Production of endoxylanase from novel *Actinomadura geliboluensis* by using agricultural wastes

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Keywords: Actinomadura, endoxylanase, agricultural wastes, fermentation.

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Abstract

Purpose: In this study, endoxylanase production from novel *Actinomadura geliboluensis* has been isolated and identified with submerged fermentation by using lignocellulosic wastes was evaluated. Also, effect of cultural conditions and medium compositions on xylanase production was studied.

Methods: After fermentation, liquid culture sample was centrifuged at 10.000 *g* for 5 min at 4 °C and supernatant was used for determination of enzyme activity and estimate total protein content. Birchwood xylan (1%; w/v) that was solved in 50 mM phosphate buffer (pH 7.0) was used as substrate in reaction mixture. Reaction was carried out at 37 °C for 10 min. Enzyme activity was detected by using DNS method. One unit of enzyme activity was defined as amount of enzyme that required to liberate 1 µmol xylose equivalent per minute at 37 °C. Total protein concentrations of supernatants were determined by the method of Bradford.

Results: Time course of endoxylanase production in different fermentation media supplemented with birchwood xylan (1%; w/v) as primary carbon and energy source was evaluated. Maximum endoxylanase production was obtained in MS-YEM medium after 3 days incubation. Wheat straw, corn stover and castor stalk (*Ricinus communis* stalk) was suitable carbon sources among lignocellulosic wastes for endoxylanase production from *A. geliboluensis*. Best particle size of carbon sources, nitrogen sources, incubation temperature, and initial pH of medium and agitation rate for endoxylanase production was also determined. Under optimum condition 200.71 U/mL, 194.73 U/mL and 148.77 U/mL endoxylanase was produced from *A. geliboluensis* by using wheat straw, corn stover and castor stalk as primary carbon source, respectively.

Conclusion: This is the first report about endoxylanase production from novel *A. geliboluensis*. Also, this report showed that *A. geliboluensis* could be used for production of significant amount (200.71 U/mL) and cheap endoxylanase by using agricultural wastes as primary carbon and energy source.

Keywords: Actinomadura, endoxylanase, agricultural wastes, fermentation.

Introduction

There has been growing interest in biological catalysts defined as enzymes that converts substrates to particular products. Because enzymes have usage potential in many industrial sectors such as food, feed, detergent, beverage, personal care, leather, textile and bioenergy industries. Compared to chemical catalysts, enzymes are environmentally friendly, highly efficient and more specific [1, 2]. Endoxylanase, one of the important industrial enzyme, cleave xylan backbone and generate xylooligosaccharides [3]. Endoxylanase are used in many biotechnological applications including biobleaching of Kraft pulp, improvement of dough and fruit juices,

degrading fiber in viscous diets, produce reducing sugar from some lignocellulosic wastes and xylooligosaccharides production as food additives [4].

There are many microorganisms such as *Aspergillus* sp., *Bacillus* sp., *Streptomyces* sp. had endoxylanase production ability [5]. But, main problem in microbial endoxylanase production is high cost due to usage of expensive inducers like pure xylan during process. Two strategies play critical role for reduce endoxylanase production cost. Improvement of enzyme performance is one of these strategies. The other is usage of cheap inducers like agricultural wastes [6]. The second approach also provide ecofriendly valuation of solid wastes remain on field after harvesting.

Actinomycetes, gram-positive, aerobic, filamentous bacteria, widely distributed in different habitats especially in terrestrial environments [7,8]. They contribute to ecosystem through degradation of organic material particularly lignocellulose and some hazardous chemicals by using extracellular hydrolytic enzymes such as cellulases, xylanases, proteases, peroxidases and amylases [9]. Consequently, actinomycetes are useful microorganisms for hydrolytic enzyme production. There are a few report about endoxylanase production from *Actinomadura* sp. in spite of the fact that considerable number of reports had been published about endoxylanase production from *Streptomyces* sp. previously, which is another genus of Actinomycetes [10, 11].

In this study, endoxylanase was produced from novel *Actinomadura geliboluensis*, which was isolated and identified previously [12], by using some agricultural wastes such as wheat straw, corn stover and castor stalk as primary carbon sources. Also, endoxylanase production was optimized by changing cultural and nutritional parameters such as incubation temperature, initial pH of fermentation medium, agitation rate, particle size of carbon source, and type of nitrogen sources.

Material and Methods

Materials and chemicals

Lignocellulosic wastes obtained from local fields in Mersin province and then these were soaked with distilled water, autoclaved and dried overnight. Birchwood xylan was purchased from Sigma. Other chemicals used in fermentation media purchased from Merck, Sigma and Alpha Easer.

