

Phorbol Esters Degradation and Enzyme Production by *Bacillus* using *Jatropha* Seed Cake as Substrate

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Abstract- The purposes of this research were to evaluate phorbol esters (PEs) degradation rate and enzyme production yield using submerged fermentation (SMF) as screening method and further using solid-state fermentation (SSF) as pilot scale-up study. SMF was carried out with 20 g seed cake in 100 ml minimal salt medium for 7 days incubation, while SSF was done with 20 g seed cake at 50% moisture content for 9 days incubation. *Bacillus* strains grew well on *J. curcas* seed cake with 10^8 - 10^{11} CFU/ml in SMF for 3 days incubation, while they were 10^8 - 10^{10} CFU/g in SSF. PEs reduced 76.5%, 77.1%, 78.4%, 85.5%, and 92.0% in SMF with *B. smithii* G16, *B. sonorensis* D12, *B. licheniformis* A3, *B. subtilis* H8 and *B. coagulans* C45 for 3 days incubation, respectively, and PEs completed degraded by these five strains for 7 days incubation. Maximum amylase, cellulase, lipase, pectinase, protease and xylanase productions in SMF were observed in *B. sonorensis* D12 (5.49 ± 0.49 U/ml; day 7), *B. subtilis* H8 (17.03 ± 4.90 U/ml; day 2), *B. licheniformis* A3 (59.03 ± 0.26 U/ml; day 7), *B. sonorensis* D12 (1.70 ± 0.04 U/ml; day 3), *B. coagulans* C45 (15.95 ± 0.35 U/ml; day 7) and *B. smithii* G16 (1.40 ± 0.01 U/ml; day 3), respectively. For SSF, PEs were reduced 86.0%, 83.2%, and 93.0% with *B. sonorensis* D12, *B. subtilis* H8 and *B. smithii* G16 for 3 days incubation, respectively. Maximum amylase, cellulase, lipase, pectinase, protease and xylanase productions in SSF were observed in *B. smithii* G16 ($16.08 \pm$

0.36 U/g; day 4), *B. sonorensis* D12 (2.94 ± 0.06 U/g; day 2), *B. smithii* G16 (3.87 ± 0.64 U/g; day 4), *B. sonorensis* D12 (8.13 ± 1.06 U/g; day 2), *B. smithii* G16 (14.13 ± 0.30 U/g; day 4) and *B. smithii* G16 (9.72 ± 0.97 U/g; day 3), respectively. *J. curcas* seed cake could be detoxified by *Bacillus* and the high-protein seed cake could be potentially used for enzyme production in industry.

Keywords: *Jatropha curcas* seed cake, phorbol esters, degradation, submerged and solid state fermentation, enzyme production.

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1. Introduction

Due to the demand of local energy production, *Jatropha curcas* has high adaption capacity for land fertilization and can grow in marginalized land. It does not competitive with other vegetation crops for agricultural lands. The oil of *J. curcas* seed can be used

as the raw materials for biodiesel production. However, biodiesel production from *J. curcas* seeds generate large quantum of residual de-oiled seed cake with an average of 700 g cake per kg of seed used [23]. The de-oiled *J. curcas* seed cakes contain high protein and other different nutrients like minerals, and amino acids etc. [1]. They can be used as the nutrient for animal feeds or production of valuable products. However, the de-oiled *J. curcas* seed cake contains anti-nutritional substances ex. trypsin inhibitors, curcins, tannins, saponins, phytates and toxic factors - phorbol esters (PEs) that restricts the uses of the seed cake. PEs have been identified as the main toxicants in cake which cannot be destroyed even by heating at 160°C for 30 min [23].

Physical methods, chemical treatments and microbial fermentation have been used to detoxify the toxic compounds in the seed cake ([4], [12], [17], [20], [22], [29]). However, the biological method would be more advantageous than the others for environmental friendly with safety and energy concerns. Especially solid state fermentation (SSF) could detoxify PEs and use for production of valuable products [17].

