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PROCEEDINGS

3RD INTERNATIONAL CONFERENCE ON BIOSCIENCES AND BIOTECHNOLOGY

MAINTAINING WORLD PROSPERITY THROUGH BIOSCIENCES, BIOTECHNOLOGY AND REVEGETATION

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BALI, SEPTEMBER 21ST-22ND, 2011

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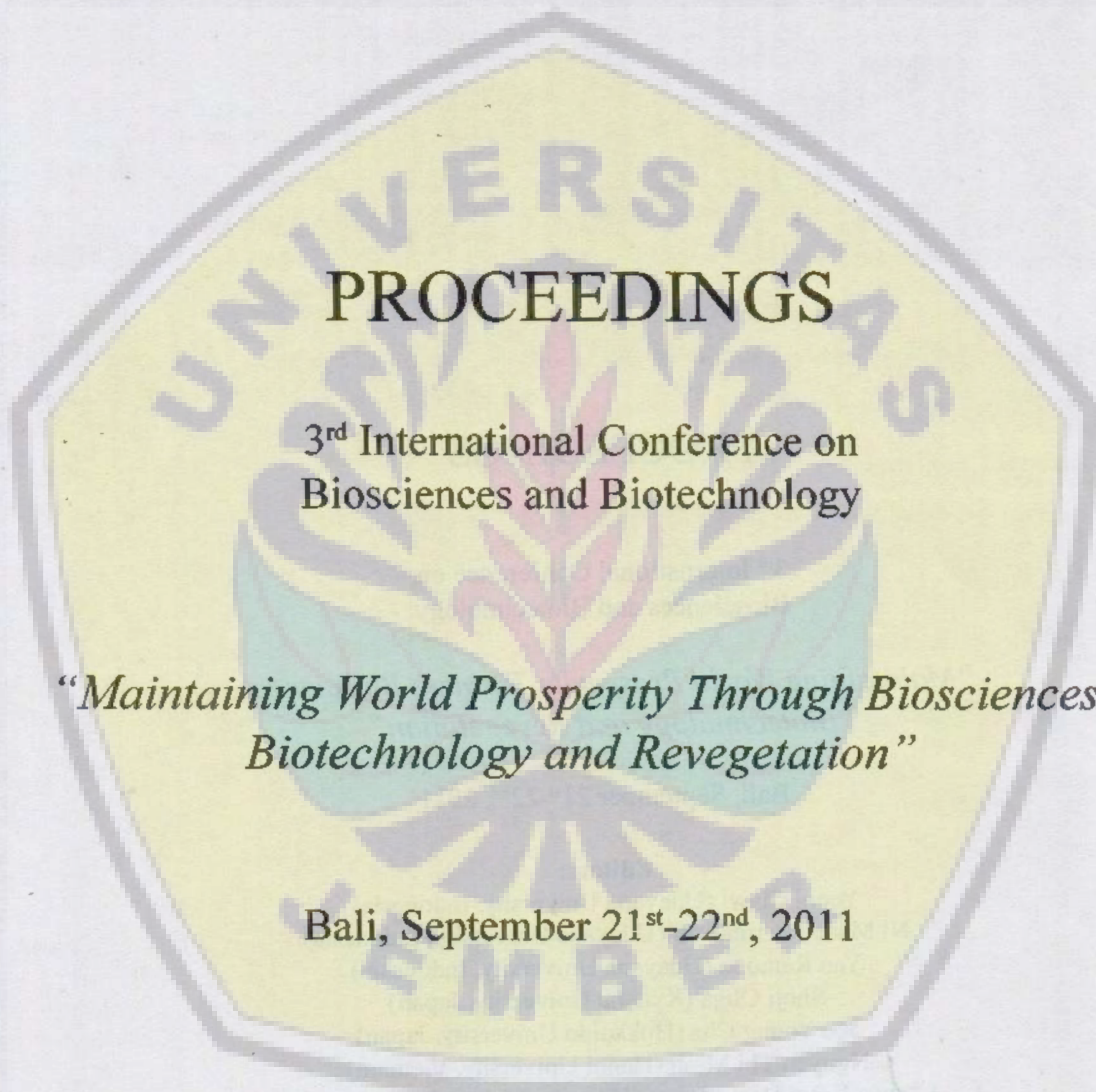