Microorganism and culture condition

A. geliboluensis was obtained from Ondokuz Mayıs University, Faculty of Art and Science, Department of Biology. *A. geliboluensis* was subcultured on ISP2 medium (4 g/L yeast extract, 10 g/L malt extract, 4 g/L dextrose, 20 g/L agar) at 30 °C for 7 days and then saved at 4 °C until used. One loop colony of *A. geliboluensis* from fresh solid medium was taken and transferred in to MS-YEM medium including 6 g/L yeast extract, 0.1 g/L ammonium sulphate, 0.3 g/L sodium chloride, 0.1 g/L magnesium sulphate, 0.02 g/L calcium carbonate, and 1 mL of trace-elements solution (1 g/L ferrous sulphate, 0.9 g/L zinc sulphate, 0.2 g/L manganese sulphate). Medium was used as seed culture for inoculation after incubated at 30 °C and 150 rpm for 7 days. One milliliter seed culture was transferred into 50 mL liquid culture in 250 mL flask as inoculum.

Determination of enzyme activities and total protein content

Suitable volume of fermentation broth was taken from fermentation flasks and centrifuged at 10 000 x *g* for 5 min at 4 °C, then supernatant was used as crude enzyme and protein sources. Birchwood xylan was used as

substrate of endoxylanase. Reaction mixture was prepared with 100 µL of 1% (w/v) birchwood xylan in 50 mM phosphate buffer (pH 7.0) and 100 µL crude enzyme. Reaction mixture was incubated at 37 °C for 10 min and then dinitrosalysilic acid (DNS) solution (400 µL) was added to reaction mixture and boiled at 100 °C for 5 min. After cooling, 1 mL distilled water was added and then absorbance of mixture at 540 nm wavelength was measured [13]. Released reducing sugar was determined using xylose standard curve. One unite endoxylanase activity was defined as amount of enzyme required to liberate 1 µmol xylose equivalent per minute under reaction conditions described above. Total protein content was calculated by using Bradford method [14].

Validation

Experiments were performed in triplicate. Graphs were drawn with standard error bars. Experimental results in tables were showed with standard deviations.

Selection of best fermentation medium for endoxylanase production

Time course of endoxylanase production by *A. geliboluensis* in different modified fermentation media such as ISP-2 liquid medium (10 g/L malt extract, 4 g/L yeast extract), MS-YEM (6 g/L yeast extract, 0.1 g/L ammonium sulphate, 0.3 g/L sodium chloride, 0.1 g/L magnesium sulphate, 0.02 g/L calcium carbonate, and 1 mL of trace-elements solution), Horikoshi Medium (1 g/L monopotassium phosphate, 0.1 g/L magnesium sulphate, 5 g/L yeast extract, 5 g/L peptone), Nutrient Broth (5,0 g/L peptone from meat, 3.0 g/L meat extract) and Minimal Medium (2.5 g/L sodium chloride, 7 g/L dipotassium phosphate, 3 g/L monopotassium phosphate, 0.1 g/L magnesium sulphate and 1 g/L ammonium sulphate) supplemented with 5 g/L birchwood xylan as a primary carbon source was evaluated. After *A. geliboluensis* inoculated, cultures were incubated at 30 °C, 200 rpm for 7 days. Endoxylanase production and total protein content was determined by taking the sample at every 24 h intervals up to 7 days.

Determination of suitable carbon sources for endoxylanase production

To produce endoxylanase, *A. geliboluensis* was cultivated in MS-YEM supplemented with 0.5 % lignocellulosic wastes that had approximately 1 mm particle size such as lentil straw, banana leaf, barley straw, wheat straw, corn stover, castor oil stalk and pine needle as primary carbon source at 30 °C, 200 rpm for 3 days. The initial pH of culture media was adjusted to 7.0 ± 0.2 before inoculation.

Improvement of endoxylanase production by optimizing of some cultural parameters

Some cultural parameters such as particle size of carbon sources (0.25; 0.5; 0.75 and 0.5 mm), nitrogen source (asparagine, glycine, casein, peptone, yeast extract, soybean meal, urea and gelatin), temperature (25, 30, 35 and 40 °C), initial culture pH (4.0; 5.0; 6.0; 7.0; 8.0 and 9.0) and agitation rate (50, 100, 150 and 250 rpm) were studied to improve endoxylanase production from *A. geliboluensis* by using 5% (w/v) wheat straw, corn stover, and castor oil plant stalk as primary carbon sources. Each parameter studied for optimization was incorporated further in the subsequent experiments.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Native-PAGE and Zymogram Analysis

Extracellular proteins that was produced by using wheat straw, corn stover, lentil straw and castor oil plant stalk under optimum conditions were concentrated to 10-fold in Amicon (Millipore, Bedford, MA, USA) stirring

ultrafiltration cell using 10 kDa cut-of ultrafiltration membrane. Concentrated samples were incubated with sample buffer in boiling water for 10 min and then loaded on stacking gel. SDS-PAGE was performed according to Laemmli [15]. Extracellular proteins were separated in 10% resolving gel using Tris-Glycine-SDS (1 X TGS) buffer. Protein bands in fermentation broth were visualized using Coomassive blue staining.

