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UTILIZATION OF ORANGE BAGASSE AND MOLOKHIA STALK FOR PRODUCTION OF PECTINASE ENZYME

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Abstract - Studies were conducted on the production and extraction of exo-polygalacturonase (PG) in solid-state fermentation (SSF) using orange bagasse (Ob) and molokhia stalks (Ms) as a new solid support by *Penicillium pinophlilum* Hedg 3503 NRRL. The parameters affecting PG production under SSF were optimized. The maximum PG activity of ~ 3270 U/ g dry solid substrate was obtained from *P. pinophlilum* Hedg 3503 NRRL grown for 7 days on Ob and Ms in the ratio 1:3 (w/w), moistened with distilled water at 68.2% initial moisture content. Highest enzyme titers occurred in SSF without added nutrients, indicating nutrient sufficiency of the Ob and MS mixture to sustain growth and a high level of pectic substances which induced PG production. The extraction of PG from the fermented biomass was optimized. Among the various solvents tested, the maximum level of enzyme activity was achieved when acetate buffer (0.05 M; pH 5.0) was used. The optimum volume of buffer was 50 mL, an extraction time of 60 min was sufficient to extract most enzyme, which yielded 3269.6 U/ g dry solid substrate of enzyme activity. Repeated washes under the optimum conditions showed that most of the enzyme (about 98%) was recovered in three repeated extractions. Moreover, 68.9% of total activity was achieved in the first wash.
Keywords: Exo-polygalacturonase; Solid-state fermentation; Solid substrate.

INTRODUCTION

Pectins are high molecular weight acid polysaccharides, primarily made up of α-(1→4) linked D-galacturonic acid residues with a small number of rhamnose residues in the main chain and arabinose, galactose and xylose in the side chain (Kumar *et al*., 2011). Pectinases constitute a group of enzymes which degrade the pectin present in most plants. Although several types can be found, polygalcaturonases are the most abundant and studied ones, representing around 25% of industrial enzymes sales (Jayani *et al*., 2005). Pectinase production has been reported from microorganisms including actinomycetes (Beg *et al*., 2000), yeast (Blanco *et al*., 1999) and fungi (Kumar *et al*., 2011). Pectinases are of significant importance in the current biotechnological area with their all-embracing applications in fruit juice extraction and clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives and in the alcoholic beverage and food industries (Jayani *et al*., 2005; Jacob and Prema, 2006; Kumar *et al*., 2011). The genus Penicillium is known worldwide for production of secondary metabolites and extracellular enzymes of commercial value, including pectinases (Banu *et al*., 2010). Solid state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence (or near absence) of free water (Sharma *et al*., 2008). This technique offers the possibility of processing agro-

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industrial residues that can be used, for instance, as substrates for enzymes production, helping in this way to minimize the pollution by removing this waste and converting it to useful products. In Egypt more than a hundred orange juice factories are widespread and produce at least 40,000 tons of orange pulps and peels annually, as highly wet wastes (El-Sheekh *et al*., 2009). Utilization of orange-canning wastes was reported by some authors (Pagán *et al*. 2006; El-Sheekh *et al*. 2009). Orange bagasse is rich in pectic substances (50.4%), so it is a good inducer of PG production. On the other hand, molokhia (Arabic common name) or Jew's Mallow (English common name) stalks agro waste from the plant (*Corchorus olitorius*) is cheap and abundantly available in Egypt. As far as we are aware, nothing has yet been reported on the use of molokhia plant in SSF. The aim of the current study was to investigate the production and extraction of exo-polygalacturonases by solid state fermentation using a mixture of orange bagasse (pulp and peels) and molokhia stalks.

MATERIALS AND METHODS

Microorganism

Penicillum pinophlilum Hedg 3503 NRRL used in this study was obtained from Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA. It was propagated on Potato –Dextrose-Agar (PDA) slants at 35 °C and transferred monthly. For short-term storage, slants were maintained at 4 °C.

Inoculum

For inoculum preparation, the microorganism grown on PDA for 5 days at 35 °C was suspended in 20 mL sterilized distilled water in a 100 mL flask (under agitation with a magnetic stirrer).

Preparation of Different Solid Substrates

Orange bagasse (pressed mixture of pulp and peels), molokhia stalks, lemon peels, pea peels and watermelon peels were obtained from the local market in Egypt, cut into small pieces (~2 mm long), washed with tap water several times in order to remove all water-soluble compounds and finally dried at 50 °C for 48 h. Corn cobs, rice straw, sawdust and onion skins were dried and ground to about 2 mm in particle size. Wheat bran was dried and used in the fermentation process.

