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Production of chitosan from local crab chitin waste enzymatically for health drink

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Abstract

The aim of the research were to produce local chitin waste to bioactive compound of chitosan enzimatically to applied in functional drink instant. The chitin waste made from crab shells as by product of canning crabs meat industry in Cirebon. Production of chitosan enzimatically used chitin deacetylase enzyme which produced by *Bacillus papandayan* isolated from Kamojang Creater, West Java, Indonesia.
The result of this research was technology of process and chitosan product which degraded enzimatically. Product was chitosan-tea drink which ready to solute in water. The conclusion of this research that fisically, chitosan-tea drink instant performance were white-brown flour mixed with dry chitosan gel (1,5 cm x 1,5 cm), soft surface, brown clear color, and smooth. Formulation of chitosan-tea drink was weight 23,7 gram perpack/once consumption (sorbitol 22,5 gram, green tea extract 0,375 gram, chitosan 0,8 gram). Chemically, chitosan-tea drink instant have water content 0,22% w/w, ash content 0,11% w/w, protein content 0,03% w/w, lipid content 0,002% w/w, calory 58,5 kkal which suitable with SNI 01-3722-1995 standard.

Keywords: chitosan, crab, chitin, enzymatically, drink

Introduction

Chitin is an insoluble polysaccharide consisting of ß(1-4) linked N-acetyl-D-glukosamine (GlcNAc) units, is the second most abundant polysaccharide in nature, after cellulose. It is widely distributed as structural component of crustaceans, insects, and other arthropods, as well as component of cell walls of most fungi and some algae. About 10^{11} tons of chitin is produced annually in the aquatic biosphere alone, however, only 0.1% of this material is currently being converted to valuable product.

Chitin waste Indonesia which has not been exploited 56.200 metric of ton per year (Department of Marine and Fishery, 2003). Chitosan is chitin which has been eliminated its acetyl group leaving free amine residue that making it as polycationic character.

Untill now, conversion of chitin to chitosan in industry is done termochemically, which is applied with strong alkali at high temperature. This process requires high energy, maintains high temperature and produces waste and basic product with high concentration so is potencial to become toxic in environment.

Alternatively, deacetylation conversion of chitin to chitosan can be done enzimatically by chitin deacetylase=CDA. This process makes chitosan easier to be controlled, more efficient, specific and safe to environment. Chitin deacetylase synthesized by various crops, bacterium, mushroom, and sea organism (Kupiec and llan, 1998). Mushroom *Colletotrichum lindemuthianum* (Tsigos and Bouriotis 1995, Tokuyasu et al. l996, Tsigos et al. 2000), *Mucor rouxii* (Kafetzopoulos et al. 1993; Kolodziejska et al, 1998), *Absidia coerulea* (Gao et al. 1995) and *Aspergillus nidulans* (Alfonso et al, 1996) proven produced CDA. Local isolate of producer CDA, *Bacillus papandayan* K29-14, has been reported by Rahayu et al (2004).

Waste treatment of rajungan chitin to improve added value need to be done. Waste treatment technology input of chitin is expected will increase its market price. On the other hand, formulation of food product bases on chitosan is needed to applied as coroner heart sickness inhibitor and as prebiotik. This thing is constituted by till now supplement of food (*neutraceutical*) is containing chitosan in international market which high, that is Rp 250.000-300.000/100 item capsule for a few certain merk. Unfortunately, neutraceutical unable to be enthused public because its form looking like drug.

Chitin deacetylase convers chitin become chitosan (E.C 3.5.1.41) degraded N-acetamido from part of N-acetyl-D-glucosamine in chitin produced chitosan compound (Tsigos et al, 2000). So far, CDA can be isolated from some moulds like, *Mucor rouxii* (Davis and Garcia, 1984), *Colletotrichum lindemuthianum* (Tokuyasu et al 1996, Tsigos et al, 2000), *Uromyces viciae-fabae* (Deising and Siegrist, 1995), and yeast *Saccharomyces cerevisiae* (Christodoulidou et al, 1999). CDA from *Bacillus papandayan* K29-14 has been reported by Rahayu et al (2004), this enzyme has optimum pH 8, optimum temperature 55 ^{o}C and activated by $MgCl_2$.

Purification with column chromatography reported by Rochima, 2004.