Native-PAGE method was used for the zymography. All gels and buffers were prepared as in SDS-PAGE without SDS. Extracellular proteins were (10-fold concentrated) directly loaded to stacking gel. After proteins separated in 10% polyacrylamide gel, gel was incubated in 50 mM phosphate buffer (pH 7.0) containing 3% birchwood xylan at 37 °C for 1 hour and then flooded with 0.1% Congo red for 5 min. For the visualizing of endoxylanase active bands, flooded gel incubated in 1 M NaCl solution for 15 min.

Results and Discussions

Suitable fermentation medium for endoxylanase production from A. geliboluensis

Among the different fermentation media, MS-YEM was more effective for endoxylanase production from *A. geliboluensis* (130.40 U/mL). Maximum endoxylanase production in Horikoshi, ISP2 liquid, Nutrient Broth and Minimal Medium were 90.35 U/mL, 81.93 U/mL, 69.78 U/mL and 58.33 U/mL, respectively (Figure 1). The maximal endoxylanase activity was detected after 3 days of incubation in all fermentation media. Endoxylanase detected in all fermentation media was significantly declined when incubation time was reached from 3 to 7 days. This reduction of endoxylanase activity detected in fermentation media could be due to proteolysis or decrease of nutrients [16, 17]. MS-YEM was used as fermentation medium and *A. geliboluensis* was incubated for 3 days in further studies.

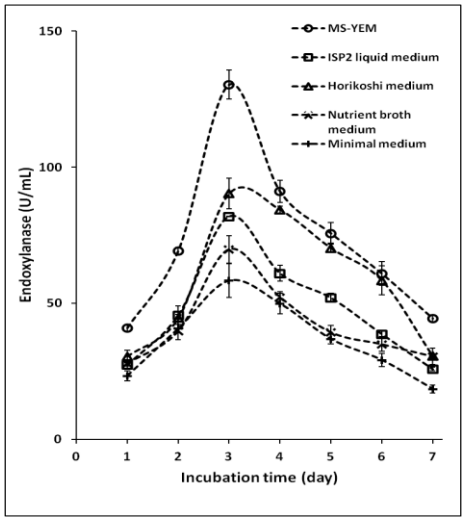

Figure 1. Time course of endoxylanase production from *A. geliboluensis* in MS-YEM, Horikoshi, ISP2 liquid, Nutrient Broth and Minimal Medium supplemented with 0.5% (w/v) birchwood xylan. Incubation was performed at 30 °C, 200 rpm.

Suitable carbon sources for endoxylanase production

A. geliboluensis was grown in MS-YEM supplemented with different lignocellulosic wastes such as lentil straw, banana leaf, barley straw, wheat straw, corn stover, castor oil plant stalk and pine needle at 30 °C, 200 rpm for 3 days for endoxylanase production. After incubation, the results indicate that wheat straw (170.91 U/mL) was the

best primary carbon source for endoxylanase production (Figure 2). On the other hand *A. geliboluensis* produce endoxylanase significantly by using corn stover (164.98 U/mL), castor oil plant stalk (122.37 U/mL) and lentil straw (79.80 U/mL). For this reason, endoxylanase production from *A. geliboluensis* by using wheat straw, corn stover, castor oil plant stalk and lentil straw was improved by changing different cultural and nutritional parameter during incubation. Different endoxylanase production might be due to structural differentiation (lignin, hemicelluloses and cellulose content) of these carbon sources [18].

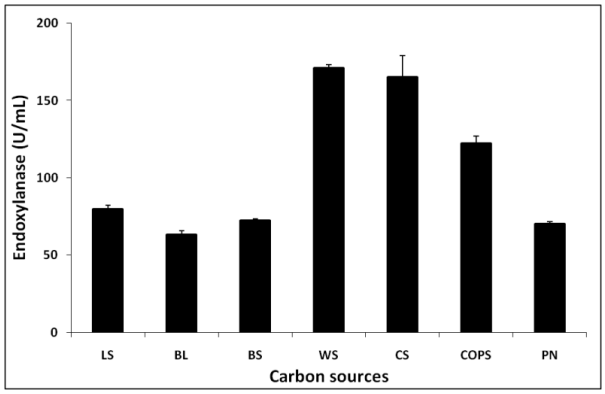

Figure 2. Effect of different lignocellulosic carbon sources (5 g/L) such as lentil straw (LS), banana leaf (BL), barley straw (BS), wheat straw (WS), corn stover (CS), castor oil plant stalk (COPS) and pine needle (PN) on endoxylanase production from *A. geliboluensis* in MS-YEM. Incubation was performed at 30 °C, 200 rpm for 3 days.