SSF and SMF techniques are common and conventional biotechnology processes for production of value-added products such as enzymes, biopharmaceuticals, organic acids, biosurfactants, vitamins, flavoring compounds, biofuels, biopesticides etc. [10]. SSF has economical and practical advantages over SMF, which includes use of raw materials as substrates, low capital cost, low energy expenditure, and less expensive downstream processing [6]. Therefore, SSF is adapted as commercial production and SMF is adapted as screening method for basic research.

In comparison with the previous works, *Bacillus* species were often subjected to the enzyme production [30] and most of *Bacillus* species are considered as Generally Regarded as Safe (GRAS) by FDA of USA. Additionally, *Bacillus* spp. was often using in production of food additives and probiotic products ([14], [32]). Therefore, *Bacillus* strains were applied to evaluate the degradation efficiency of PEs toxins factors in *J. curcas* seed cake, and *J. curcas* seed cake was used as the substrate for enzyme production in this paper.

2. Materials and Methods

2.1. *J. curcas* Seed Cake and Tested Microbes

De-oiled *J. curcas* seed cake was kindly provided by Shin-Feng Energy Technology Co., LTD (Pingtung, Taiwan), then ground and pressed the defatted *J. curcas*

seed cake. Five *Bacillus* spp. (*B. coagulans* C45, *B. licheniformis* A3, *B. smithii* G16, *B. sonorensis* D12, and *B. subtilis* H8) isolated from different compost plants and biofertilizers were used for PEs degradation and enzyme production [7].

2.2. Biodegradation of PEs and Enzyme Production of *J. curcas* Seed Cake by *Bacillus*

For SMF, one ml of inoculums (about 10^8 CFU/ ml) was inoculated to 100 ml of mineral salts medium (MSM). Each liter of MSM medium contained seed cake 200 g, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 6.7 g, KH_2PO_4 1.5 g, $(\text{NH}_4)_2\text{SO}_4$ 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, ferrous ammonium citrate 0.06 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01 g and trace-element solution 1 ml. The trace element solution contained H_3BO_3 0.3 g, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.03 g, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.03 g, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.02 g and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.01 g in 1 liter of 0.1N HCl [28]. The cultures were incubated in rotary shaker at 25°C, 150 rpm for 0, 1, 2, 3 and 7 days. PEs degradation and enzyme production were determined.

For SSF, 1ml of freshly prepared bacterial cells (about 10^9 CFU/ ml) was transferred to each of 250-ml Erlenmeyer flasks containing 20 g sterile seed cake at 50% initial moisture content. The flasks were then manually shaken well and incubated at 25°C for 0, 1, 2, 3, 5, 7 and 9 days. PEs degradation and enzyme production were determined.

2.3. Phorbol Ester Extraction and Analysis

PEs were extracted and determined by the modified method of Hass and Mittelbach [13]. The mixture of *J. curcas* crushed seed cake 5 g and 95% ethanol 20 ml was shaken at 200 rpm for 5 min, then centrifuged at 14,000 *g* for 5 min. The residue was extracted two additional times with 95% ethanol. The extract fractions were combined and dried under vacuum at 50°C. The dried extract was dissolved in 1 ml 95% ethanol and passed through a 0.2- μm membrane filter. Phorbol esters were analyzed by HPLC system (Thermo Separation Products, U.S.) consisted of an AS1000 autosampler, P2000 pump and UV1000 detector. The solvents were water and acetonitrile: start with 60% water and 40% acetonitrile for 15 min, then 25% water and 75% acetonitrile for the next 20 min, and finally 100% acetonitrile for the next 20 min. Separation was performed at room temperature (25°C) with flow rate 1.3 ml/ min. The detector wavelength was set at 280 nm. The results were expressed as

equivalent to phorbol-12-myristate-13-acetate (PMA) (Sigma, U.K.) used as an external standard.

2.4. Enzyme Extraction and Assay

For enzyme assay in SMF, the sample was centrifuged at 12,000 *g* for 30 min and the supernatant was used for enzyme activities determination. While in SSF, 5 g fermented substrate was extracted with 20 ml 0.1 M Tris-HCl buffer pH 8.0 at 200 rpm orbital shaking for 30 min. The suspension was then centrifuged at 12,000 *g* for 30 min and the supernatant was used for enzyme assay.

