Bioeconomy: Pectinases Purification and Application of Fermented Waste from Thermomyces Lanuginosus

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***Abstract*—Malaysia takes a step forward to promote and support the development of sustainable and green technology by adopting and master the technology holistically. Bioeconomy refers to all economic activity derived from scientific and research activity focused on biotechnology. Pectin degradation enzymes are widely used in the industrial processing of fruits and vegetables in order to decrease the viscosity of juices and thus to facilitate extraction, maceration, liquefaction, filtration and clarification processes. Sugar-cane bagasse (SCB) is agro-waste produced in large quantities in our environment used as the substrate for Exo-polygalacturonase (Exo-PG) from *Thermomyces lanuginosus* at 55℃ under solid-state fermentation (SSF) culture. Exo-PG was purified using Gel-filtration chromatography and exhibited only one sharp peak. Biotechnological applications of fermented waste obtained, mixed with the fungal biomass at 55℃ as biofertilizer at different concentrations (w/w) were cultivated with *Zea mays* for 30 days to indicate the plant growth on sandy soil and succeeded to induce the growth which may give an indication of applying the present biofertilizer particularly in the reclaimed sandy soils. Only 14 amino acids were detected and obviously that Exo-PG was typical proline containing enzyme.**

***Index Terms*—bioeconomy, polygalacturonase, thermomyces lanuginosus, biofertilizers, solid-state fermentation**

I. Introduction

Researches all over the world today are focusing on ways of utilizing either industrial or agricultural wastes as a source of raw materials for the industry. These wastes utilization would not only be economical, but may also result to foreign exchange earnings and environmental pollution control [1]. Pectinases are a group of enzymes involved in degradation of pectin, which includes various enzymes classified into various classes and subclasses depending on the substrate specificity and mode of action, for example, methyl deesterases, hydrolases, and lyases. According to the cleavage site, pectinases are divided into three groups: (i) hydrolases consisting of polygalacturonase, PG (EC 3.2.1.15); (ii) lyase/trans-eliminases comprising pectin lyase, PNL (EC 4.2.2.10), and pectate lyase, PL (EC 4.2.2.2); (iii) pectin esterase, PE (EC 3.1.1.11) [2]. Pectinases are widely distributed in higher plants and microorganisms. They are today one of the upcoming enzymes of the commercial sector. It has been estimated that microbial pectinases account for 25% of the global food enzymes sales. Among industrial applications of pectinases are using these enzymes as an animal feed supplementation [3]. In this study, we reported the purification of Exo-PG enzyme, then evaluating the fermented waste as fertilizer for the purpose of desertification combat of soil and then detection the amino acids containing of these enzymes.

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II. Materials and Methods

A. Growth Medium and Exo-PG Assay

The medium used for fungal induction and growth under SSF conditions contained basically 5g of dried and ground sugar cane bagasse (SCB) supplemented with only 25ml mineral salts of Starch-nitrate yeast-extract (SNY) medium. Exo-pectinolytic productivity in cell free filtrate (CFF) was assayed by quantification of reducing sugar using 3, 5-dinitrosalicylic acid (DNS) reagent [4]. The reaction mixture of enzyme assay was described in details according to [5].

B. Purification of Exo-PG Enzyme

The following techniques were performed during the course of pectinase purification enzyme produced by *Thermomyces lanuginosus* at 55℃ incubation temperature due to the growth on the dried SCB at optimal static natural substrate under SSF conditions. At the end of incubation period, the CFF was saturated with ammonium sulphate [6] as mentioned by [7], then centrifugation at 15,000rpm for 15min and the pellet was resuspended in acetate buffer pH 5.0 to determine both the enzyme activity and protein content according to [8].

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The obtained enzyme was dialyzed against sucrose 30% (w/v), this dialyzed enzyme (1ml) subsequently loaded on a gel filtration of Sephdex G-200 (mesh, 200 µ).