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3rd International Conference on
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"Maintaining World Prosperity Through Biosciences, Biotechnology and Revegetation"

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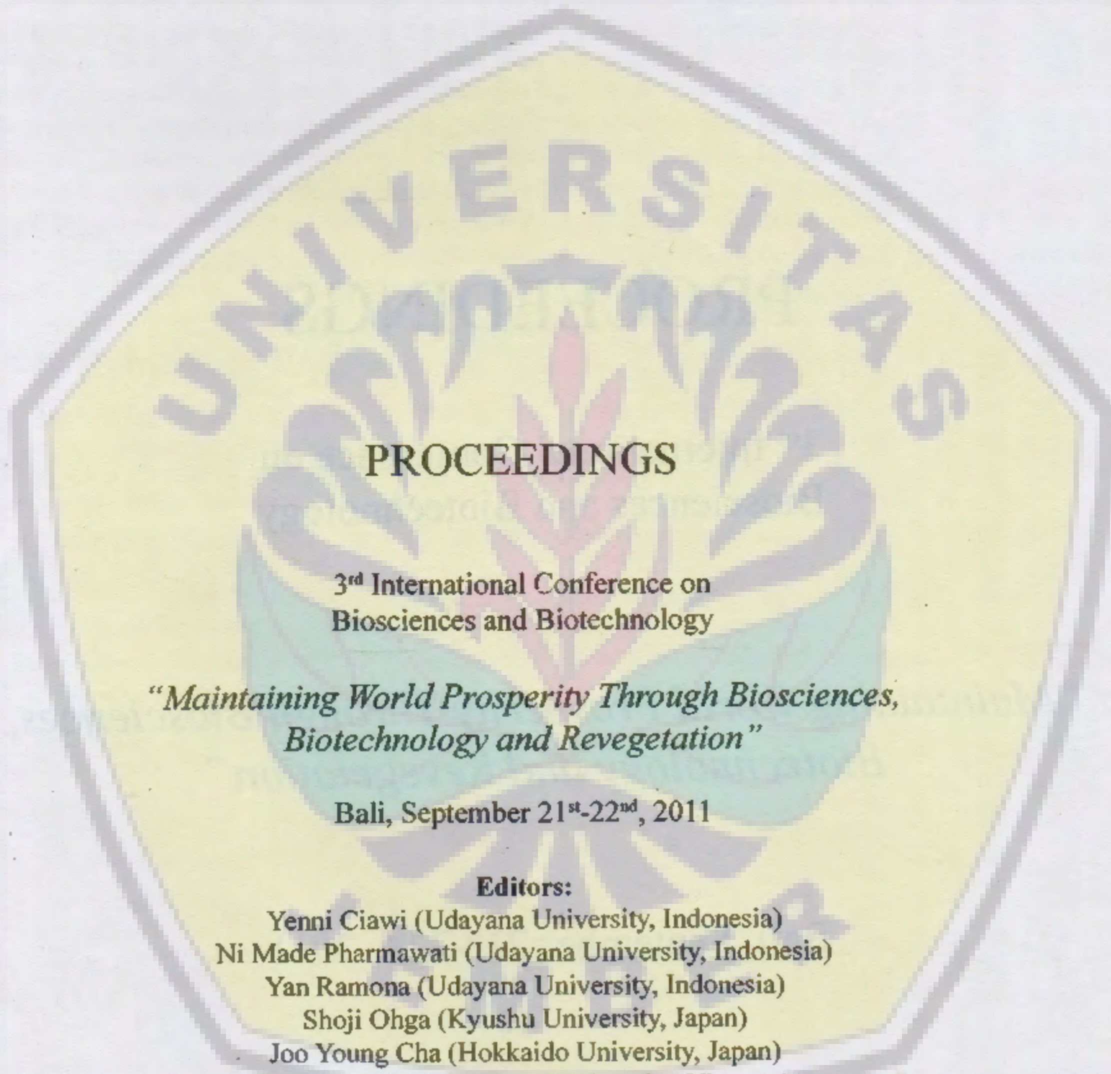

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FOREWORDS-HEAD OF ORGANIZING COMMITTEE

I would like to sincerely thank all of the authors who contribute their papers in this proceedings. I would therefore give my high appreciation on all of those effort and dedication.

