Screening of Culture Conditions for Production of Xylanase from Landfill Soil Bacteria

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Abstract: *Culture conditions including initial pH media, incubation period, inoculum size, type of carbon source, type of nitrogen source and its concentration, which affect xylanase production were screened via the one-factor-at-a-time approach. The bacteria used in the production of xylanase was isolated from the landfill site at Sg. Ikan, Kuala Terengganu, Malaysia. Three characterizations of the landfill soil were investigated for their moisture content, ash content, and pH. The culture conditions range used in the experimental work were between 6–30 h for the incubation period, with initial pH between 5–9, inoculum size between 1–20% v/v, carbon, nitrogen sources, and nitrogen source concentration between 1–5% w/v. Xylanase activity was estimated using dinitrosalicylic acid (DNS) based on the release of xylose under standard assay conditions. The landfill soil was observed to have pH between pH 3.4–7.2 with a moisture content between 12.4–33.7% and ash ranged between 3.5–4.3%. Results showed that the highest xylanase activity within studied ranges was recorded at 25.91±0.0641 U/mL with 10% (v/v) inoculum size, 1% (w/v) xylose as sole carbon source, mixture of 1% (w/v) peptone and 0.25% (w/v) ammonium sulphate as nitrogen sources, which was carried out at initial pH of 8.0 for 24 h incubation.*

Keywords: *xylanase; bacteria; landfill soil; screening parameter*

■ INTRODUCTION

Xylan is a major constituent of hemicellulosic polysaccharides found in plant's cell wall which represent up to 20–30% of the total dry weight in plant tropical plant biomass [1]. Besides, 9–12% of municipal solid waste is composed of hemicellulose on dry weight basis [2]. The enzymatic method can be used for the degradation of these materials involving the use of microbial enzymes [3] that are less polluting, environmental friendly, energy saving and lower disposal problems [4]. Microorganisms such as bacteria, fungi, actinomycetes, and yeast are found to be the rich sources of xylanases [5-6]. Although many microorganisms have been isolated and exploited for enzyme production, there is still a need for novel strains capable in producing enhanced levels of the enzyme [7]. The bacteria used in this work was previously isolated from landfill soil [8] in Malaysia which is the natural occurring physical covering of the earth's surface and are home to a diversity of microorganisms [9]. The landfill is a site for disposal of solid waste that comprises a high composition of xylan [8] and the high possibility of the xylanase to degrade them. Thus, it would be the right place to discover new strain for xylanase producer from the landfill area. The potential ability of Isolate C3 to produce xylanase is yet to be discovered in this current work.

Xylanase is a biocatalyst which specifically degrades xylan into reducing sugars such as xylose and xylobiose [5]. It has been used in many important industrial applications such as paper, pulp, juice, bakery and beer industries [10]. This enzyme has been employed in the manufacturing of paper to bleach paper pulp and increase the paper pulp brightness instead of using toxic and expensive chemicals [5]. Xylanase also being used in the pre-treatment of lignocellulose biomass for bioethanol production and used in the treatment of barley and wheat to improve the properties of animal diet in animal feed industries [1]. Besides, this

enzyme can be applied for conversion of xylan into xylose in agricultural wastewater and to clarify fruit juices in beverage industries [11].

Enzyme synthesis is a complex process, where the final quality and quantity of the product yield is incredibly relying upon microorganism strains, metabolic pathways, fermentation parameters, enzyme generation stages, carbon and nitrogen sources, and nutrient consumption conditions. It is necessary to determine the parameter and conditions which affected the enzyme secretion when a new potential strain was identified. This information is important to further the works into the optimization stage to give positive impact in the growth of microorganisms and to synthesize the desirable xylanase at maximum level with improvement in its production process [12]. Previously, xylanase was produced either by using commercial substrates such as xylan [13], birchwood xylan [14-15], and glucose [7] or by utilizing cheaper substrates such as rice straw [16], rice bran [17], wheat bran [5,17-20], wheat straw [21], orange peel, banana peel, mango peel, apple pulp, oil cake [7], grass extract [22], sugarcane sheath leaf extract [22], *Prosopis juliflora* pods [23], olive mill waste, olive leaves, sawdust, corn cobs [18], barley husk [6], sugarcane bagasse [24] and apple pomace [25] supplemented with other nutrient sources in submerged fermentation [7,13-17,20-24] or in solid state fermentation [5,18-19,25] modes. The xylanase is produced by microorganisms such as *Streptomyces thermovulgaris* TISTR1948 [16], *Bacillus* sp. PKD-9 [19], *Bacillus subtilis* [6,13,24], *Bacillus megaterium* [7,24], *Bacillus arseniciselenatis* DSM-15340 [5], *Bacillus tequilensis* [15], Recombinant *Pichia pastoris* [20], *Aspergillus flavus*, *Cladosporium sphaerospermum* and *Epicoccum purpurascens* [18], *Streptomyces* sp. [14], *Cellulosimicrobium* sp. [25], *Stenotrophomonas maltophilia* [17,21], a mixed culture system of *Bacillus polymyxa* and *Cellulomonas uda* [22] and a mixed culture system of *Acetobacter xylinum* and *Cellulomonas uda* [23].