Improvement of endoxylanase production from A. geliboluensis by using wheat straw as primary carbon source

Wheat (*Triticum aestivum* L) is widely cultivated crop in world. It can be grown under wide range of environmental conditions. In Turkey, wheat is cultivated on 8.5 million ha and produced 20-24 million tons annually [19]. Ratio of waste produced from wheat is 1.3 of product [20]. Despite the fact that small part of wheat straw remain on field after harvesting use as feedstock, it was burned generally [21]. Therefore, we improved endoxylanase production from *A. geliboluensis* by using wheat straw which is abundant and inexpensive agricultural waste.

Particle size of wheat straw affected the endoxylanase production. The endoxylanase production increased from 173.86 U/mL to 192.76 U/mL with particle size decreased from 0.5 mm to 0.25 mm (Figure 3a). The endoxylanase production decreased significantly from 173.86 U/mL to 77.15 U/mL with particle size increased from 0.5 mm to 1 mm. Optimum particle size of wheat straw for endoxylanase production was 0.25 mm.

To optimize the effect of nitrogen sources for endoxylanase production, different nitrogen sources added by replacing the yeast extract from MS-YEM supplemented with 5% wheat straw (particle size 0.25 mm). Yeast extract (193.26 U/mL) was the best nitrogen sources for endoxylanase production from *A. geliboluensis* (Figure 3b). Endoxylanase production significantly decreased with use of other nitrogen sources such as asparagine (62.64 U/mL), glycine (80.94 U/mL), casein (76.25 U/mL), soybean meal (101.19 U/mL), urea (107.04 U/mL) and gelatin (46.27 U/mL) substituted for yeast extract. Endoxylanase production was moderately (144.55 U/mL) when pepton was used as nitrogen source.

Maximum endoxylanase production (189.26 U/mL) found at 30 °C (Figure 3c). The highest endoxylanase production (200.71 U/mL) was observed when initial pH of medium was adjusted to 6.0 ± 0.2 (Figure 3d). About 75-95 % production was retained at pH 7.0-8.0. But, endoxylanase activity decreased drastically at initial pH 5.0 (55.35 U/mL) and 4.0 (5.11 U/mL). Maximum endoxylanase production was obtained when incubation carried out at 200 rpm (Figure 3e). Consequently, endoxylanase production by using wheat straw was improved from approximately 170 U/mL to approximately 200 U/mL by optimizing nutritional and cultural parameters. Similarly, Sanghvi et al. [22], Katapodis et al. [23], Ghanem et al. [24] and Bakri et al. [25] have shown use of wheat straw as a primary carbon sources for endoxylanase production. Higher endoxylanase production by using wheat straw from some microorganisms such as *Melanocarpus albomyces* IIS68 [26] and *Paecilomyces themophila* [27] was reported. But, a few study about endoxylanase production by using wheat straw from bacteria had been reported previously [28, 29].