Chitosan is composed primarily of GlcNAc and GlcN (2-amino-2-deoxy-ß-D-glucopyranose) residu. Unlike most polysaccharide, chitosan has three types of reactional functional groups, an amino group as well as both primary and secondary hydroxyl groups at C-2, C-3, and C-6 position respectively. Amino group (NH_2) what causes chitosan to have the character of dissolving water so that easy to be application. (Bastaman, 1989)

This positive charge makes chitosan can tie compound around which haves negative charge, like cholesterol, fat, bile acid, and some other fat generations at the time of passing alimentary canal, and releases it through faeces (Furda, 1980). Chitosan can absorb 97% body fat, binding ability of fat by chitosan had been proved by Japan researcher long time ago (Sugano et al, 1980). N-acetylglucosamine residu in chitosan can stimulate growth of bifidobakteria, which is good microbe against pathogenic bacteria in intestine microflora. Study to chicken as attempt animal indicates that giving of diet containing whey 20% and chitin component 0.5-2% can improve intestine mikroflora (Austin et al, 1989).

Edible Chitosan Film

Edible film is a continued thin layer, made from edible material, formed above food component (coating) or among food component (film) which is functioning as barrier to mass transfer, and or as food-stuff carrier and additive, and easier for handling (Caner et al, 1998). The character of edible chitosan film are strong, elastic, flexible, hard to tear (Buttler et al., 1996).

Mechanism of formation edible film with polysaccharide material is with disconnection of polymer chain becomes film matrix or gel influenced by evaporation of hydrophilic condensation, hydrogen bond, elektrolik, and tying ionik (Buttler et al., 1996). Nurdiana (2002) express that formula edible best chitosan film with sorbitol as plasticizer is 1% b/v chitosan in 1% v/v acetic acid with addition of sorbitol 0,25% b/v. Research of edible chitosan film with CMC (Carboxy Methyl Chitosan) as plasticizer yields best formula of edible chitosan film as pengemas consisted of 1% chitosan in 1% v/v acetic acid with addition of CMC 0,1% b/v (Sitanggung, 2002)

Green tea

Based on its production process, tea can be divided into 3 types, that are green tea, black tea and tea olong. Green tea is made without fermentation process, black tea is made with full fermentation, while tea oolong, is combination black tea and green tea, that is fermentation that shorter than black tea (Hartoyo, 2003).

Polyphenol which implied in tea has proven gives positive effect in the form of disease prevention of heart and stroke. Tea also contains a number of vitamins (like vitamin B1 and B2); ascorbic acid, vit E, and K), and mineral (manganese, potasium, and fluor). The ascorbic acid content is higher from orange, two small cups of green tea has same as ascorbic acid of orange juice.

Sorbitol

Substitution sweetener of sucrose that can be divided into two types is low calorie sweetener and reduced-calorie sweetener. Low calorie sweetener is higher sweetener than sucrose, (sucrose equivalent) required number of very few, so that low calorie. It is usually applied in food industry like saccharin, aspartame, K-acesulfame, and sucralose. Reduced-calorie sweetener is sweetener which has lower calorie and sweetness than sucrose (Lindsay, 1996). Sorbitol (D-Glusiol) be sweetener having the character of bulky (low density), easy to dissolve in water, very stable to acid, enzyme and temperature 140 ^{o}C (Chandra, 1997), as humectans, not easy to metabolism by bacterium in mouth so that not easy to generate dental caries (Almatsier, 2002). Sorbitol is including substitution of sugar that is component is looking like sugar (sucrose and fructose) but in metabolism of body doesn't influence insulin (Belitz and Grosch, 1999).

Materials and Method

Time and Place

This work was conducted from January 2007 to October 2007 in Laboratory of Tecnology of Industry of Postharvest Fishery, Fishery and Marine Science Faculty of Padjadjaran University and Laboratory of Microbiology and Biochemistry Biotechnology Research Center Bogor Agricultural University.

Material and Equipment

Bacillus papandayan K29-14 isolate was collection of Laboratory of Microbiology and Biochemistry Biotechnology Research Center Bogor Agricultural University. Growth isolate media (Sakai et al 1998) i.e: Bacto Agar, Ammonium sulphate, K_2HPO4, NaCl, $MgSO_4$. $7H_2O$, Yeast Extract, Bacto trypton, Coloidal chitin. Coloidal chitin made of chitin powder Sigma based on Arnold and Solomon method (1986). Chitin glycol made of chitosan glycol based on Trudel and Asselin method (1989).