The conference was held in relation to the 49th Udayana University Anniversary and in collaboration with Yamaguchi University, Japan. This conference was aimed to gather scientists, academics, engineers and industries in biological related areas to discuss and share their expertise and ideas in the field of Biosciences and Biotechnology. The conference theme **"Maintaining World Prosperity through Biosciences, Biotechnology and Revegetation"** has appealed participants presenting their studies on four major fields of **Health, Agriculture, Agricultural Technology and Food Sciences, and Environment and Biodiversity**. This 3rd ICBB also focused on revegetation as one way to prevent global warming and conserve biodiversity. The conference was financially supported by Rector of Udayana University and several sponsors.

I hope this International Conference has created an international networking and collaboration and open up new ideas in maintaining world prosperity in all aspects in Biotechnology and Biosciences.

I will use this opportunity to invite you again to join us in The 4th International Conference on Biosciencesand Biotechnology which will be held on 21st - 22nd September 2012 in conjunction with the golden anniversary of Udayana University.

Last but not least, I will highly appreciate all of the members of the Organizing Committee for the good teamwork to make the 3rd International Conference on Biosciences and Biotechnology (ICBB-Bali 2011) possible and the team of editors for the hard work compiling and editing 123 papers presented in this book.

See you again in Bali at ICBB 2012

Dr. dr. I Dewa Made Sukrama, M.Si.,SpMK(K)

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AN ENDO-B-D-1,4-GALACTANASE SECRETED BY *ASPERGILLUS NIGER* DURING SOLID STATE FERMENTATION ON SOYBEAN PULP

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ABSTRACT

Production of protein soybean curd tofu is one of the important food industries in Indonesia. However, huge materials as soybean pulp (SP) wastes were produced without any economic value. We examined, this material consist of around 60% total sugars, 30% of crude proteins and 10% lipids. Through GC analysis, SP mainly composted by galactose (40.3%), arabinose (21.1%), xylose (11.7%) and mannose (10.9%). Solid state fermentation of soybean pulp by introducing *Aspergillus niger* revealed that this fungus could grew well, indicated by the bulky black spores emerged after four days solid state fermentation. An extracellular galactanase was harvested by water extraction containing 1% NaCl and 0.1% toluene (v/v). For the purification, the crude enzyme was precipitated using 70% saturated and further chromatographed on a DEAE Butyl Toyopearl 650M, Q-Sepharose and Mono-Q column chromatography. This purification steps, resulted in 3.1% yield and 3,330 fold purification of enzyme. Estimated by SDS-PAGE, galactanase has molecular weight 44 KDa approximately. The enzyme exhibited maximum activity at pH 3.6 and 55°C, and retained nearly 100% activity in a pH range of 3-6 and below 55°C after 30 minutes exposure to respective temperatures. By TLC analysis, it was detected to be capable of releasing galactose and galactosyl oligomers (dimer, trimer, tetramer, pentamer, etc) from soybean pulp alkali extract substrate, in similar manner as from citrus pectin galactan which is known to have backbone chain of β-D-(1,4) linked. The results, confirmed that this galactanase is an Endo-β-D-1,4-Galactanase (E-βGAL) which specifically hydrolyzed internal β-D-(1,4)-galactopyranose linkage at random.

Keywords: galactanase, *Aspergillus niger*, soybean pulp

INTRODUCTION

Cellulose which occurs as microfibrils embedded in pectic and hemicellulolytic materials in cell wall are most important component of plant biomass, while the hemicellulose (xylans, arabinans, galactans, glucans and mannans) rank next to cellulose as the second most abundant group of the renewable polysaccharides in agriculture biomass (3, 4,8,10). They also found in food industry where generally some of the hemicelluloses cannot be processed, and a significant amount remains as organic wastes. For example, the protein soybean curd *tofu* which was known as one of the important traditional food industries in Indonesia also discarded plenty of secondary organic products as soybean pulp (SP) wastes. The usage of this waste is very limited with no economical value.