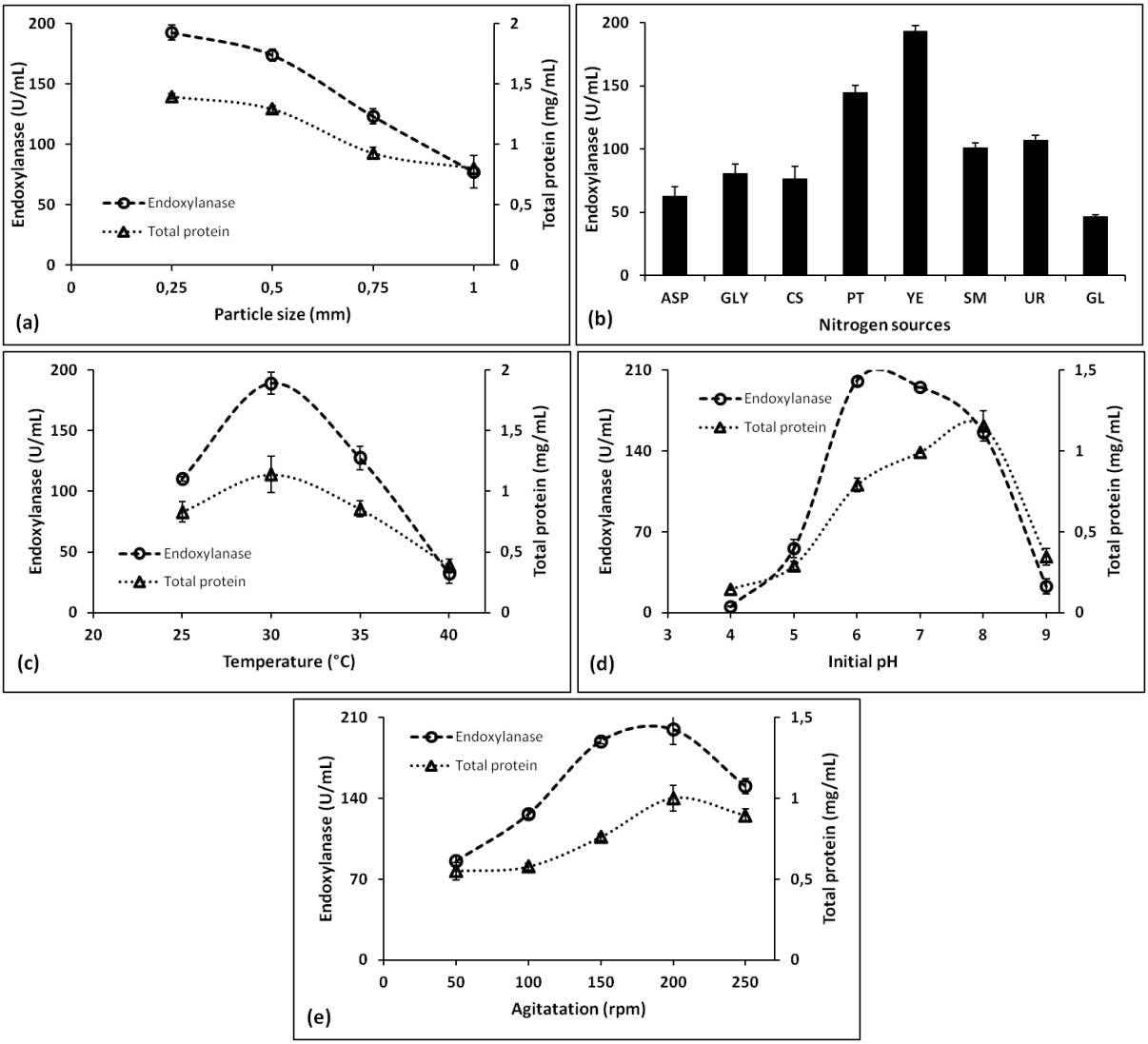

Figure 3. Optimization of **(a)** particle size of wheat straw, **(b)** nitrogen sources, **(c)** incubation temperatıre, **(d)** initial pH of medium and **(e)** agitation rate for endoxylanase production from *A. geliboluensis* by using wheat straw as primary carbon source in MS-YEM.

Improvement of endoxylanase production from A. geliboluensis by using corn stover as primary carbon source

Corn stover comprise stalks, leaves and cobs that remain in field after the corn harvest. One kg corn stover remain for obtain one kg corn grain. Also, approximately 70% of corn stover compose of cellulose and hemicellulose. Therefore, corn stover is an abundant and cheap substrate for enzyme production especially lignocellulose degrading enzyme such as endoxylanase.

Particle size of corn stover affects endoxylanase production from *A. geliboluensis* significantly. The highest endoxylanase (172.15 U/mL) was produced with corn stover had 0.25 mm particle size whereas least endoxylanase (96.63 U/mL) was produced with corn stover had 1 mm particle size (Figure 4a). Endoxylanase production increased from 170.32 U/mL to 175.10 U/mL with using peptone substituted for yeast extract (Figure 4b). Endoxylanase production decrease significantly when other nitrogen source was used in fermentation medium. The least endoxylanase (34.82 U/mL) production was obtained when gelatin was used as nitrogen source. Optimum incubation temperature was 30 °C for endoxylanase production by using corn stover (Figure 4c). Endoxylanase production decrease slowly with decreasing incubation temperature to 25 °C (121.65 U/mL). The decrease in endoxylanase production was drastic when incubation temperature was increased to 35 (94.17 U/mL) and 40 °C (31.56 U/mL). Highest endoxylanase production was obtained when initial pH of medium was adjust to 6.0 (151. 95 U/mL), 7.0 (165.49 U/mL) and 8.0 (95.95 U/mL) (Figure 4d). Maximum endoxylanase was obtained after *A. geliboluensis* was incubated at 200 rpm (Figure 4e). When agitation rate was alter, endoxylanase production decrease. Endoxylanase production from *A. geliboluensis* by using corn stover was reached up from 164.98 U/mL to 194.73 U/mL. Similarly, some researcher had been published some report about endoxylanase production by using corn stover. Panagiotou et al. determined that 1840 U/g substrate xylanase was produced from *Fusarium oxysporum* grown on corn stover [30]. Bhalla et al. recorded that *Geobacillus* sp. WSUCF1 produced approximately 20 U/mL endoxylanase by using corn stover [31].