Chitosan production was made of Cirebon crabshell, Formulation of tea instant covers green tea. Chitosan making apparatus, capillary viscometer Ubbelohde, sentrifugator, analytical balance, oven, spectrometer Perkin Elmer Lambda 25 UV/VIS, spectrometer First Derivative Ultra Violet (FDUV),

PH METER, shaking incubator, incubator, vortex, glass equipments, vortek.

Method

Chitin deacetylase production

Culture of *Bacillus papandayan* K29-14has been fermented in Sakai media at pH 8,0 and 55 ^oC for 2 day. After completed, enzyme is harvested by sentrifugation 8000 rpm for 15 min. Supernatant dissociated and tested activity of chitin deacetylase according to Tokuyasu et al., 1996.

Ammonium sulfate precipitation (Copeland, 2000)

Ammonium sulphate was added slowly into harsh extract of enzyme, stirred until 80% saturation Mixture was precipitated over night at 4^oC then centrifugated 8000 rpm for 15 min. Pellet dissolved in 0.02 M borate buffer then kept at 4^oC.

Protein measurement by Lowry method (Copeland, 2000)

This method based on ability of protein ties special colourant. A number of 0.1 ml sample, 0.9 ml akuades and 10 ml reactant Lowry placed in tube then is vortex. Tube incubation 15 min. in room temperature. A number of 30 ml reactant folin-fenol ciocalteau which has been dissolved 10 times is added into tube, vortex then is more incubation 45 minutes in room temperature. Sample absorbance measured at wavelength 540 nm. Absorbance value compared to standard curve which has been made before all with bovine albumin serum (BSA) as standard. As blanko, applied akuades for substitution of condensation of protein.

Chitin deacetylase assay (Tokuyasu et al., 1996)

Mixture consists of 50 µL chitin glycol 1%, 100 µL 0.2 M borate buffer pH 8.0 and 150 µL enzyme. Incubation it for 30 min. at 55 ^oC. Inactivation enzyme in 100 ^oC for 15 min. 200 µL of mixture was added 200 µL $NaNO_2$ 5%, 200 µL acetic acid 33%, then vortex and let 10 min. After that, is added 200 µL ammonium sulphamate 12.5% then shaker 30 min at room temp. Then is added 800 µL HCl 5% and 80 µL 0.1% indol in absolute ethanol (prepared when will be applied). Boiling it for 5 min. then cooling., It is added 800 µL absolute ethanol before measurement of absorbance at wavelength 492 nm

Chemically and deacetylation enzymatic of chitosan

Crabshell waste, source of chitin, obtained from Bondet Cirebon Indonesia. It is washed and sun-drying for two-day, then flouried until 177 mm to 325 mm particles. Demineralization by addition of HCl 1 N 1:7 ratio, heated 90 ^oC 1 h, decantation, then cleaned again until pH 7 and dried. Deproteination by added of NaOH 3,5% ratio 1:10, then heated 90 ^oC 1 h, more decantation, washed until pH 7, then dried. Bleaching by addition of H_2O_2 2% ratio 1:10 till get is white chitin flour (Suptijah, 1992)

Deacetylation of chitin flour chemically by soaking in NaOH 50% (1:10) at 80 ^oC for 1 h. Chitosan formed then rinsed with water until neutral, then is measured degree of deacetylation. Enzymatic deacetylation by CDA which precipitated ammonium sulfate. 1 ml soluble chitosan 1% incubated with CDA 0.04 U/mg chitosan at 55 oC for 24 hours (Rochima, 2005).

Chitosan characterization

Degree of deacetylation (Muzzarelli et al, 1997)

Curve First Derivative is obtained with slid width 1 nm, and scanning speed 30 nm/menit. Calibration curve by prepared acid solution of acetate at some concentrations: 0.01, 0.02, 0.03, 0.04 and 0.05 M then read by spektra First Derivative at 250-190 nm. Cut point of all spektra becomes zero crossing point (ZCP) for acetic acid.

Chitosan viscosity (Hwang et al, 1997)

Chitosan viscosity is determined with Ubbelohde dilution viscometer. Viscometer is cleaned with akuades and dried. Soluble chitosan made in different concentration in acetate acid solvent aqueous 0.1 M and acetate sodium 0.25 M. Each sample is placed in viscometer a number of 10 ml.

