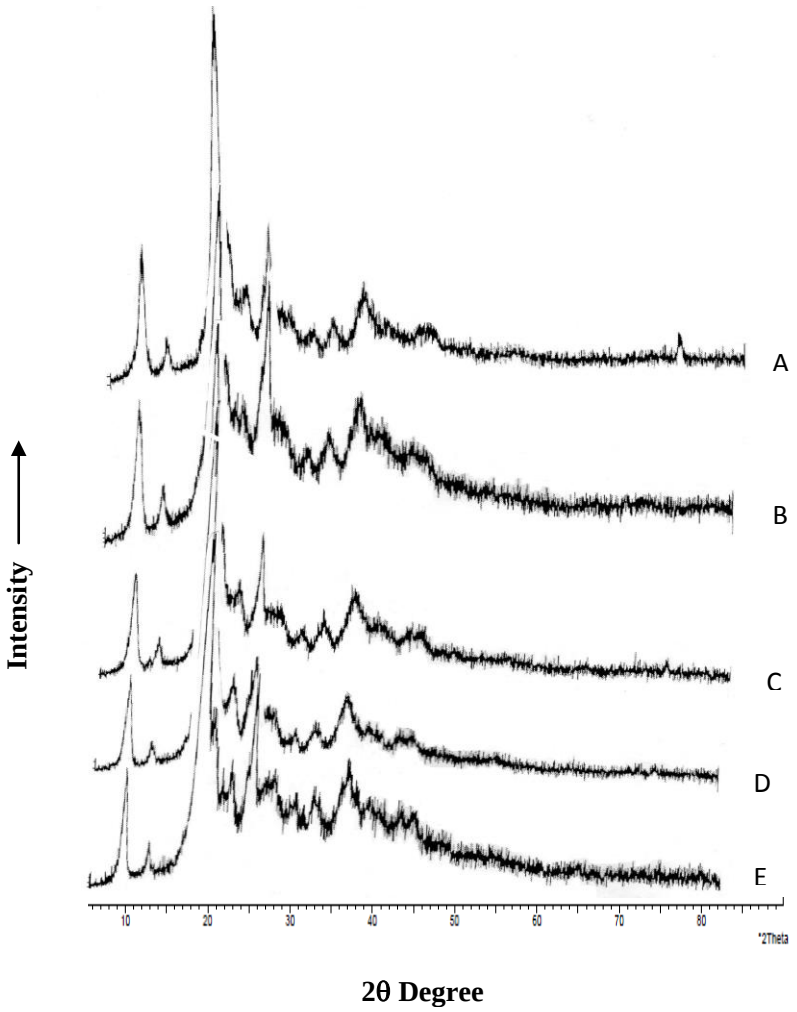

Figure 3. Difractograme of powder chitin (A), colloidal chitin (B), amorphous chitin (C), bead chitin (D) and superfine chitin (E).

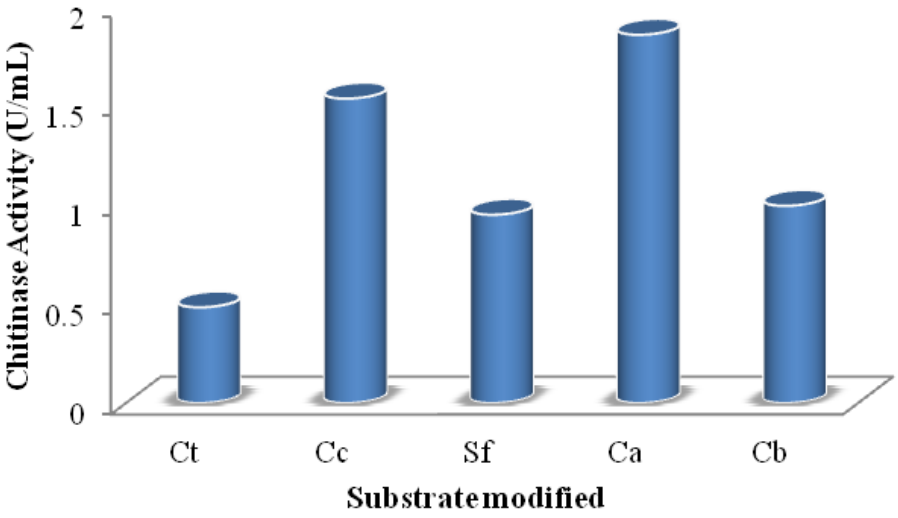

Figure 4. Chitinase activity with substrate types. Ct, Powder chitin; Cc, colloidal chitin; Sf, Superfine chitin; Ca, amorphous chitin; Cb, bead chitin.

likely to facilitate interaction with chitinase that can provide higher chitinase activity than other substrates. Ilankovan (2005) reported that among the chitinolytic activities of the commercial enzymes investigated with amorphous chitin as substrate, the bovine pepsin had the highest chitinolytic activity.