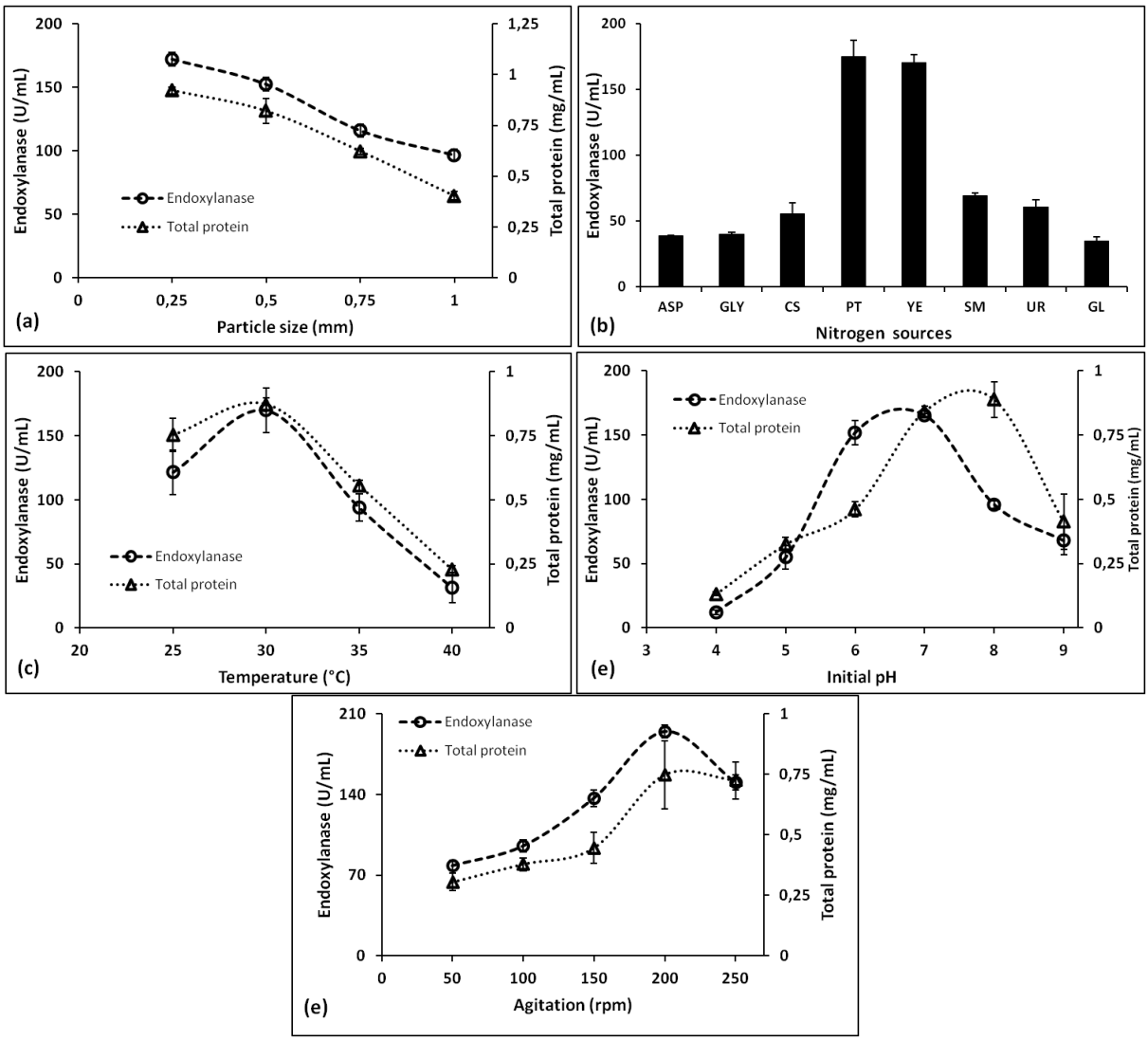

Figure 4. Optimization of **(a)** particle size of corn stover, **(b)** nitrogen sources, **(c)** incubation temperatıre, **(d)** initial pH of medium and **(e)** agitation rate for endoxylanase production from *A. geliboluensis* by using corn stover as primary carbon source in MS-YEM.

Improvement of endoxylanase production from A. geliboluensis by using castor oil plant (Ricinus communis) stalk as primary carbon source

The castor oil plant (*Ricinus communis*) is fast growing tree in the wild. It reach up to 12 m and has 10-40 cm leave [32]. It is not effected so much any environmental condition such as saline and alkaline soils, cold and hot climate [33]. Castor oil plant was planted for obtain oil from its seed. Castor oil use in many industrial application such as coating fabrics, high grade lubricant, printing inks and polyamide nylon type production [34]. Approximately 10 ton dry stalks are obtained from castor cultivate on 1 ha field [35]. After harvesting of seed, stalks are available for use in some biotechnological application such as enzyme production and lignocellulose bioconversion. Therefore, endoxylanase production from *A. geliboluensis* by using castor stalks as carbon source was enhanced with optimization tests in this study.