Microbial utilization of SP may possible to increase the value of this waste. In this research we evaluated the solid state fermentation of SP by introducing *Aspergillus niger* and its possibility to produce an endo-β-D-(1,4)-galactanase (E-βGAL). Isolation and characterization of this enzyme was reported.

MATERIALS AND METHOD

Materials

Fresh soybean pulps were collected from local industry at Jember. The chromatography media DEAE Toyopearl 650 M and Butyl Toyopearl were supplied by TOSOH Corp. (Tokyo, Japan). Q-Sepharose High Performance and Mono-Q HR 5/5 were produced of Pharmacia, (Uppsala, Sweden). The molecular weight marker was obtained from Daiichi Pure Chemicals Co., Ltd. Citrus pectin polygalacturonic was obtained from Sigma (St. Louis, Mo., USA).

Cultivation and optimization of crude enzyme production

Aspergillus niger was used for producing crude enzyme by inoculating to 500 g of sterilized SP in a 5 liter Erlenmeyer flask and incubating at 30°C. After 4-5 days, the culture was stopped by adding 500 ml water containing 1% NaCl and 0.1% toluene (v/v), followed by shaking at room temperature for 9 hours. The suspension was centrifuged to recover the supernatant as a crude enzyme. Then the crude enzyme was concentrated to about one-tenth of the initial volume by ammonium sulfate precipitation at 70% saturation.

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The precipitate was dissolved and dialyzed against water for 3 days. This solution was stored at 4°C till used for SP hydrolysis. For optimization of cultivation period, 10 g of SP in 100 ml Erlenmeyer flasks were inoculated with *A. niger* and the cultures were stopped by adding 10 ml containing 1% NaCl and 0.1% toluene (v/v) daily. Then, the crude enzyme was obtained with the same procedure above.

Degree of hydrolysis and total sugar content analysis

The degree of hydrolysis was examined by incubating the reaction mixture of concentrated crude enzyme and substrate at 37°C. The hydrolyzate was obtained by centrifugation and the release of reducing sugars measured by the method of Nelson (7) as modified by Somogyi (9) using glucose as a standard sugar for calibration. The degree of hydrolysis was calculated as follows:

$$\text{Degree of hydrolysis}(\%) = \frac{\text{Total reducing sugar of hydrolyzate } (w/v)}{\text{Total substrate } (w/v)} * 100\%$$

The total sugar content of hydrolyzate was also measured by the phenol-sulfuric acid method (1).

Analysis of SP components

Twenty mg pulverized-dried SP was hydrolyzed with 4 ml of 1 N sulfuric acid at 100°C for 2 hours and total carbohydrate was measured by the phenol-sulfuric acid method. Total lipid was measured by ether refluxing of 9 g freeze-dried SP for 16 hours at 50°C. Lipid content was calculated as percentage of SP. The crude protein content was analyzed by gas chromatograph (GC 8A, Shimadzu, Tokyo, Japan) equipped with combustion chamber (NC 80, Sumigraph, Tokyo, Japan). Fifteen milligrams of freeze-dried samples were ashen at 830°C and the released nitrogen was measured to estimate crude protein content, using acetanilide as for calibration. All measurements were done in duplicates.

Analysis of sugar composition was performed by using gas chromatograph (G-3000, Hitachi, Tokyo, Japan) as alditol acetates. A sample of 20 mg of SP was fully hydrolyzed with 2 ml of 2 N HCl for 6 hours at 100°C. The hydrolyzate was filtered, evaporated to dryness, mixed with 1 mg of 2-deoxy-D-glucose as an internal standard, and reduced with 2 ml of 0.2 M $NaBH_4$ at room temperature overnight. Five to six drops slurry of dowex resin H type 100-200 mesh (Bio-Rad Laboratories, CA) were then added to the mixture and incubated at room temperature for 1 h, followed by filtration. The filtrate was evaporated to dryness and any residual boric acid removed by repeated evaporation with methanol. The sugar alcohols obtained were acetylated in 2 ml of acetic anhydride:pyridine (1:1) at 100°C for 10 min. The mixture was then diluted with chloroform:water (1:4), shaken well and the upper layer removed by centrifugation at 2000 rpm for 10 minutes. Remaining pyridine was removed from the chloroform extract by washing with water, followed by centrifugation. The resulting alditol acetates were dried and dissolved using chloroform. Gas chromatograph (GC) analysis was performed on a stainless steel column, 2 mm I.D. x 1.83 m, packed with 3% (w/w) ECNSS-M on Gas Chrom Q 100-120 Mesh (GL Sciences, Tokyo, Japan). Nitrogen gas flowing at 30 ml min^{-1} was used as carrier gas with the initial column temperature of 190°C for 5 minutes and then increased to 210°C at a rate of 1°C/min. The sugar component of SPAE, residual SP, soybean arabinogalactan, coffee bean arabinogalactan and citrus pectin galactan were analyzed also with the same procedure above.