Hence, this study was aimed to investigate the effect of culture conditions on the production of xylanase by the newly isolated bacteria from landfill soil using one-factor-at-a-time (OFAT) approach. The studied conditions were initial pH of media, incubation period, inoculum size, carbon source, nitrogen source, and nitrogen source concentration.

■ EXPERIMENTAL SECTION

Materials

All chemicals used were analytical grade. Nutrient agar, nutrient broth, peptone, and Congo red were purchased from Merck (Germany) while agar bacteriological from Oxoid (UK). Xylan from corn core was purchased from TCI (Tokyo, Japan), 3,5-Dinitrosalicylic acid was from Sigma Aldrich (USA), D-xylose, D-glucose, glycerol, ethyl alcohol, glycine, potassium chloride, and sodium dihydrogen phosphate were from R&M Chemicals (UK).

Instrumentation

The instruments used throughout this study were UV-vis spectrophotometer (Varian Cary® 50, Australia), pH meter (Mettler Toledo, USA), and mass balance (A&D, Japan). While the equipment used were autoclave (Hirayama, Japan), water bath (Memmert, Germany), incubator (Memmert, Germany), incubator shaker (Infors, Switzerland), freezer (Heraeus, German), chiller (Thermo Fisher Scientific, USA), centrifuge (Eppendorf, Germany) and laminar flow cabinet (Esco, Singapore).

Procedure

Soil sample collection

Soil samples were collected from surface, 10 cm depth and near the leachate pond of the landfill site at Sg. Ikan, Kuala Terengganu, Malaysia (latitude 5°19'07.3"N and longitude 102°59'32.0"E). Approximately, 1 kg of each sample was scoped with a sterile spatula and stored in sterile polythene bags maintaining aseptic conditions and marked accordingly to its source and location. The three collected samples were transported to the Bioprocess Laboratory of Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang for characterization and further work.

Soil characteristic study

Collected soils were subjected to the analysis for moisture content, ash content, and pH. The moisture

content of the soil was analyzed by measuring the change in soil mass before and after drying at 110 °C. Landfill soil samples were kept in aluminum weighing placed inside an incubator at 110±5 °C. During the drying process, the weight of the samples was measured a few times until a consistent weight was obtained [26]. The percentage of moisture content was determined by the following Eq. 1 [27]:

$$\mathrm{MC}(\%) = \frac{\mathrm{W} - \mathrm{w}}{\mathrm{w}} \times 100\% \quad (1)$$

where MC is moisture content, W is the initial weight and w is the constant weight after oven drying.

The ash content was examined based on ash formation at 440 °C. The oven-dried landfill soil from the above procedure was placed in the porcelain dish, and the initial weight of the soil was determined. The temperature of the furnace was gradually brought to 440 °C and hold until it was completely turned to ash indicates by no change of weight after a period of heating. The dishes were cooled in the desiccator, weighed and the percentage of ash content were determined by the following Eq. 2 [28]:

$$\text{Ash content}(\%) = \frac{\text{Mass of ash}}{\text{Mass of oven} - \text{dried soil}} \times 100\% \quad (2)$$

pH of the soil was measured using its suspension. Ratio 1:1 of soil in water was prepared as suggested by Hubbard [29] by mixing 10 g of soil into 10 mL deionized water and mechanically shaken at 15 rpm. Once the soil settled at the bottom of the beaker, the pH value was recorded by immersing pH electrode into soil suspension [30].