Amylase, cellulase, lipase, pectinase, protease, and xylanase activities were determined by the methods of Bernfeld [5], Hu et al. [15], Kilcawley et al. [19], Janani et al. [16], Shimogaki et al. [31] and Joshi and Khare [18], respectively.

2.5. Chemical Analysis

Moisture, pH, protein, fat, and ash contents were analyzed by the standard methods of Association of Official Analytical Chemists [3]. Total organic carbon (TOC) was determined by TOC-5000A total organic carbon analyzer (Shimadzu, Japan).

3. Results and Discussion

3.1. Chemical Composition and Phorbol Ester Content in *J. curcas* Seed Cake

The chemical compositions of *J. curcas* seed cakes from Pingtung County, Taiwan are shown in Table 1. *J. curcas* seed cakes had pH 6.58 ± 0.02 , moisture $5.92 \pm 0.10\%$, ash $6.15 \pm 0.20\%$, protein $24.24 \pm 2.13\%$, fat $7.72 \pm 0.26\%$, TOC $44.41 \pm 0.01\%$, C/N ratio 11.02 ± 1.23 and PEs 0.60 ± 0.06 mg/g dry sample.

PEs concentrations in seed cakes were lower than those of unshelled seed cakes from Nicaragua (1.78 mg/g dry sample) [2], and Zimbabwe (0.70 mg/g dry sample) [8], but they were higher than seed cakes from four provinces of Thailand (0.21-0.47 mg/g dry sample) [11]. This is possibly caused by various amounts of residual oil left in the samples and the variations of *J. curcas* in cultivation areas, soils, and climatic conditions ([4], [23], [26]).

Table 1. Chemical composition of *J. curcas* seed cakes.

Composition	Amount
pH	6.58 ± 0.02
Moisture (%)	5.92 ± 0.10
Ash (%)	6.15 ± 0.20
Protein (%)	24.24 ± 2.13
Fat (%)	7.72 ± 0.26
TOC (%)	44.41 ± 0.01
C/N ratio	11.02 ± 1.23
Phorbol ester (mg g ⁻¹ dry sample)	0.60 ± 0.06
Means \pm S. D. (n=3)	

3.2. Degradation of Phorbol Esters and Enzyme Production by Submerged Fermentation

The *J. curcas* seed cake 20 g in 100 ml MSM medium was fermented with 5 *Bacillus* strains at 25°C. The *Bacillus* strains had initial cell numbers 10^4 - 10^6 CFU/ml and then increased to 10^8 - 10^{11} CFU/ml after 7 days incubation. While the PEs contents reduced 76.5%, 77.1%, 78.4%, 85.5%, and 92.0% with *B. smithii* G16, *B. sonorensis* D12, *B. licheniformis* A3, *B. subtilis* H8, and *B. coagulans* C45 for 3 days incubation, respectively (Figure 1).

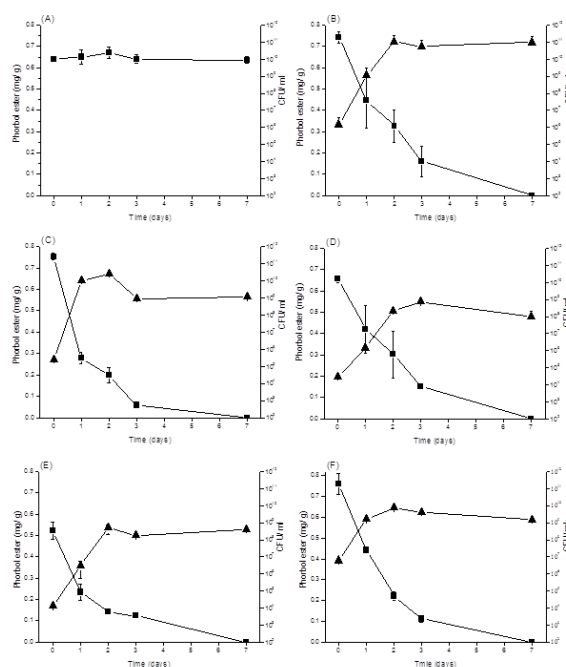


Figure 1. Phorbol esters contents (■) and *Bacillus* populations (▲) in *J. curcas* seed cake submerged fermentation. (A) Blank; (B) *B. licheniformis* A3; (C) *B. coagulans* C45; (D) *B. sonorensis* D12; (E) *B. smithii* G16; and (F) *B. subtilis* H8.