Analysis of enzymatic hydrolysis product

The enzymatic hydrolysis product was analyzed by TLC on a silica gel plate (silica gel 60 F254, Merck, Darmstadt, Germany). Butanol:ethanol:chloroform:25% ammonia (4:5:2:8) was employed as a solvent system. Sulfuric acid with 1% vanillin was used for detection.

Preparation of substrates

Soybean Pulp Alkali Extract (SPAE) was prepared as follows: 500 g of SP were suspended in 10% NaOH and shaken overnight. The mixture was filtered and the filtrate adjusted to pH 5 with acetic acid. Filtrate was made to 50% in ethanol and the precipitate was obtained by centrifugation at 12000 rpm, for 20 minutes. The pellet was dried under reduced pressure at 40°C. The quantity of the dry powder was about 3% of the starting material.

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The citrus pectin galactan (1,4)βgalactan was prepared as described by Labavitch et al. (5). Forty grams of citrus pectin were dissolved in 1.5 liters of 5 N NaOH containing 100 mM NaBH4. The solution, in 5 liter round bottom flask, was stirred while being heated at 90oC. The solution was refluxed for 20 hr, cooled, and adjusted to pH 5.0 by the addition of glacial acetic acid. Solid residues were removed by centrifugation. The resulting supernatant was dialyzed for 3 days against running tap water and for 1 day against distilled water. The dialyzed preparation was made 10 mM in potassium phosphate, pH 7.0. The buffered preparation was passed through a column (5 x 25 cm) DEAE-cellulose that had been equilibrated in 10 mM potassium phosphate, pH 7.0. The solution which passed directly through the column and 10 mM phosphate wash were combined, concentrated at 40oC under reduced pressure. The concentrated preparation was dialyzed against deionized water at 4oC and then made 50% in ethanol by the addition of absolute ethanol. The precipitate that formed was collected by centrifugation, dissolved in deionized water, and lyophilized. The final recovery of citrus pectin galactan was about 2% of the total citrus pectin polygalacturonic. The arabinogalactan of coffee bean was prepared by the method of Emi et al. (2). Coffee beans were pulverized and successively extracted with 2 portions of a mixture of ethanol and benzene (1:2), 10 portions of water (25°C) and 20 portions of 1.0% sodium hypochlorite. The residue obtained was boiled with 20 portions of water for 8 hr. After concentration to an adequate volume under reduced pressure, the supernatant was mixed with 3 volumes of cold ethanol. The precipitate obtained was dried by washing it successively with ethanol and ether. Final yield was 2% of the starting material.

Enzyme assays

Enzyme activities were determined by incubating the reaction mixture at 37°C and measuring the released of reducing sugars by the method of Nelson (7) as modified by Somogyi (9). One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugar at rate of 1 μmol per minute. For pH range 1-6 and 6-8, acetate and phosphate buffer were used respectively. All assays were performed in 1 ml total volume.

Purification E-βGAL

All purification steps were carried out at room temperature, using 20 mM acetate buffer, pH 5. Details of the purification are mentioned under 'Result and Discussion'.

Enzyme Properties

The effect of pH and temperature on activity and stability of these enzymes were determined at the pH range of 1 to 8 and at a temperature range of 20 to 70°C. The molecular weight of enzymes was estimated by sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weights of the denatured enzymes were estimated by comparison of their migration rates with those of protein standards. Proteins standard (in Dalton) used, were phosphorylase (97,400), albumin (66,300), α-L-Arabinofuranosidase (64,400), aldolase (42,400), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and lysozyme (14.400).

