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International Conference on Sustainable Agriculture and Food Security: *Challenges and Opportunities*

Bandung-Indonesia, 27-28 September 2011

Proceeding

(Oral Papers)

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UNIVERSITAS PADJADJARAN

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Universitas Padjadjaran
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FOREWORD

Agriculture as one of leading economic sectors in some countries, is currently facing many problems. This situation could be overcomed by policy and institutional environment which is condusive to increase agricultural productivity while maintaining a sustainable agriculture development and food security. According to this, it is required to develop strategies, a new paradigm, and holistic approach to support the agricultural growth continuum.

In order to make a significant contribution to the better understanding of sustainable agriculture for meeting food security needs and addressing climate change challenges, an International Conference on Sustainable Agriculture and Food Security was held in Bandung Indonesia on 27-28 September 2011. This conference was organized by collaboration of four faculties in Universitas Padjadjaran: Faculty of Agriculture, Faculty of Animal Husbandry, Faculty of Fishery and Marine Science, and Faculty of Agricultural Industrial Technology. Ministry of Agriculture of Republic Indonesia and internationally well-known experts from USA, Finlandia, Singapore, Germany, Malaysia, Romania, Republic of Serbia, China as well as Indonesia were invited as resource speakers.

More than 250 participants from 15 countries attended the conference. The conference shared experiences and views regarding agricultural production in a changing environment towards sustainable agriculture development to maintain food security, and stimulated cooperative research among participating institutions.

About 180 papers are presented and the committee hopes that these papers will be a lsating record of the contributions to this conference and a useful reference for all practitioners in the fields of agriculture in general. Some of the topics presented include critical issues dealing with sustainable agriculture and food security, agrosocio-economy, agritechnology, plant sciences, animal production, and food technology. The committee would like to thank the many reviewers of the papers for their contribution to these proceedings.

The conference and proceeding would have not been accomplished without the support of many individuals, groups and academic units. We owe our gratitude to those who commit and dedicate their self to this conference.

Benny Joy
Chair of ICSAFS

CONTENTS

INVITED SPEAKERS

Anticancer Activity of Chitosan from Local Chitin Waste of Fishery Products In Vitro

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Abstract

The aim of the experiment was to produce bioactive compound of chitosan enzymatically from local chitin waste to be applied in functional instant drinks. The activity of anticancer in this product was determined using in vitro assay. The chitin waste was obtained from crab shells as by- product of canning crabs meat industry in Cirebon West Java Indonesia. Production of chitosan enzimatically was using chitin deacetylase enzyme produced by Bacillus papandayan isolated from Kamojang Creater, West Java. This experiment resulted the technology of process and production of chitosan which degraded enzimatically. The product was chitosan-tea drink which is ready to be dissolved in water. The physical characteristics of Chitosan-tea drink instant which is mixture between flour and dry chitosan gel (1.5 cm x 1.5 cm) were having soft surface, brown-clear color, and smooth. Formulation of chitosan-tea drink (23.7 g per pack/one serve) consisted of 22.5 g sorbitol, 0.375 g green tea extract, and 0.8 g chitosan). This chitosan-tea drink instant contained 0.22% w/w water, 0.11% w/w ash , 0.03% w/w protein, 0.002% w/w lipid, and has 58.5 kkal calory which is suited SNI 01-3722-1995 national standard. The product was then tested for toxicity by in vitro using AH 109 cancer cells. The test showed that chitosan was cytotoxic to cancer cells AH109 with a value of KI_{50} (tg/mL) that was equal to 189.00 for exposure for 8 hours and the value of 1.20 for 24 hours.

Keywords: chitosan, anticancer, in vitro

Introduction

Chitin is an insoluble polysaccharide consisting of ß(1-4) linked N-acetyl-D-glukosamine (GlcNAc) units that 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. Indonesia chitin waste 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.

Conversion of chitin to chitosan in industry is generally done using termochemical technique, that use 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). Purification with column chromatography reported by Rochima (2004).

Waste treatment of crab 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. 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.

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).

Cancer is one disease that can cause death and the existing treatment methods still have several weaknesses, among others because it has a selective toxicity is low, so these drugs also attack normal body cells resulting in side effects serious enough. Search of new drugs that effectively and safely continue through the synthesis or the use of natural resources. The search for anticancer drugs can be done using various methods of testing as a tool to detect the presence of the anticancer activity of the material under study. The balance of apoptosis and cell proliferation is a key determinant of growth in all normal tissues. Apoptosis is also an important phenomenon in the destruction of tumor cells by a chemotherapeutic, γirradiation and immunotherapy that works by stimulating the onset of apoptosis in target cells while the previously known that a direct cytotoxic effect of chemotherapy on tumor cells (Kaufmann and Earnshaw, 2000; Herr and Debatin, 2001; Hu and Kavanagh, 2003).