Bacteria isolates from landfill soil

Landfill isolate, known as Isolate C3 was obtained from our collection in Bioprocessing Laboratory, Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Malaysia. This isolate was locally isolated from landfill soil as described in Masngut et al. [8] and maintained on nutrient agar (Merks, Germany) slants at 4 °C. Isolate C3 was selected as xylanase producer via submerged fermentation. In the previous study, Isolate C3 has shown the highest xylanase activity among other isolates from landfill soil after qualitative and quantitative analysis for xylanase production [8]. Isolate C3 is a gram-negative bacteria and has bacilli shape observed under a microscope. The morphological characteristics of colony Isolate C3 were in punctiform form, convex elevation, and entire margin.

Inoculum preparation

An inoculum of Isolate C3 was prepared by transferring the colony from agar slant into broth medium containing 1% peptone, 0.25% ammonium sulfate, 0.2% dipotassium phosphate, 0.03% magnesium sulfate and enriched with 1% glucose at initial media pH of 7.0. The mixture was incubated for 24 h at 37 °C and agitated at 150 rpm. After 24 h incubation, these exponential growth cells were utilized as a source of inoculum all through the study [31].

Experimental design of screening of culture conditions for xylanase production

The classic "one-factor-at-a-time" (OFAT) approached was utilized to assess the effect of culture conditions (initial pH media, incubation period, inoculum size and nutritional conditions including carbon sources, nitrogen sources and initial concentration of nitrogen source) to xylanase production. The dependent variable for this study was xylanase activity, expressed in U/mL. The growth media contained 1% peptone, 0.25% ammonium sulfate, 0.2% dipotassium phosphate, 0.03% magnesium sulfate and enriched with 1% of xylose and at pH 7.0. It was prepared and sterilized in each cotton plugged 250 mL Erlenmeyer flask. The fermentation precedes at initial pH of 7.0, 37 °C and an agitation rate of 150 rpm as recommended by Masngut et al. [8]. The OFAT running strategy was divided into six experimental runs as summarized in Table 1. The best condition found to produce the highest xylanase activity by the preceding run, were then be applied in the subsequent run.

The experiment started by varying the initial media pH to pH 5, 6, 7, 8 and 9 by the addition of 1 M of sodium hydroxide or 1 M of hydrochloric acid to the medium, before sterilization. Other fermentation factors (incubation period, inoculum size, carbon sources, nitrogen sources, and nitrogen source concentration) were fixed, according to the values stated in Table 1, Run 1. Next, the best value of initial pH media from Run 1, will be used in Run 2. Run 2 was carried out by varying

Table 1. Summary of experimental runs

Run	Initial pH media	Incubation period	Inoculum size	Carbon source	Nitrogen source	Nitrogen source concentration
1	Varies: pH 5, 6, 7, 8, 9	24 h	10% v/v	Xylose	Peptone + ammonium sulphate	1% peptone + 0.25% ammonium sulphate
2	Max from Run 1	Varies: 6, 12, 18, 24, 30 h	10% v/v	Xylose	Peptone + ammonium sulphate	1% peptone + 0.25% ammonium sulphate
3	Max from Run 1	Max from Run 2	Varies: 1, 3, 5, 7, 9, 10, 15, 20% (v/v)	Xylose	Peptone + ammonium sulfate	1% peptone + 0.25% ammonium sulfate
4	Max from Run 1	Max from Run 2	Max from Run 3	Varies: xylose, glucose, sucrose, CMC	Peptone + ammonium sulfate	1% peptone + 0.25% ammonium sulfate
5	Max from Run 1	Max from Run 2	Max from Run 3	Max from Run 4	Varies: peptone, ammonium sulfate, sodium nitrate, urea, ammonium chloride	1% of each nitrogen sources
6	Max from Run 1	Max from Run 2	Max from Run 3	Max from Run 4	Max from Run 5	Varies: 1, 2, 3, 4, 5% (w/v)

Table 2. Characteristics of landfill soil

Type of landfill soil	Moisture content (%)	Ash content (%)	pH
Leachate soil	22.96±0.3041	4.16±0.3794	7.2±0.1697
10-cm depth soil	12.45±0.2564	3.55±0.1869	3.6±0.2132
Surface soil	33.71±0.7356	4.23±0.3143	3.4±0.1768

the incubation period, while other factors were fixed (Table 1). Subsequent run (Run 3, 4, 5, and 6) will follow the same rules.