RESULTS AND DISCUSSION

The successful of growth of *A. niger* proved SP could be used as carbon and nitrogen sources for this organism during solid state fermentation. We investigated that fresh SP contained around 75-85% water content, while dried SP consist of around 60% total sugar, 30% of crude protein and 10% lipid. The gas chromatograph analysis of sugar components showed that fresh SP mainly consists of galactose (40.3%), arabinose (21.1%), and also the minor components were xylose, mannose, rhamnose, fucose, and glucose (Table 1). It is also reported by Morita (6) that the major component soybean arabinogalactan contains galactose and arabinose in the approximate proportion of 2:1. Furthermore, the SPAE and residual SP also contain galactose and arabinose in a ratio nearly 2:1.

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Table 1. Gas Chromatograph Analysis Sugar Components of SP and Various Substrates

Substrates	Sugar compositions (%)						
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
Fresh SP	3.5	2.7	21	12	11	40	9.8
SP alkali extract (SPAE)	2.7	2.3	13	20	19	24	20
Residual SP	6.3	2.5	27	10	4	46	4.5
Soybean arabinogalactan	6.9	1	4.1	17	0.2	68	3.2
Coffee bean arabinogalactan	5.7	-	25	-	10	59	-
Citrus pectin galactan	0.3	-	2.7	-	-	91	6

During fermentation, the appearance of liquefied forms as hydrolyzed product during cultivation started between fourth or fifth day. As the cultivation time increased, the liquefied forms also increased. It is believed that these fungi secrete some potential liquefying enzymes on SP solid media. The optimum cultivation period was 4 days, optimized by daily harvesting (Figure 1). The crude enzyme had maximum digestion (50% as monosaccharides) of SP after 35 hours at 37°C.

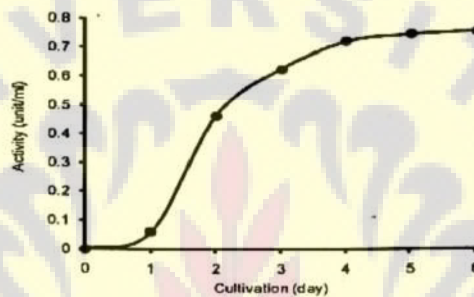

Figure 1. Optimum Cultivation of *Aspergillus niger* on SP Medium. The optimum cultivation period was determined by daily monitoring activity of crude enzyme at 37°C in 1 ml total volume of 0.1 M acetate buffer pH 5 containing 1% SPAE for 10 minutes.

Crude enzyme properties

The effect of pH and temperature on the enzyme activity was measured after 10 minutes incubation at 37°C of each enzyme in 1 ml 1% substrate at various pH and temperatures. Optimal pH and temperature ranges for assay of crude enzyme activity were pH 4 and 60°C, respectively (Figure A and B). The stability pH and temperature of crude enzyme were 2.5 – 7.5 and below 55°C after incubated 30 min to the corresponding pH and temperatures.

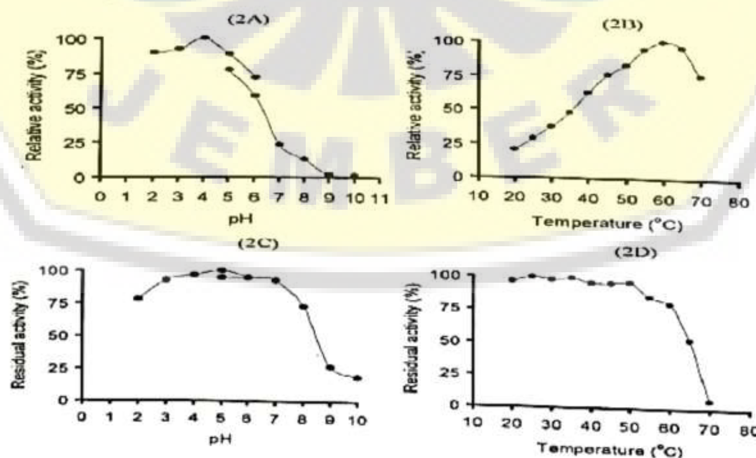

Figure 2. Effect of pH and Temperature on activity and stability of crude enzyme. The crude enzyme was obtained from 4 days cultivation at 30°C and the optimum pH (2A), optimum temperature (2B), pH stability (2C), and temperature stability (2D) of crude enzyme were assayed for 10 min at 37°C in 1 ml total volume of 0.1 M acetate buffer pH 5 containing 1% SPAE.