C. Factors Affecting the Purified Enzyme Activity

The reaction mixture incubated intervals from 10 to 60min at 55 ℃ of purified enzyme. At the end of incubation times, the enzyme was determined after each particular factor. To study the relation of different pH values to purified Exo-PG enzyme was incubated at different pH values viz. 3.0, 4.0, 5.0, 6.0 and 7.0 using acetate buffer for 20min. Effect of different enzyme concentrations to its activity in range of (50-300 µl) also studied. For thermal stability of Exo-PG activity, the experiment carried out by incubating the purified enzyme for 3 hours at different temperatures in range of 10-80 ℃. At the end of treatment, the replicate tubes were cold and assayed for enzyme activity. The relation of different substrate concentrations (pectin) to purified enzyme (w/v, %): 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 was done [9].

D. Biotechnological Application and Amino Acid Analysis

The fermented waste of bagasse (FB) were dried and used for the growth of *Zea mays* as compost at different concentrations viz: 0.0. 0.5, 1.0, 1.5, 2.0, 2.5 and 3% (w/w) into sandy soil for 30 days. Quantitative determination of chlorophyll (a), (b) and total chlorophyll was done according to Vernon and Seely, (1966). Amino acid analytical data of the purified enzyme was carried out using concentration; LC 3000 standard program H1, Readymade buffers H1 (4-buffer system), Column type H 125 ×4mm, Per-Column type 60 ×4mm.

III. Results

A. Enzyme Production and Preparation of CFF

TABLE I. The Optimum Nutritional and Environmental Parameters Controlling Exo-PG Productivity by T. *Lanuginosus* under SSF Conditions

Parameters	***Exo-PG Enzyme***
Temperature (°C)	55
Bagasse conc. (g)	4
Inoculum size (disc)	3
Incubation period (day)	4
pH-factor	5.4
Flask volume (ml)	2000
Carbon sources	Cont.
Nitrogen sources	Amm. sulphate.
Hormones	GA_3
Vitamins	L-asco.
Mineral salts	23
Mean of O.D	2.420
(U/ml)	4.033±0.004

The fungal strain was allowed to grow on SCB as solid substrate supplemented with MS under optimal static SSF conditions Table I. About 1,350ml of Exo-PG crude enzyme were extracted and collected, centrifuged at 5,000rpm for 15min at 10 ℃, the precipitate was collected and tested for both enzyme activity and protein content as well as corresponding specific activity (sp. act.) was calculated up to (1.404Umg^{-1}) at 55 ℃ incubation temperature.

B. Fractional Precipitation with Ammonium Sulphate

Results represented graphically in Fig. 1 indicated that the most active protein preparation was obtained at ammonium sulphate level of 60%. Data recorded in Table II exhibited that Exo-PG enzyme activity was reached up to (0.775±0.003U/ml) and protein content of (0.87mg/ml) corresponding to sp. act. (0.891Umg^{-1}). Only 59ml were obtained at the end of the process of dialyzation against tap water and dialyzed again against sucrose crystals until a volume of 0.5ml obtained and specific activity was determined as (1.844Umg^{-1}) Table II.

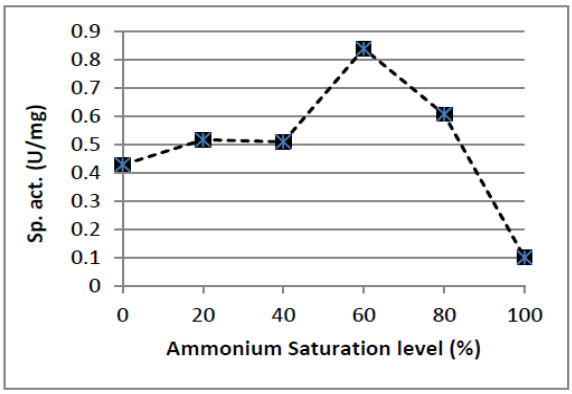

Figure 1. Ammonium sulphate fractionation levels

C. Sephadex G-200 Gel Filtration Column

Data represented in Fig. 2 revealed that fifty fractions of Exo-PG enzyme were collected and the enzyme fractions activities were appeared from fraction 24 to 42 and the fraction number 38 was reached the maximum specific activity up to (5.812Umg^{-1}).