PEs contents in deoiled *J. curcas* seed cake were extracted by ethanol and analyzed by HPLC. The chromatogram (Figure 2) shows four major peaks of phorbol esters at retention times of 29.09-32.74 min, closely related to the reported by Makkar et al. [23]. PEs could be completely degraded by these five strains for seven days cultivation (Figures 1 and 2).

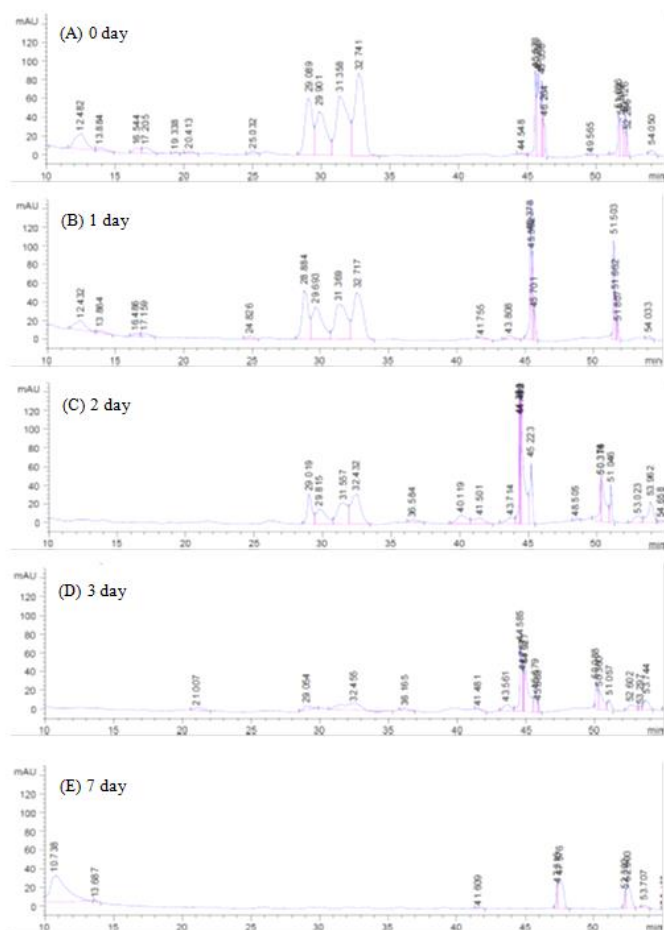


Figure 2. HPLC chromatograms of phorbol esters (PEs) biodegradation by *B. coagulans* C45 for 0-7 days (A-E).

Enzyme productions of *Bacillus* strains in SMF at 25°C were shown in Figure 3. *B. sonorensis* D12 had the maximum amylase production 5.49 ± 0.49 U/ ml for 7 days cultivation. *B. subtilis* H8 had the maximum cellulase production 17.03 ± 4.90 U/ ml for 2 days cultivation. *B. licheniformis* A3 had the maximum lipase production 59.03 ± 0.26 U/ ml for 7 days cultivation. *B. sonorensis* D12 had the maximum pectinase production 1.70 ± 0.04 U/ ml for 3 days cultivation. *B. coagulans* C45 had the maximum protease production 15.95 ± 0.35 U/ ml for 7 days cultivation. *B. smithii* G16 had the

maximum xylanase production 1.40 ± 0.0 U/ ml for 3 days cultivation.