Solid State Fermentation

It was carried out using a 250-mL Erlenmeyer flask containing 2 g from each previous solid substrate moistened with 15 mL of distilled water (initial pH 5.0) before sterilization. The production medium was inoculated with 1 mL of spore suspension (prepared as described above). The flasks were incubated in a rotary shaker (150 rpm) at 35 °C for 7 days.

Moisture Content

A known weight of each sample (dry orange bagasse or molokhia stalks) was heated at 105 °C till constant weight, the values were calculated on the dry basis (Ismail, 1995).

Ash Content

This was carried out according to the method of Abdel-Fattah *et al*. (1976). It was determined by heating solid substrate to constant weight at 800 °C.

Pectic Substance

It was determined according to the method of Abdel-Fattah *et al*. (1976).

Enzyme Extraction

This was done according to the method of Xiros *et al*. (2008). After the fermentation period, the enzyme was extracted by suspending the fermented material (10 g) in 50 mL distilled water (1:5 w/v) and shaking (150 rpm) for 1 h at 35 °C. Following extraction, the suspended materials and fungal biomass were separated by centrifugation at 5000 rev/min for 20 min at 4 °C. The clarified supernatant was used as the source of the crude enzyme.

Assay of Polygalacturonase Activity

This was performed according to the method of Silva *et al*. (2005). Exo-polygalacturonase (exo-PG) activity was evaluated by mixing 0.2 mL of enzymatic extract and 0.8 mL of citrus pectin solution (0.5% w/v pectin in 0.05 M acetate buffer, pH 5.0). Samples were incubated at 50 °C for 10 min and the reducing sugar was determined by Somogyi method (1952). One unit of exo-PG activity was defined as the amount of enzyme releasing 1 μ mol of reducing sugars (as galacturonic acid) per min per mL of enzyme solution.

RESULTS AND DISCUSSION

Enzyme Production by Solid State Fermentation

Polygalacturonase (PG) is generally considered to be synthesized in the presence of an inducer. Due to the composition of orange bagasse (Ob), which contains pectin substance (50.4%), protein (5.1%), ash (4.0%) and moisture (4.0%), it was selected and tested initially alone as a solid substrate for the production of PG. *Penicillium pinophilum* Hedg NRRL 3503 was capable of growing on Ob as substrate in SSF, and produced PG in the absence of supplemented nutrients only with distilled water as moistening agent. Enzyme production by SSF in a rotary shaker (to prevent the solid substrate from agglomerating) was analyzed during 11 days, indicated that maximum PG activity (82.23 U/ g dry solid substrate) was obtained after 7 days, thereafter the enzyme activity declined (data not shown). Similar observation was reported for the production of pectinase and cellulase by fungi on SSF (Desgranges and Durand, 1990). However, when the time was increased or decreased to other than 7 days, the production of PG gradually decreased. This might be due to the fact that cultivation of fungi for an extra time could affect the pH of the medium, which may favour a limited growth rate and PG production by reducing accessibility of pectic substances (Arabi *et al*., 2011). The activity obtained in this study was very high compared to those reported for pectinolytic strains such as *Penicillium frequentans* (3.4 U/ g dry ss)*, Aspergillus niger* (25 U/ g dry ss), *Penicillium viridicatum* RFC3 (12 U/ g dry ss) and *Penicillium* sp. (64.5 U/ g dry ss), (Garzón and Hours, 1992; Castilho *et al*., 1999, Silva *et al*., 2002 and Patil and Chaudhari, 2010). The results showed that SSF was suitable for pectinase production by *P. pinophilum* Hedg NRRL 3503 using agricultural and agro industrial wastes. Orange bagasse has been used as a fermentable substrate to induce pectinase production by some authors (Silva *et al*., 2005 and El-Sheekh *et al*., 2009).

Orange Bagasse Loading

Study the effect of orange bagasse (Ob) quantity on the production of PG (with the same ratio of solid to liquid) is presented in Figure 1. The results indicated that 1g Ob was sufficient as a rich medium for the highest PG yield (139.13 U/ g dry ss), which represented 69.20% increase in enzyme production compared with control. Giese *et al*. (2008) found that orange bagasse at a solids concentration of 16% (w/v) was sufficient to sustain *Botryosphaeria rhodina* MAMB-05 and produced high pectinase titres.