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Purification

The crude enzyme was concentrated with 70% saturated ammonium sulfate and centrifuged at 12000 rpm for 20 min. The precipitates were dissolved in buffer. Remaining ammonium sulfate was removed by overnight dialysis against the same buffer at 4°C. This was followed by DEAE Toyopearl 650M ion exchange column pre-equilibrated with buffer. The column was eluted by 0–0.5M NaCl linear gradient. The fractions which actively hydrolyzed SPAE were pooled separately, dialyzed against buffer to remove NaCl. This dialyzed active fractions was then reloaded on a DEAE Butyl Toyopearl 650M column, followed by using Q-Sepharose column chromatography. Finally, Mono-Q (anion exchange) was used. All of the column were pre-equilibrated with buffer and eluted with linear gradient 0-0.5 M NaCl. This purification scheme resulted in 3.1% yield and 3,330 fold purification of E-βGAL.

Tabel 2. Purification of E-βGAL

Purification Step	Total ABS-280	Total Activity (unit)	Spec. act. (unit) / ABS280	Yield (%)	Fold
Crude enzyme	54,340	1,700	0.03	100	1
Ammonium sulfate Precipitation	10,560	910	0.09	54	3
DEAE Toyopearl 650M	73	185	2.53	10.8	84
DEAE Butyl Toyopearl 650M	15	85	5.7	5	190
Q-Sepharose-HP	1	71.4	74.3	4.2	2,480
Mono-Q	0.52	51.9	99.8	3.1	3,330

Enzymes properties

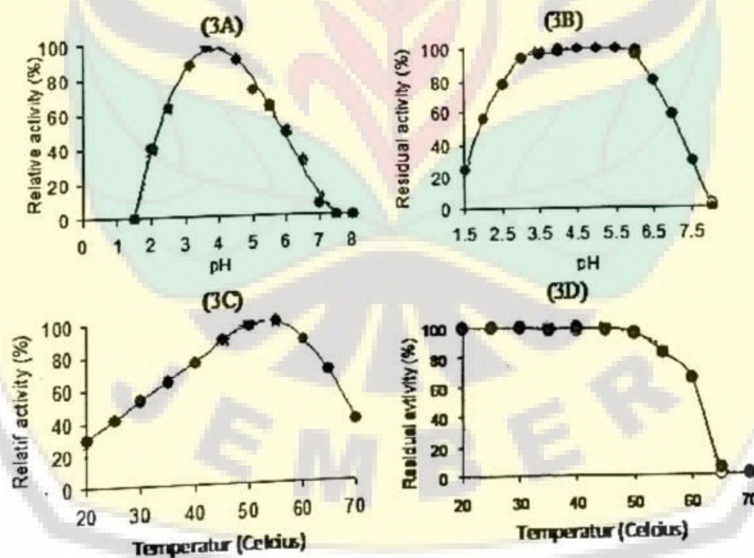

Figure 4. Effect of pH and Temperature on Activity and Stability. The optimum pH (3A), pH stability (3B), optimum temperature (3C) and temperature stability (3D) of E-βGAL (•) were assayed in 1 ml total volume containing 1% SPAE at 37°C.

The effect of pH on the enzyme activity was measured after 10 minutes incubation at 37°C of each enzyme in 1 ml 1% substrate at various pH values. E-βGAL exhibited maximum 3.6 (Figure 3A) and retained nearly 100% activity in a pH range of 3-6 respectively after 30 minutes exposure to corresponding pH values (Figure 3B). E-βGAL showed optimum activity at 55°C respectively (Figure 3C), and is nearly 100% stable below 55°C after 30 minutes exposure to respective temperatures (Figure 3D). As shown in Figure 5 the molecular weight of E-βGAL was 44 KDa approximately.