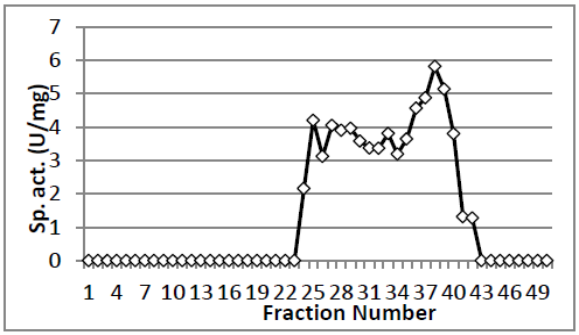

Figure 2. Fractions of sephadex G-200 chromatography

D. Characterization of the Purified Enzyme at 55 ℃ under SSF Conditions

For the purpose of investigating the various properties of the purified Exo-PG enzyme towared various parameters that control the enzyme activity, the relation of different incubation periods towared the enzyme activity, pH-values, enzyme concentration, thermal stability and substrate concentration have been studied.

TABLE II. SUMMARY OF PURIFICATION STEPS OF EXO-PG ENZYME PRODUCED BY *T. LANUGINOSUS* AT 55 ℃

NO	*Purification step*	*Volume (ml)*	*Enzyme activity (U/ml)*	*Protein content (mg/ml)*	*Specific activity (Umg^{-1})*	*Protein Fold*	*Yield (%)*
1.	CFF	1350	0.538±0.023	0.383	1.405	1.00	100
2.	$(NH_4)_2SO_4$ (60%)	100	0.775±0.003	0.87	0.891	0.634	144
3.	Dialysis against sucrose	59	3.112±0.002	1.688	1.844	1.312	578
4.	Sephadex G-200	5	0.558±0.002	0.096	5.813	4.137	104

Results represented in Fig. 3 revealed that the highest Exo-PG enzyme activity was obtained after 30min incubation time of the reaction mixture and reached up to (4.753±0.002U/ml) above this time the activity was gradually decreased when the time increased. Concerning pH-values, as shown in Fig. 4 the best pH value that fulfill the highest Exo-PG enzyme activity was 5.0 and reached up to (4.486±0.004U/ml), below and above this particular pH the enzyme activity was decreased gradually. Data represented in Fig. 5 emphasized that the continuous increasing of Exo-PG enzyme activity due to the increase of enzyme concentration until 300 µl reached up to (5.215±0.002U/ml). While Fig. 6 showed that the maximum Exo-PG enzyme activity reached up to (4.433±0.001U/ml) was attained at 50°C, below and above this particular degrees of temperature the enzyme activity was decreased, although the enzyme exhibited its ability to work at an incubation temperature of 80°C. Results recorded in Fig. 7 showed that 1.5% pectin fulfilled the maximum activity up to (4.713±0.004U/ml), below and above this particular concentration the enzyme activity decreased gradually.