Chitosan-Tea Drink Instant

Formulation of chitosan-tea drink refers to Palupi , 2006. Chitosan 1 g dissolved in 100 mL acetic acid 1% (b/v), added sorbitol. 0,25 % (b/v), heated at 85 ^oC, then put into petridisk (D=10 cm). After printed, chitosan layer dried in oven. Formulation of green tea ehich acceptable organoleptically is consisted of green tea extract 0.25% b/v and sorbitol 1.5% b/v, and chitosan instant (chitosan 1% b/v, acetic acid 1% v/v, and sorbitol 0.25% b/v).

Results and Discussion

Chitin deacetylase production

Morphology and characterization of Bacillus papandayan K29-14, producer CDA, according to Rahayu et al. 2004 as follows: Gram-positive bacteria bar shape, having spore, and motile. Optimum pH=7 at 55 ^oC. Free filtrate cell harvested at phase stationer on 28-32 incubation periods. Optimum CDA activity at pH=8, 55 ^oC, and activated by $MgCl_2$ 1 mM.

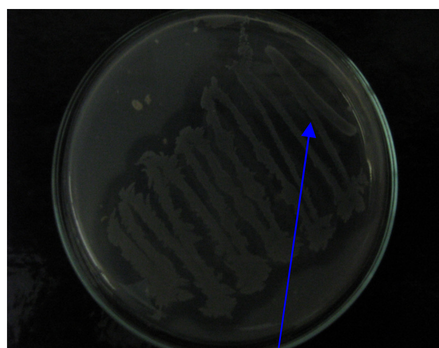

Figure 1 *Bacillus papandayan* K29-14 in chitinolityc media

Free filtrate activity of cell CDA at this research is 0.005 U/ml, smaller than CDA activity from *M. Rouxii* 0.0305 unit/ml (Kafetzopoulos et al. 1993) and CDA from *C. Lindemuthianum* 0.0195 unit/ml (Tokuyasu et al. 1996). Tsigos and Bouriotis (1995) tested activity CDA from different strain of *C. Lindemuthianum*, it's activity 0.002 unit/ml. From third of above researcher, only Tokuyasu et al (1996) what applies the same method like in this research, while Kafetzopoulos et al. And Tsigos and Bouriotis applies method is having Bergmeyer (method reaction of three enzymes). This research applies method Tokuyasu et al., because easy practically. Method Tokuyasu et al. Also applies a few of raw material, and low cost.

Chitosan solubility

Soluble chitosan in acetic acid solution influenced by the soaking time in NaOH aq. (Table 1). Acetic acid containing carboxyl group (- COOH). It contains a carbonyl and hydroxyl residu. Boiling point 118 oC and aroma is very sharply, respectively (Fessenden & Fessenden 1984).

Chitosan can only dissolve in dilute acid, such as acetic acid, formic acid, citrate except chitosan which has been substitution can soluble in water. Existence of carboxyl group in acetic acid will facilitate solubility of chitosan because the happening of interaction of hydrogen between carboxyl groups with amine bunch from chitosan (Dunn et al. 1997).

Tables 1 Effect of soaking time to condensation of chitosan

Temperature (^{o}C)	Time (hour)	Solubility (%)
80	0.25	38.62
	1	79.39
	2	85.52
	3	97.15

In acid solution, very compatible free amine group as polycationic can chelat of metal or form dispersion. Because in acid solution of chitosan will become polymer with diametrical structure so its very good for flocculation, formation of film or enzyme immobilization (Ornum 1992). Sanford (1989) said that, in acid situation, amine free of proton chitosan would form amino group kationik (NH3+). This cation can reacts with other polymer anionik complex electrolite.

Enzymatic deacetylation

Enzymatic deacetylation increases degree of deasetilation 5-30%, depend on degree of initial deasetilation (Tables 2). The higher degree of initial deacetylation, smaller improvement degree of deasetilation. Degree of deacetylation above 90% can only be reached at sample with degree of initial deacetylation above 75%. Longer initial passed to sample, conformation of sample would increasingly estranged that enzyme is easy to deacetylate. Chemical chitosan dissolved in acetic acid 0.1 M until 1% concentration pH 4. Addition of Na-asetat 0.25 M to increase pH becomes 6 to get soluble formation. Rahayu et al. (2004) express that Na-asetat doesn't pursue activity CDA.