The research objective was to produce chitosan from chitin waste enzymatically small crab is formulated in the instant drink product, characterize the physio-chemical properties, and then tested its activity against cancer cells.

Materials and Methods

This work was conducted from January 2010 to October 2010 in Laboratory of Technology 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.

Chitin preparation

Bacillus papandayan 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).

Chitin deacetylase production

Culture of *Bacillus papandayan* has been fermented in Sakai media at pH 8.0 and 55 ^{o}C for 2 day. After completed, enzyme is harvested by sentrifugation 8000 rpm for 15 min. Supernatant dissociated and tested activity of CDA according to Tokuyasu *et al*., 1996.

Chitin deacetylase assay

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 ^{o}C. Inactivation enzyme in 100 ^{o}C 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 (Tokuyasu *et al*., 1996).

Chemicall technique and deacetylation enzymatic of chitosan

Crabshell chitin waste was 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 ^{o}C 1 h, decantation, then cleaned again until pH 7 and dried. Deproteination by added of NaOH 3,5% ratio 1:10, then heated 90 ^{o}C 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 ^{o}C 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 ^{o}C for 24 hours (Rochima, 2005)

Formulation of Chitosan-Tea instant drinks

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 ^{o}C, 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).

Preparation of cultured cancer cells

The prepartion of cultured cancer cells was done according to Ananta (2000). Cell suspension: AH109 cancer cells in a frozen state stored in a tank containing liquid nitrogen after being expelled, experience the process of thawing, in advance, ie ampoules containing the cells were incubated at 37 ° C pads or held by hand until the content of it melts. Centrifuged at 228 XG for 10 minutes, the supernatant discarded and the pellet plus basal medium, then centrifuged again at 228 XG for 5 minutes, so preservative material of cells and cells that have died can be removed from the cell culture. Pellet cell growth media was then added and homogenized, then the cell suspension was transferred into a flask with 5 ml of growth medium, then incubated in an incubator with 5% CO_2 at 37 ° C. Maintenance of cell turnover or laundering is done by the media every 3 days or if the suspension has changed color from red to yellow to indicate there has been a decrease in pH due to metabolic activity of cells, used for culturing cells that are in the logarithmic phase of growth curve .

Monolayer: Maintenance of a layer of cells (AH109 cells) with cell suspension, washing or replacement only in the media in the flasks added by enzyme solution 0.02% trypsin in 0.5% EDTA-PBS, for the removal of the cells attached to the flask wall. Media that will be replaced at first discarded all so that only the remaining cells attached to the wall flask, then added to 500 mL of trypsin solution (for 5 ml culture volume) and incubated in the incubator for 8 (eight) minutes. Cells that are attached to will be detached, then add just enough PBS before the cell suspension was transferred into centrifuge tubes and performed the cell washing procedure as has been done on cell suspensions.

Cytotoxicity activity assay

Chitosan at various concentrations to test the activity of cytotoxicity against AH109 cancer cells by MTT assay method. The principle of measurement is the ability of living cells to change compounds the pale yellow MTT into blue formazan compound. Cancer cells (2 x 10^4) grown in 96 well plates, as many as 100 mL per well. After 24 hours, against cancer cells were given various concentrations of test compound and incubated again for 24 hours. 10 mL MTT was added into the test plate and to control, and then incubated in an environment with 5% CO_2 at 37 º C for three hours. In each well, which is formed formazan was dissolved with 100 mL 1 N hydrochloric acid. Absorbance of the dissolved formazan was measured at 450 nm (Yang *et al*., 2005).

Results and Discussion

The condition of industrial crab waste

The main source of chitin for this research came from a small crab waste is a byproduct of small crab meat canning industry as shown in Figure 1 below. Waste is processed by a small crab is an industry miniplan households assisted small crab meat canning industry is located in the area Bondet, Cirebon regency. Miniplan scattered throughout the Cirebon area totaling about 20 pieces, and the total production of small crab shell waste waste about 10 tons perday.

Small crab waste treatment process begins with washing, boiling, peeling and sorting. Small crab body parts were separated into the main part of the meat, and the waste includes

shells, claws large and small claws. Small crab meat from miniplan will immediately be packed to be sent to a small crab meat canning industry Philips Pemalang PT Central Java to be exported to various countries, while the waste is dried and packaged for direct sale or processed into flour chitin. Waste of small crab claw shells and dried sold in local market at a price of Rp 1800.00 per kilo, while the waste of small pincers USD 1500.00

2. Crabs

1.Crabs miniplan Cirebon

3.Washing

4.Boiling

5.Sorting

5a.Crab shell

5b.Big tweezers

5c. Small tweezers

6. Packing

7.Chitin flour

Chitin Deacetylase production

Morphology and characterization of *Bacillus papandayan* producer CDA, according to Rahayu *et al*. 2004 as follows: Gram-positive bacteria bar shape, having spore, and motile. Optimum pH=7 at 55 ^{o}C. Free filtrate cell harvested at phase stationer on 28-32 incubation periods. Optimum CDA activity at pH=8, 55 ^{o}C, 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) who applied 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. It also applies a few of raw material, and low cost.