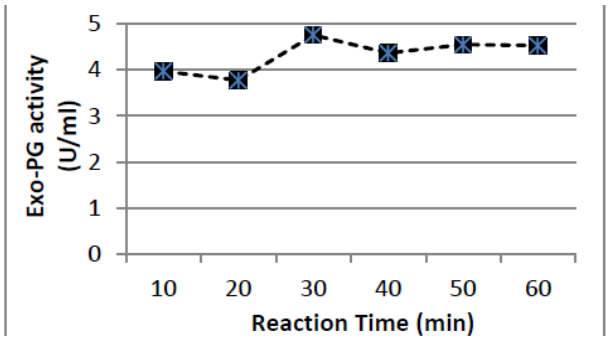

Figure 3. Effect of time progression

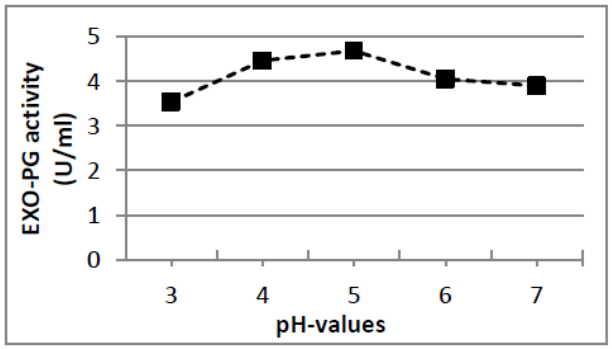

Figure 4. Relation of different pH values

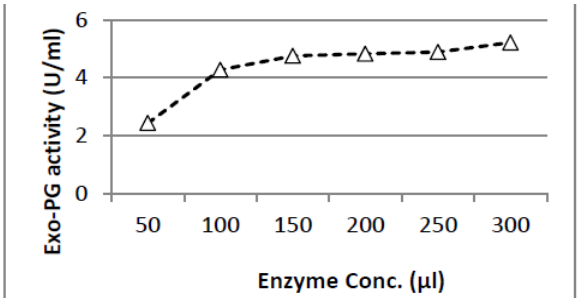

Figure 5. Effect of purified enzyme concentrations

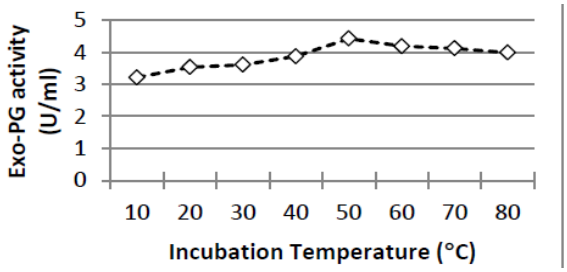

Figure 6. Effect of thermal stability

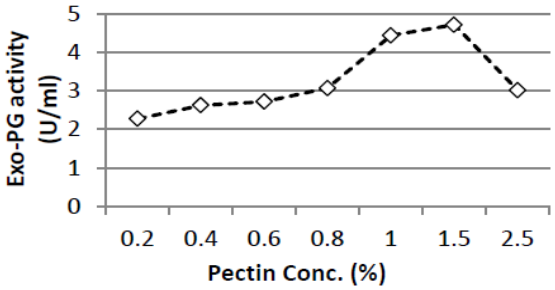

Figure 7. Effect of different substrate concentrations

E. Biotechnological Application of Fermented Bagasse

Data recorded in Table III showed that the fermented bagasse (FB) obtained from the processes of Exo-PG enzyme purification at different concentrations were cultivated with *Zea mays* for 30 days that was able to indicate the growth of *Zea mays* on sandy soil as evidenced by data of root length (RL), shoot length (SL), fresh (FW) and dry weights (DW), determination of chlorophyll (a), (b) as well as total chlorophyll (a+b). It could be concluded that there is a specific concentration of the introduced organic manure (biofertilizer) which is responsible for plant growth and/or related parameters. These particular results are great values from the economic point of view.

TABLE III. BIOTECHNOLOGICAL APPLICATION OF FERMENTED BAGASSE OBTAINED FROM EXO-PG ENZYME PRODUCTION AS ORGANIC MANURE IN CULTIVATION OF ZEA MAYS IN SANDY SOIL

FB (%w/w)	*RL (%)*	*SL (%)*	*FW (%)*	*DW (%)*	*Chl. a (%)*	*Chl. b (%)*	*Chl. (a+b) (%)*
Cont.	100	100	100	100	100	100	100
0.5	149.2	99.2	85.7	104.8	95.4	123.6	98.2
1.0	154.9	99.2	82.4	103.0	60.2	61.0	57.3
1.5	170.5	113.2	87.7	99.3	62.0	61.9	58.8
2.0	213.9	109.9	90.8	75.4	83.8	83.5	79.4
2.5	195.1	115.6	82.1	92.2	94.3	93.2	89.2
3.0	160.6	121.0	83.9	82.6	109.8	109.2	104.1

RL: root length; SL: shoot length; FW: fresh weight; DW: dry weight.

F. Amino Acids Analytical Data of Purified Enzyme

As represented in Fig. 8, it is obviously that 14 amino acids were detected in addition to ammonium sulphate and proline was represented by the highest value up to 53.54% of purified Exo-PG enzyme. Interestingly, it could be notices that the amino acids analytical data of investigated purified enzyme refers to the fact that only one of typical proline containing enzyme i.e. Exo-PG. This means simply that the structure of the hydrolytic enzyme not only depend on the kind of the producing strain but also depends on the substrate used for its production as well as other factors controlling the nature of the fermentation process.