Quantification of xylanase activity

Xylanase activity was estimated by the method suggested by Kim et al. [32] with some modification. The activity of xylanase was assayed using dinitrosalicylic acid (DNS) reagent by estimation of reducing sugars released from oat spelt xylan. The fermented medium was centrifuged at 4,000 rpm and 4 °C for 15 min to discard the bacterial cells. The clear supernatant collected after centrifugation was used as a source of crude enzyme. A reaction mixture containing 0.2 mL of crude enzyme, 0.3 mL of phosphate buffer (pH 6.0) and 0.5 mL of 1% xylan solution in 0.05 M phosphate buffer (pH 6.0) was incubated at 50 °C for 10 min. After incubation, the enzymatic reaction was stopped by adding DNS reagent, boiled in a capped glass tube at 100 °C for 5 min and cooled in cold water for color stabilization. Xylose released was estimated by measuring absorbance at 540 nm. D-xylose was used as a standard for the preparation of a calibration curve. One unit of xylanase activity was defined as the quantity of enzyme which required to release 1 μmol of xylose per minute under standard assay conditions. All experimental works were performed in duplicate.

■ RESULTS AND DISCUSSION

Characterization of Landfill Soil

The landfill soil characterization result is shown in Table 2. It was observed that the pH was 3.4 to pH 7.2 with moisture content percentage ranged between 12.5–33.7% and ash content percentage ranged between 3.6–4.2%. Study by Bahaa-Eldin et al. [33] reported that the pH of landfill soil in Dengkil, Selangor, Malaysia were acidic which pH ranging between 2.44 to 3.01 with the