Best endoxylanase production (125.75 U/mL) was obtained when 0.25 mm castor oil plant stalk was used as primary carbon sources. However, there was no significant change in endoxylanase production after substrate has 0.25-0.75 mm particle size was used in fermentation medium (Figure 5a). Endoxylanase production (76.95

U/mL) decreased significantly with using 1 mm castor oil plant stalk as primary carbon sources. To determine alternative nitrogen source for endoxylanase production, different nitrogen sources was used in fermentation medium. Highest endoxylanase production was obtained with yeast extract (125.16 U/mL) as a nitrogen source followed by peptone (119.53 U/mL) and soybean meal (94.50 U/mL) (Figure 5b). The least endoxylanase activity was achieved with gelatin (32.86 U/mL) that was used as nitrogen sources. When incubation temperature decreased from 30 °C to 25 °C, endoxylanase production decreased 124.71 U/mL to 76.73 U/mL (Figure 5c). After incubation was carried out at 35 °C, endoxylanase production was 103.24 U/mL. Endoxylanase production by using castor oil plant stalk increased from 124.33 U/mL to 139.80 U/mL after initial pH of production medium was adjust to 6.0 from 7.0 (Figure 5d). It was found that maximum activity (148.77 U/mL) for endoxylanase production from *A. geliboluensis* was obtained when agitation was adjusted to 150 rpm (Figure 5e). After optimization, endoxylanase production by using castor stalk improved 23% approximately (122.37 U/mL-148.77 U/mL). This is the first report about endoxylanase production by using castor stalk.

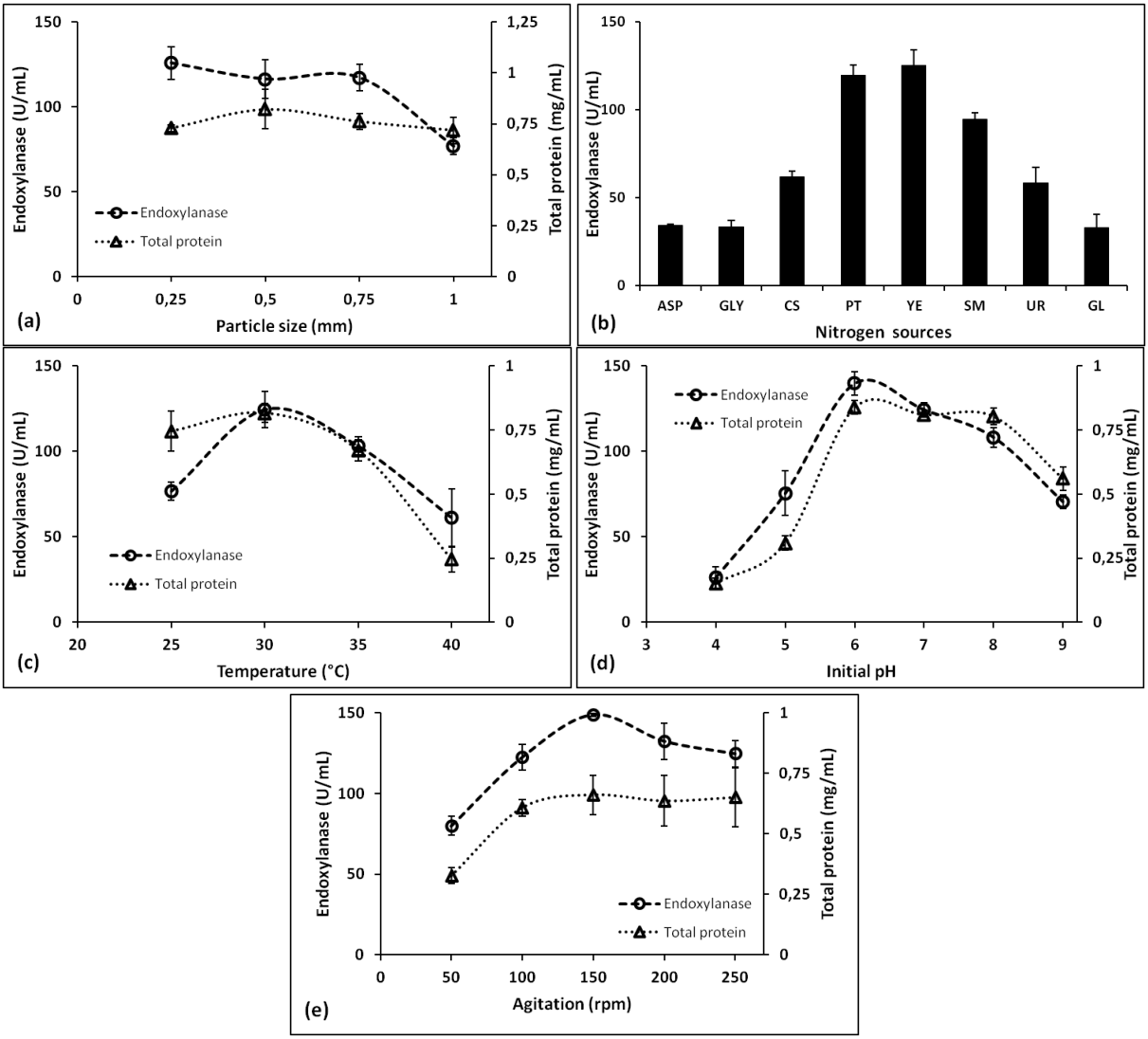

Figure 5. Optimization of **(a)** particle size of castor oil plant stalk, **(b)** nitrogen sources, **(c)** incubation temperatıre, **(d)** initial pH of medium and **(e)** agitation rate for endoxylanase production from *A. geliboluensis* by using castor oil plant stalk as primary carbon source in MS-YEM.