Formulation of Green Chitosan-tea drink instant

The result of quality product analysis in Tables 1 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).

MTT assay for in vitro

In vitro testing that can either guess the response of tumors to the drug and the results of these estimates will be invaluable. Test whether a chemical component has anti-tumor activity can be done through two ways, namely in vivo and in vitro, because the test in vivo is very costly and time it was developed in vitro assays using cultured cancer cell lines such as cultured strains KR-4 (lymphoblastoid B humans). Testing the activity of proliferation of cancer cells and normal cells using the Alamar blue method or methods of MTT (3 - [4,5-dimethylthiazol-2-yl] -2.5 diphenyl-tetrazolium bromide) in 96 wells flat plate.

This observation is based on MTT reduction by mitochondrial succinate dehydrogenase of living cells provide formazam blue color that can be measured with a spectrophotometer.

Based on the amount of chitosan is given, ie at concentrations of 0, 10.20, 50, 100 and 200, and then exposed for 24 hours with MTT method, the obtained data as follows:

Table 1. 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	^{o}Brix	-	1.354	-
Vitamin C	mg/100g	-	-	Min 300
Antioxydan act.	mM Trolox® % b/b	-	8.41	-
Tot. Glucose (as sucrose)	Kcal	-	-	Max.78
Calory	-	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)

Table 2. Cytotoxic activity of chitosan after 24 hours of contact with cancer cells AH109

Observation	Absorbance of control cell	Absorbance of control medium	Formazan absorbance of AH109 cells after contact with chitosan for 24 hours at certain concentration (µg/mL)				
	(+)	(-)	12.5	25	50	100	200
1	0.628	0.124	0.536	0.531	0.518	0.149	0.134
2	0.621	0.117	0.529	0.550	0.545	0.120	0.137
3	0.641	0.114	0.508	0.510	0.573	0.133	0.140
Means	0.630	0.118	0.524	0.530	0.545	0.134	0.137
IC_{50} (%)			16.772	15.820	13.439	78.730	78.254

Cell culture is a method of studying the behavior of animal cells free of systemic diversity that usually appears in animals during normal homeostasis and under the pressure of the experiment. Cells used may be a flow cell, namely cell population derived from a particular network resource that has cultured further, until it reaches the sub culture. There are two types of cultured cancer cell line that is attached to form a layer of culture (monolayer) on a solid substrate, or a suspension in culture media. Both of these cell types have different properties, where the cell suspension does not require support or supporting material to stick, otherwise the cell layer requires support. The suspension usually from hemopoetik, blood cells or cells from malignant tumors, whereas monolayer cells normally to cells derived from tissue (Freshney, 1994).

The observations in this study is the proliferation of cells. Cancer cells are not normal cells, therefore it does not follow the normal rules of normal cell division. Normal cell cycle is controlled by a group of cyclin proteins takes place through a phase of mitosis (M), gap-1 (G1), DNA synthesis (S phase), gap-2 (G2), mitosis (M) and so on. Daughter cells of mitosis results regularly into the cycle in the G1 phase, some daughter cells enter the resting phase (G0). Cells at G0 phase can be actively re-entered the G1 phase of the cell cycle (Slinerland and Tannock, 1998). The speed of proliferation of tumor cells is different. Proliferating cells are not often encountered because of cell death at high speed.

Cell response to a compound depends on the type of cell, the weight of pressure (compound concentration) and duration of contact with the compound. To see the effect of length of exposure to levels of cytotoxicity, chitosan exposure performed at different times, ie 8 and 24 hours of exposure and the value of KI50 obtained results that exposure for 8 hours obtained KI50 (µg / mL) at 189.00, while if exposed for 24 hours to reach 1.20. This suggests that the longer the contact with the chitosan compound trials of cancer cells, the greater the effect sitotoksiknya, as indicated by the declining value of KI50 in a longer contact time.

Conclusion

Product yielded in the form of instant tea-chitosan drink readily dissolved in water to be consumed. 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 kcal which suitable with SNI 01-3722-1995 standard. Chitosan are cytotoxic to cancer cells AH109 with the KI50 (µg / mL) of 189.00 for exposure for 8 hours and the value of 1.20 for 24 hours.

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