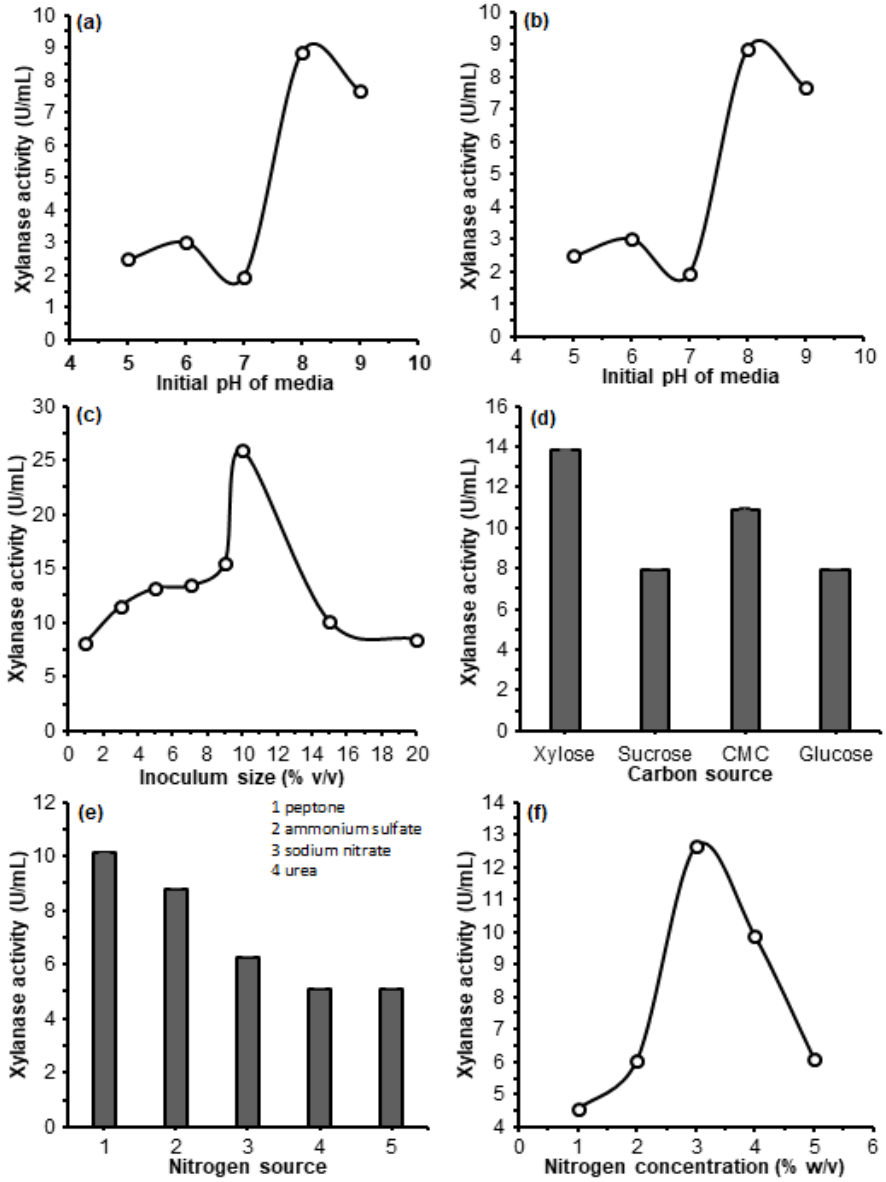

Fig 1. Xylanase activity affected by the variation of (a) initial pH media, (b) incubation period, (c) inoculum size, (d) carbon source, (e) nitrogen source, (f) nitrogen concentration. Results are the mean of duplicate flasks with standard deviation represented by error bars

percentage of organic matter of 2.50% while a research conducted by Whalen et al. [34] indicated that the moisture content of landfill soil was 11% with pH of 5.4 and organic content of 4%.

Effect of Culture Condition on Xylanase Production

Effect of initial pH media

Effect of initial pH media on xylanase production was shown in Fig. 1(a). It was observed that the xylanase activity by Isolate C3 favored alkaline range. The highest xylanase activity was observed at pH 8 (8.87±0.0068 U/mL) followed by pH 9 (7.67±0.0004 U/mL), pH 6 (3.02±0.0006 U/mL), pH 5 (2.47±0.0024 U/mL) and finally pH 7 (1.96±0.0017 U/mL). pH factor played an essential role in the xylanase production by bacteria due to substrate binding and catalysis that are often reliant on charge distribution on the substrate and in particular enzyme molecules [15]. A similar finding had been reported for maximum xylanase production by *B. subtilis*

[24], *B. megaterium* [24], *Cellulosimicrobium* sp. CKMX1 [25] and *S. maltophilia* AG137 [17] happened to be initial pH 8. According to Panwar et al. [19], the production of xylanase from *Bacillus* sp. PKD-9 was reported to increase with the increase of the initial pH medium from pH 5 to 8 with the maximum xylanase activity of 55,819 U/g which was attained at pH 8.

Effect of incubation period

The effect of incubation period on the xylanase production was displayed in Fig. 1(b). The highest xylanase activity was detected after 24 h incubation period at 10.86±0.0482 U/mL, followed by 30 h (8.26±0.0704 U/mL), 18 h (8.07±0.0919 U/mL), 12 h (4.62±0.0569 U/mL) and finally 6 h (4.28±0.0576 U/mL). This current result was in a good term with a finding by Khusro et al. [15] who reported the maximum xylanase production was obtained at 24 h incubation of *B. tequilensis*. Maximum enzyme production which was affected by the incubation period deviated among various bacteria and dependent upon the organism type, source of isolation, its enzyme production pattern, cultivation conditions and genetic makeup of the organism [15,35].

Effect of inoculum size

The result was shown in Fig. 1(c). From the results, it can be seen that the xylanase activity was gradually increased from 1% (v/v) to 9% (v/v) before dramatically increased afterward. Beyond 10% (v/v) of inoculum size, the xylanase activity was markedly decreased. This may due to the increasing demand for nutrient as the inoculum size getting bigger until the nutrient-depleted [35]. Xylanase production was at its minimum when using a small inoculum size. However, as the inoculum size increased, the xylanase production also increased until the nutrient depletion limited its production. The highest xylanase activity was recorded at 10% (v/v) of inoculum size with 25.91±0.0641 U/mL. The size of inoculum contributed to the fermentation profile of an organism as it helps to minimize the lag phase of fermentation [17]. The result was similar to the study conducted by Walia et al. [25] and Kareem et al. [7] where the maximum xylanase yield by *Cellulosimicrobium* sp. CKMX1 (530 U/g DBP) and *B. megaterium* (743 U/mL), respectively, were obtained at 10% (v/v) inoculum. In contrast with Raj et al. [17], the maximum xylanase production of 21.2±1.0 U/mL by *S. maltophilia* AG137 was observed at inoculum concentration of 1% (v/v). Again, the variety of the inoculum size was probably due to different genus and species of the microorganisms.