Extracellular protein profile and zymogram analysis of A. geliboluensis fermentation broths

To demonstrate extracellular protein profiles produced from *A. geliboluensis* by using wheat straw (Line 1), corn straw (Line 2) and castor stalk (Line 3), proteins were running on 10% polyacrylamide gels. Protein bands were similar on all lines. However, distinction was observed on line 3. The band in the midst of line 3 was quite sharp (Figure 6a). After zymogram analysis, one activity bands that was shinier was obtained from extracellular proteins of *A. geliboluensis* that was grown in MS-YEM containing wheat straw (Figure 6b). After zymography of *A. geliboluensis*, that was cultivated in MSYEM contain corn stover and castor stalk, extracellular proteins, one endoxylanase activity band was obtained (Figure 6b).

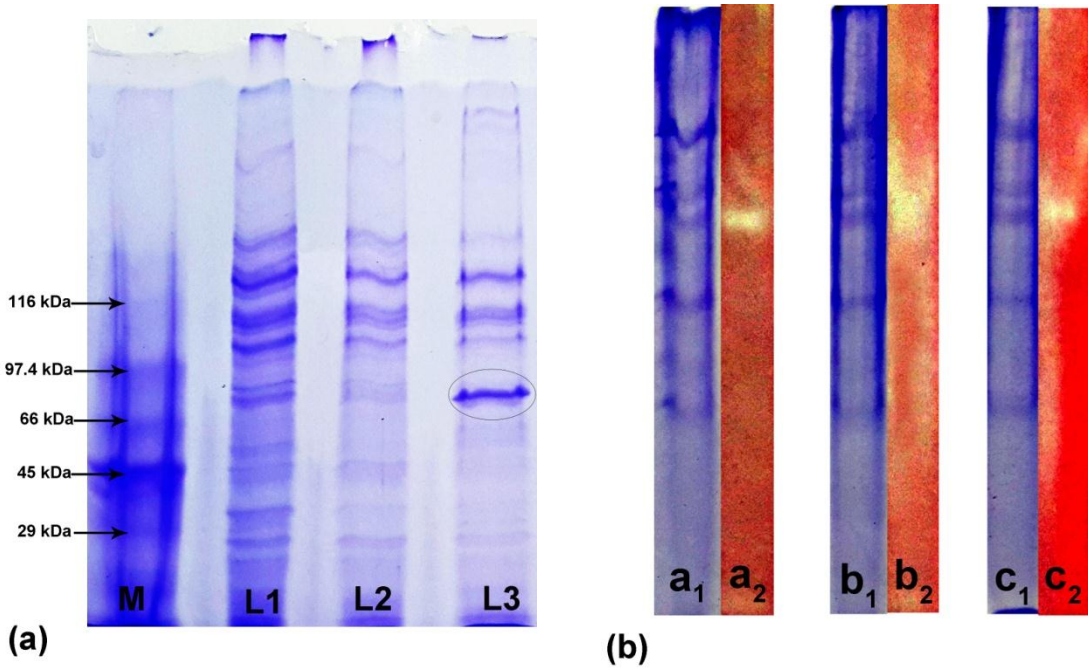

Figure 6. (a) SDS-PAGE profile of extracellular proteins in fermentation liquid after incubation of *A. geliboluensis* by using wheat straw (L1), corn stover (L2) and castor oil plant (L3) in MS-YEM. Moleculer marker (M) included carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), rabbit phosphorylase b (97.4 kDa) and β-galactosidase (116 kDa). **(b)** Native-PAGE and zymography of extracellular proteins in fermentation liquid after incubation of *A. geliboluensis* by using wheat straw (a_1: native PAGE, a_2: zymography), corn stover (b_1: native PAGE, b_2: zymography) and castor oil plant (c_1: native PAGE, c_2: zymography) in MS-YEM.

Conclusion

Suitable fermentation medium was MS-YEM for endoxylanase production from *A. geliboluensis* while wheat straw, corn stover and castor stalk was good endoxylanase inducer. After nutritional parameter such as particle size of carbon sources and nitrogen sources, cultural parameter such as incubation temperature, initial pH of medium and agitation rate, *A. geliboluensis* produced 200.71 U/mL, 194.73 U/mL, 148.77 U/mL endoxylanase by using wheat straw, corn stover and castor stalk as primary carbon sources, respectively.

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