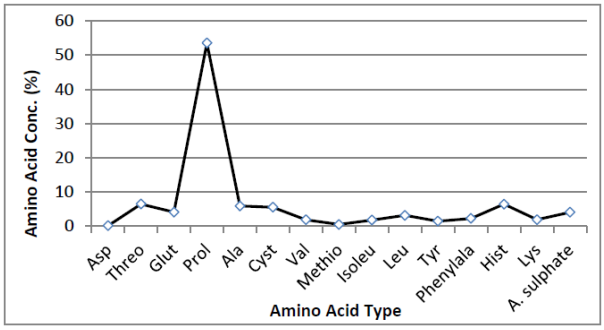

Figure 8. Amino acids analytical data of purified Exo-PG enzyme

IV. DISCUSSION

Bagasse, the fibrous residue after sucrose extraction, consists of 46-49% cellulose, 25-27% hemicellulose and 20-22% lignin [10]. The use of enzymes from thermophilic microbes offers important advantages over those of mesophiles in large-scale biomass conversion processes [11]. Bagasse was evaluated as a sole carbon source for production of thermostable extracellular enzymes by *Thermomonospora curvata*, which establishes itself as the dominant population during the high temperature composting of a variety of lignocellulosic material [12]. According to [13], application of SCB to areas planted with sugar cane increased root growth due to an increase in the amount of mycorrhizal hyphae. Comparing the present recorded, [14] reported an optimum pH of 6.0 for maximum production of endo and exoglucanase by *A. fumigatus* 1M1 (246651). The optimum pH of PG activity produced by *Aspergillus niger* from orange peel was reported to be 5.0 whereas that *A. niger* isolate from tamarind and *A. niger* MTCC strain have also been reported to be 5.4 [15]. Polygalacturonase (PG) production by *Thermoascus aurantiacus* was carried out by means of SSF using orange bagasse, SCB and wheat bran as a carbon source. PG had optimum activity at pH 5.0 [16]. In contrast, optimum pH of PGII activity has also been reported by [17] between 3.8-4.3 and that of PGIV between 3 and 4.6. The differences in pH optima could be due to the differences in the enzyme type and mode of action. The enzyme was found to have a half-life of 5 hours at 42 ˚C and 2 hours at 50 ℃ [18]. According to [19] PG was purified up to 116.77 fold from *Rhizoctonia solani* Kühn (AG2-2) by dialysis, precipitation with 0.7 saturation ammonium sulfate, gel filtration Sephadex G-100 and ion-exchange on diethylaminoethyl cellulose with a yield of 72.397% and sp. act. of 32.5Umg^{-1}. Quantitative estimation of amino acids obtained from *R. solani* showed that it contained 17 amino acids [20]. Worked on pectinase activity in Micorrhizal fungi reported that when assay mixtures consisted of 4ml 1% polygalacturonic acid, a linear increase in enzyme activity was observed by increasing substrate up to 0.8 (w/v) pectin concentrations for the crude of both strain tested. These results were in accordance with the results on *T. ressei* [21]. Of the most fascinating data in this work those belonging to the biotechnologcial application of the feremented bagasse mixed with the fungal biomass. According to [22] he isolated petinase(s) from Marjoram fermentation under SSF and applied the fermented material as a biofertilizer for *Eruca sativa* cultivation. A biotechnology for aerobic conversion of food waste into organic fertilizer under controlled aeration, stirring, pH and temperature at 55-65 ℃ was reported by [23]. The addition of 4% organic fertilizer to the subsoil increased the yield and growth of *Ipomoea aquatica* (Kang Kong) by 1.5 to 2 times. The addition of phosphorus is required to enhance the positive effect of organic fertilizer on plant growth. Similar data were recorded by [24] who found that the amino acid content of the isoenzymes of endo-polygalacturonase was very similar with Glyine containing enzymes.

V. CONCLUSION

The results of the present study clearly indicate the Exo-PG enzyme was successfully produced and purified by *T. lanuginosus* at thermophilic conditions when grown on solid wastes as the sole carbon source and displays good results after purification. The amino acids revealed that the Exo-PG was typical proline containing enzyme Moreover, the fermented wastes obtained used as biofertilizers and induced that the growth of *Zea mays* when cultivated on sandy soil.

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