Effect of nutritional sources

In the present study, the effect of nutritional sources as shown in Fig. 1(d) demonstrated that among the tested carbon sources, xylose showed the highest xylanase activity at 13.86±0.0384 U/mL followed by carboxymethyl cellulose (10.91±0.0865 U/mL), glucose (7.97±0.0041 U/mL) and lastly sucrose (7.96±0.0041 U/mL). Similarly, among the tested nitrogen sources, as shown in Fig. 1(e), peptone displayed the highest xylanase activity at 10.13±0.0001 U/mL followed by ammonium sulfate (8.81±0.0052 U/mL) and sodium nitrate (6.29±0.0002 U/mL). The lowest xylanase activity was recorded when using urea and ammonium chloride with similar xylanase activity at 5.10±0.0103 and 5.09±0.0003 U/mL, respectively. Therefore, peptone was furthered for nitrogen concentration study.

The carbon source is an important nutrient for the growth and metabolic process of microorganisms. A study conducted by Irfan et al. [24] and Adhyaru et al. [35] showed similar results with the present study. The used of xylose as sole carbon source was reported to produce maximum xylanase in submerged fermentation by *B. subtilis*, *B. megaterium* and *Bacillus altitudinis* with 48.30±0.61, 43.06±1.10, and 145.13±3.85 U/mL, respectively, of xylanase activity. Kapoor et al. [36] reported a study using a combination of nitrogen sources of yeast extract and peptone. They found out that the combination resulted in high xylanase activity up to 1288±75 U/mL using *Bacillus pumilus*. However, the result of the current study was contradicted with Raj et al. [17]. They reported a high xylanase activity when using bran xylan as a carbon source and yeast extract as the nitrogen source on the fermentation of *S. maltophilia*. The contradiction might because of different microorganisms used in the current study than that the others.

Effect of initial nitrogen source concentration

The result was shown in Fig. 1(f). Xylanase activity was increased as the peptone concentration increased from 1 to 3% (w/v). However, beyond 3% (w/v), the xylanase activity started to decrease. The highest xylanase activity was recorded at 12.64±0.0243 U/mL. The previous study by Battan et al. [37], reported a lower range of nitrogen source (0.10% w/v to 1% w/v) than that this study. They recorded a maximum xylanase activity of 2500 U/mL by *B. pumilus* ASH at 0.75% (w/v) of peptone. Another study by Gupta et al. [21] who used peptone on *Melanocarpus albomyces* reported maximum xylanase activity at 173.7±2.9 U/mL, obtained at initial peptone concentration of 1.5 g/L. The differences in the xylanase activity might relate to the different species used in each study.

■ CONCLUSION

The effect of six culture conditions on production of xylanase by landfill bacteria C3 was investigated including initial pH of media, incubation period, inoculum size, type of carbon source, type of nitrogen source and nitrogen source concentration. The highest xylanase activity within studied ranges was recorded at 25.91±0.0641 U/mL with 10% (v/v) inoculum size, 1% (w/v) xylose as sole carbon source, mixture of 1% (w/v) peptone and 0.25% (w/v) ammonium sulfate as nitrogen sources, carried out at initial pH of 8.0 for 24 h. This investigation has established the influence of culture conditions on the xylanase production by bacterial Isolate C3 from landfill soil. All the factors above might significantly affect the xylanase production by the isolate, individually or by the interaction between factors. The latter is advisable to be considered in the future work, to analyze the significance of the interaction between multiple factors in xylanase production and further to optimization the cultivation conditions. Central Composite Design of Response surface methodology (RSM) is suggested to be applied to carry out this work.

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