Conclusion

Modification of chitin by adding detergents causes characteristic changes in its physical properties and the structure becomes more open than chitin powder, thus causing its interaction with the enzyme chitinase. Amorphous chitin can be used as an alternative substrate or inducer for chitinase enzyme indicated by higher chitinase activity than using chitin powder.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Acosta N, Jimenez C, Borau V, Heras A (1993). Extraction and characterization of chitin from crustaceans. Biomass Bioenergy 5:53.

Chernin LS, Winson MK, Thompson JM (1998). Chitinolytic Activity in Chromobacterium violaceum : Substrat analysis and regulation by quorum sensing. J. Bacteriol. 180: 17.

Coutin˜o LR, Marı´a del Carmen MC, Huerta S, Revah S, Shirai K, (2006). Enzymatic hydrolysis of chitin in the production of oligosaccharides using *Lecanicillium fungicola* chitinases. Process Biochem. 41: 1106–1110.

Dai DH, Wei-lian H, Guang-rong H, Wei L (2011). Purification and characterization of a novel extracellular chitinase from thermophilic *Bacillus* sp. Hu1, Afr. J. Biotechnol. 10:2476-2485.

Das MP, Rebecca LJ, Sharmila S, Anu, Banerjee A, and Kumar D (2012). Identification and optimization of cultural conditions for chitinase production by *Bacillus amyloliquefaciens* SM3. J. Chem. Pharm. Res. 4(11):4816-4821

Guo SH, Chen JK, Lee WC (2004). Purification and Characterization of Extracellular Chitinase From *Aeromonas schubertii*. Enzyme Microb. Technol. 35: 550-556.

Herdyastuti N, Raharjo TJ, Mudasir, Matsjeh S (2009). Kitin dari limbah cangkang udang sebagai media untuk bakteri kitinolitik yang diisolasi dari lumpur sawah, Jurnal Manusia dan Lingkungan. 16 : 115-121

Hsu SC, Lockwood JL (1975). Powdered Chitin Agar As a Selective Medium for Enumeration of Actinomycetes in Water and Soil. Appl. Microbiol. 29:422-426.

Ilankovan P, Hein S, Chuen-How Ng, Trung TS, Stevens WF (2005). Production of N-acetyl chitobiose from various chitin substrates using commercial enzymes. Carbohydr. Polym. 63(2):245-250.

Kuddus SM, Ahmad RIZ (2013). Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. J. Genet. Eng. Biotechnol. 11:39-46

Majtán J, Bíliková K, Markovic O, Gróf J, Kogan G, Simúth J (2007). Isolation and characterization of chitin from bumblebee (*Bombus terrestris*). Int. J. Biol. Macromol. 40:237-241

Monreal J and Reese, ET (1969). The Chitinase of Serratia marcescens. Can. J. of Micro., 15:689-696.

Rostinawati T (2008). Skrining dan Identifikasi Bakteri Penghasil Enzim Kitinase dari Air Laut di Perairan Pantai Pondok Bali. Jatinagor: Universitas Padjajaran.

Sharaf EF, El-Sarrany AEQ, El-Deeb M (2012). Biorecycling of shrimp shell by *Trichoderma viride* for production of antifungal chitinase. Afr. J. Microbiol. Res. 6(21):4538-4545

Singh AK (2010). Optimization of culture conditions for thermostable chitinase production by *Paenibacillus* sp. D1. Afr. J. Microbiol. Res. 4: 2291-2298

Suraini AA, Sin TL, Alitheen N, Shahab N, Kamaruddin K (2008). Microbial Degradation of Chitin Materials b*y Trichoderma virens* UKM1. J. Biol. Sci. 8:52-59

Tamimi M, Herdyastuti N (2013). Analysis functional groups using FT-IR spectroscopy of chitin variation as *Pseudomonas sp*. TNH-54 substrate's. UNESA J. Chem. 2:47-51.