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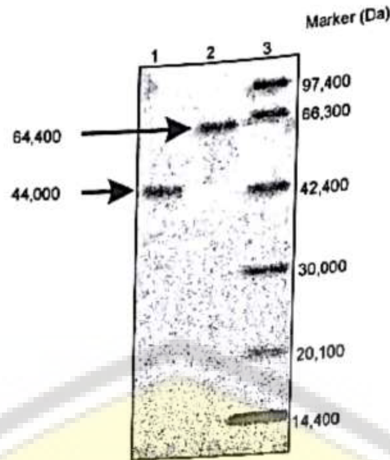

Figure 5. SDS-PAGE of Purified Enzymes. Lane 1 is purified E-βGAL, lane 2 and 3 are markers

Substrate specificity and analysis of hydrolysis product.

The substrate specificity had been identified toward various substrates for purified enzymes E-βGAL. SPAE was hydrolyzed by and E-βGAL to 7.4% (Table 3). TLC analysis revealed E-βGAL released galactose as well as some oligomers (Figure 5).

Tabel 3. The Degree of Hydrolysis (%) towards Various Substrates

Substrates	Degree of Hydrolysis (%)
Soybean pulp alkali extract (SPAE)	7.4
Soybean-arabinogalactan	20.3
Coffebean-arabinogalactan	2.1
Citrus pectin galactan	37.4

E-βGAL besides active on SPAE also effectively hydrolyzed soybean arabinogalactan or citrus pectin galactan with degree of hydrolysis 20.3 % and 37.4% respectively. However, it hydrolyzed coffee bean arabinogalactan with low efficiencies (Table 3). By TLC analysis,it was detected to be capable of releasing galactose and galactosyl oligomers (dimer, trimer, tetramer, pentamer, etc) from SPAE (Figure 6), in similar manner as from citrus pectin galactan or soybean arabinogalactan (data not shown) which they were known (3, 9) to have similar backbone chain of β-D-(1,4) linked. The results, confirmed that the E-βGAL is an endohydrolase which specifically hydrolysis internal β-D-(1,4)-galactopyranose linkage at random.

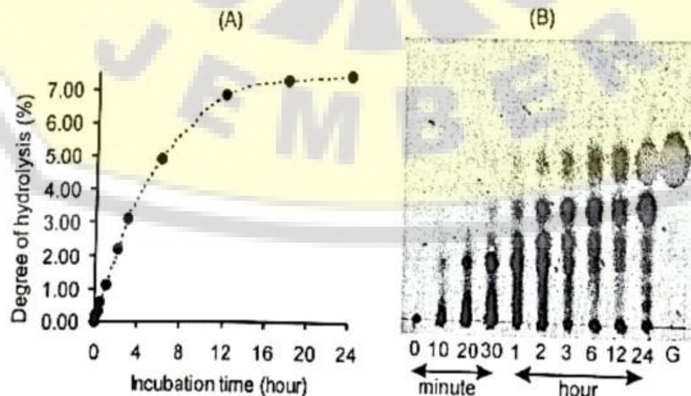

Figure 6. Hydrolysis of SPAE by E-βGAL. 1% SPAE was used as substrate in 50 mM acetate buffer, pH 4. The mixtures were incubated at 37°C for different duration as mentioned. The degree of hydrolysis measured as percentage of substrate hydrolyzed (A) and the products were analyzed by TLC, using galactose (G) as standard (B).

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CONCLUSION

Microbial utilization of SP was investigated. During SP fermentation a liquefying enzymes were produced and identified as E-βGAL with molecular weight 44 KDa approximately. The enzyme exhibited maximum activity at pH 3.6 and 55°C, and retained nearly 100% activity in a pH range of 3-6 and below 55°C after 30 minutes exposure to respective temperatures. By TLC analysis, it was detected to be capable of releasing galactose and galactosyl oligomers (dimer, trimer, tetramer, pentamer, etc) from SPAE substrate. Suggested, this galactanase is an Endo-β-D-1,4-Galactanase which specifically attacked internal β-D-(1,4)-galactopyranose linkage at random.

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