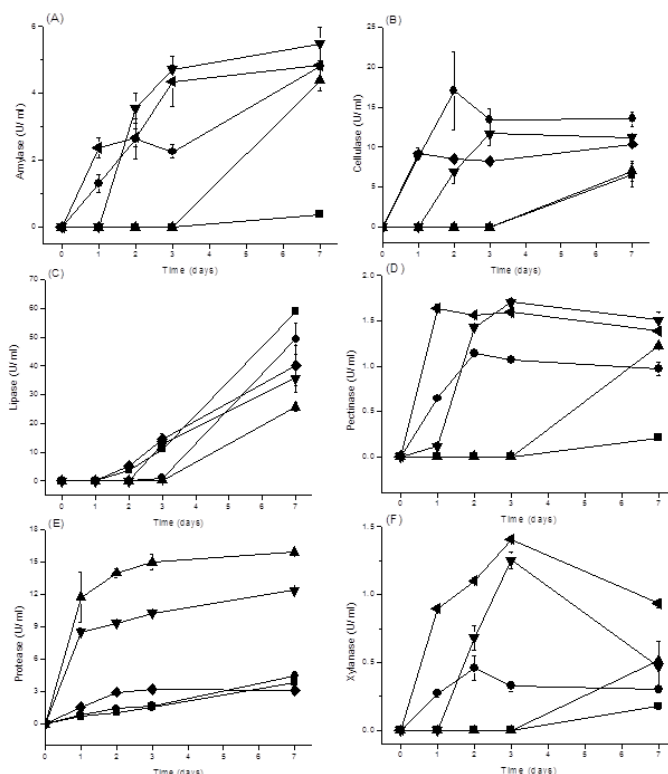


Figure 3. Enzyme production of *J. curcas* seed cake with submerged fermentation by *Bacillus* spp. (A) Amylase, (B) Cellulase, (C) Lipase, (D) Pectinase, (E) Protease, (F) Xylanase. A3 (■), H8 (●), C45 (▲), D12 (▼), and G16 (◆).

3.3 Degradation of Phorbol Esters and Enzyme Production by Solid-state Fermentation

For SSF, 20 g seed cake with 50% initial moisture content at 25°C was fermented with 3 *Bacillus* spp. (H8, D12 and G16) had high enzyme production in SMF. The *Bacillus* strains had initial cell numbers 10^4 - 10^6 CFU/ g and then increased to 10^8 - 10^{11} CFU/ g after 7 days incubation. The PEs contents in seed cake reduced 86.0%, 83.2% and 93.0% with *B. sonorensis* D12, *B. subtilis* H8, and *B. smithii* G16 for 3 days incubation at 25°C, respectively (Figure 4).

The enzyme productions of tested microbes increased with cultivation and had the maximal production for 2-4 days cultivation (Figure 5). *B. smithii* G16 had the maximum amylase production 16.08 ± 0.36 U/ g for 4 days cultivation. *B. sonorensis* D12 had the maximum cellulase production 2.94 ± 0.06 U/ g for 2 days cultivation. *B. smithii* G16 had the maximum lipase production 3.87 ± 0.64 U/ g for 4 days cultivation. *B. sonorensis* D12 had the maximum pectinase production

8.13 \pm 1.06 U/ g for 2 days cultivation. *B. smithii* G16 had the maximum protease production 14.13 \pm 0.30 U/ g for 4 days cultivation. *B. smithii* G16 had the maximum xylanase production 9.72 \pm 0.97 U/ g for 3 days cultivation.

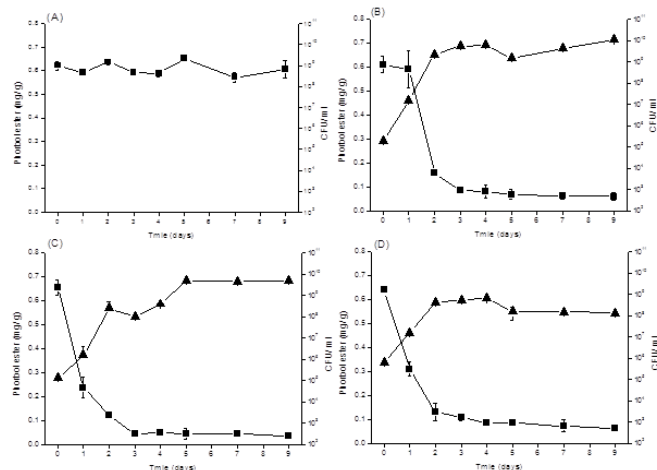


Figure 4. Phorbol esters contents (■) and *Bacillus* populations (▲) in *J. curcas* seed cake by solid state fermentation. (A) Blank; (B) *B. sonorensis* D12; (C) *B. smithii* G16; (D) *B. subtilis* H8.