Table 2 Deacetylation degree of enzymatic chitosan by ammonium sulfate precipitation

Temperature (^{o}C)	Time (hour)	Degree of Deacetylation (%)			
		Chemically	Enzimatically (U/mg sol kit 1%)		
			0.008	0.04	0.1
80	0.25	65.65	74.85	75.85	78.75
	1	70.7	86.66	87.81	91.6
	2	99.3	99.33	99.36	99.82
	3	99.41	99.5	99.47	99.09

Chitosan Viscosity

Measurement of viscosity by viscometer Ubbelohde which is including capillary viscometer type. For determination of polymer solution viscometer, capillary viscometer that is most precise is viscometer Ubbelohde(Harrington, 1984).

Table 3 Effect of enzymatic deacetylation chitosan viscosity

Temperature (°C)	Time (hour)	Specific viscosity		
		0.008 U/mg	0.04 U/mg	0.1 U/mg
80	0.25	0.215	0.193	0.210
	1	0.269	0.253	0.213
	2	0.267	0.226	0.238
	3	0.256	0.240	0.220

Specific viscosity at 80 oC is 1.3% (for 0.008 and 0.04 U/mg) then more increased by 0.1 U/mg equal to 0.7%. Increasing of viscosity because of the height of acetyl content in chitosan so that with increase of temperature more acetyl is dissolved, so that degree of deacetylation and viscosity increases too (becomes gel). Time of deacetylation relates to depolymerization process where longer deacetylation chitosan hence depolymerization higher too so that viscosity declines (Bastaman 1989).

Formulation of Green Chitosan-Tea Drink Instant

The result of quality product analysis in Table 4 has suitable to standard SNI 01-3722-1995 (orange taste drink powder). SNI orange taste drink selected as comparator because until now hasn't standard for chitosan. SNI 01-3722-1995 assumed to be nearest of instant drink product of chitosan-green tea. With the same analytical method, functional drink of tomato-cinnamon chosen beloved has antioxidant activity 5,44 mM Trolox ® (Radianti, 2005). Thereby, instant drink of chitosan-green tea showed high antioxidant activity (8,41 mM Trolox ®) if compared to instant drink of tomat-kayu manis one, tomato is rice of licopene have a good antioxidant activity and cinnamon that is also rich phenol.

Characteristics of chitosan-green tea powder such as white-brown colour of gel (1,5 cm x 1,5 cm) which has smooth surface and shiny and soft. Its weight is ± 23,7 grams per pack/once consumption (sorbitol 22,5 grams, green tea extract 0,375 grams, chitosan 0,8 grams).

Conclusions

The conclusion of this research that fisically, chitosan-tea drink instant performance were white-brown flour mixed with dry chitosan gel (1,5 cm x 1,5 cm), soft surface, brown clear color, and smooth. Formulation of chitosan-tea drink was weight 23,7 gram perpack/once consumption (sorbitol 22,5 gram, green tea extract 0,375 gram, chitosan 0,8 gram). Chemically, chitosan-tea drink instant have water content 0,22% w/w, ash content 0,11% w/w, protein content 0,03% w/w, lipid content 0,002% w/w, calory 58,5 kkal which suitable with SNI 01-3722-1995 standard.

Tables 4 Result of achitosan-green tea drink instant product analysis (150 mL water per serving)

Parameter	Unit (b.b.)	Chitosan-green tea Drink instant		Comparation[b]
		Dry[a]	After boiling	
Water content	% b/b	0,22	85,30	Max.0,5
Ash content	% b/b	0,11	0,04	Max. 0.1
Protein content	% b/b	0,03	0,94	-
Fat content	% b/b	0,002	0,08	-
pH	-	-	5	-
TAT	%	-	1,73	-
Tot. Carbohyd.	% b/b	0,37	13,64	-
Tot. Susp. Solid	°Brix	-	1,354	-
Vitamin C	mg/100 g	-	-	Min 300
Antioxydan act.	mM Trolox ®	-	8,41	-
Tot. Glucose (as sucrose)	% b/b	-	-	Max.78
Calory	Kkal	58,5	58,5	Max. 312
Shynthetic sweetener	-	-	-	no saccharine and syclamate

a Chitosan-green tea instant product which has not been poured boiling water into
b SNI 01-3722-1995 (SNI orange taste drink powder which has not been poured boiling water)

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