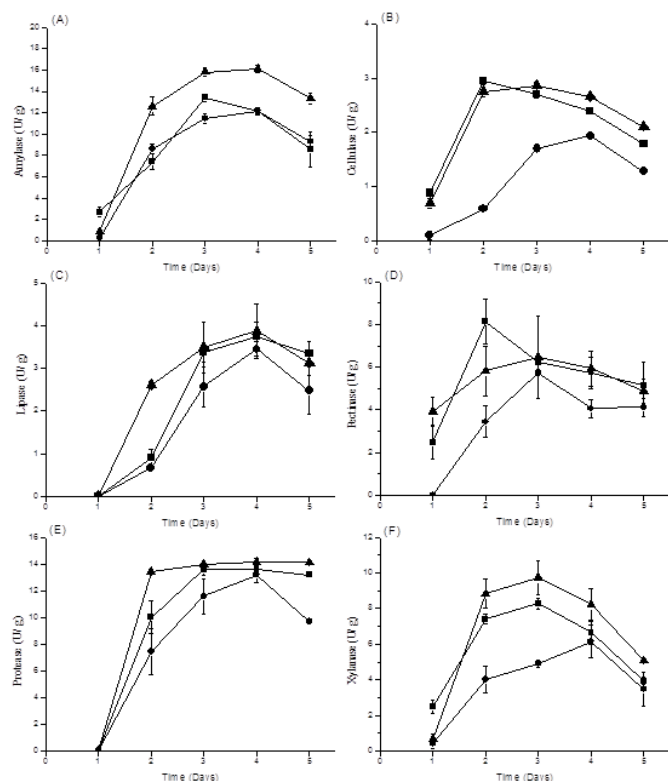


Figure 5. Enzyme production of *J. curcas* seed cake with solid-state fermentation by *Bacillus* spp. (A) Amylase; (B) Cellulase; (C) Lipase; (D) Pectinase; (E) Protease; (F) Xylanase. D12 (■), H8 (●), and G16 (▲).

The world market of industrial enzymes is estimated to be 1.6 billion \$US, including food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%). *Bacillus* can secrete a variety of soluble extracellular enzymes [27] and most of *Bacillus* species are considered as GRAS. It is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market around the world [25]. Therefore, *Bacillus* is a good candidate microbe for enzyme production of *J. curcas* seed cake. Each *Bacillus* strain in this study has different capacities of enzyme production, and can be applied by SMF or SSF for enzyme production in the future.

Phengnuam and Suntornsuk [26] reported that *B. licheniformis* and *B. subtilis* could degrade PEs 60% and 40% for 5 days incubation with SMF. In this study, 5 *Bacillus* strains can degradation PEs 76.5-92.0% for 3 days incubation and 100% for 7 days incubation by SMF. Joshi et al. [17] reported that *Pseudomonas aeruginosa* degraded 60-73% phorbol esters in the seed cake for 6 days incubation with SSF, and Belewu and Sam [4] also reported that *Aspergillus niger* degraded 77% phorbol esters in the seed cake for 7 days incubation by SSF. In this study, 3 *Bacillus* strains can degrade PEs 83.2-93.0% for 3 days incubation by SSF. Both of SMF and SSF can be used for PEs degradation [26].

Jatropha seed oil has been used extensively for biodiesel production. The residue of *J. curcas* seed cake can be used as substrate in SMF or SSF for production of useful substances. In SMF, Choudhury et al. [9] used *J. curcas* seed cake as nutrients for pullulan production by an osmotolerant yeast *Aureobasidium pullulans*. Phengnuam and Suntornsuk [26] used *J. curcas* seed cake as nutrients for protease, phytase, and esterase production by *Bacillus licheniformis*. In SSF, different kinds of microbes like *Aspergillus niger*, *Aspergillus oryzae*, *Pseudomonas aeruginosa*, and *Scytalidium thermophilum* were used for xylanase, cellulase, protease, and lipase production ([18], [21], [24], [33]). From this study, it showed that SMF and SSF could degrade PEs efficiency, and the high-protein seed cake could be used for enzyme production or other valuable products production in the future.

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