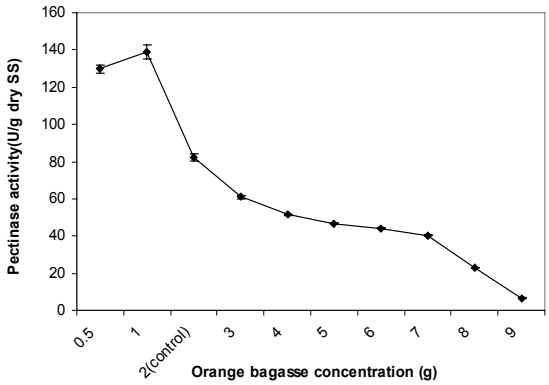
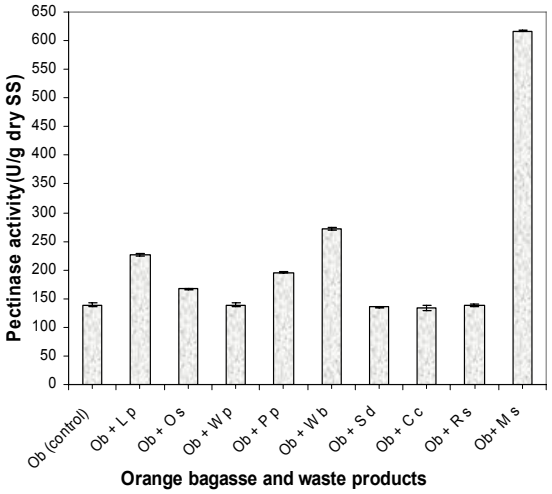


Figure 1: Effect of orange bagasse concentration on the production of pectinase enzyme by the solid state fermentation technique.

Supplementation with Natural Substrates

Orange bagasse constitutes an excellent inducer substrate for PG production by *P. pinophilum* Hedg NRRL 3503 under SSF. It was supplemented with an equal amount of different natural substrates, i.e., lemon peels (Lp), onion skins (Os), watermelon peels (Wp), pea peels (Pp), wheat bran (Wb), sawdust (Sd), corn cobs (Cc), rice straw (Rs) and molokhia stalks (Ms). Among all agricultural wastes and agro-industrial by-products tested (50% w/w), the Ob and Ms mixture was the most suitable for maximal PG production (616.86 U/ g dry ss) without nutrients addition. However, it is interesting to notice that no previous studies have been reported on enzyme production using Ms as solid substrate. Besides, the efficiency of enzyme production depended on the bare chemical composition of the raw material, accessibility of various components and their chemistry or physical association. Molokhia plant is an annual herb in the Middle East and dry samples are rich in mineral salt (16.45%) such as iron, phosphorus, calcium, sodium, magnesium, potassium, etc. Moreover, Molokhia contains pectic substance (21.20%), protein (22.0%), ash (18.0%), moisture (10.0%), fat, fiber and vitamins (vitamins A, B …etc). From the results presented in Figure 2, it may be concluded that addition of some agricultural wastes to Ob improved the production of PG. Silva *et al*. (2002) reported that, among all agricultural wastes and agro-industrial by-products, an orange bagasse and wheat bran mixture 50% (w/w) was the most proper for the maximal pectin lyase production in SSF cultures of *Penicillium viridicatum* Rfe3.



Lemon peels (Lp), onion skin (Os), watermelon peels (Wp), pea peels (Pp), wheat bran (Wb), sawdust (Sd), corn cob (Cc), rice straw (Rs) and molokhia stalk (Ms).

Figure 2: Effect of mixing of orange bagasse with different waste products (0.5/ 0.5 w/w) on the production of pectinase enzyme.

Effect of Different Ob and Ms Ratio

In the comparative studies, mixed substrate fermentation was carried out using Ob and Ms with different ratios (Figure 3). The mixture which contained one part of Ob and three parts of Ms showed a significant increase in enzyme yield (111.94%).

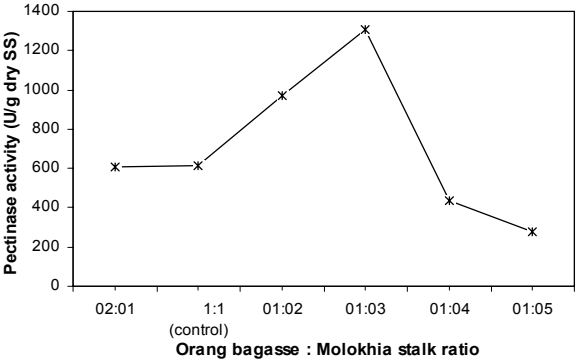


Figure 3: Effect of different ratios (w/w) of orange bagasse and molokhia stalks on the production of pectinase enzyme by the SSF technique.

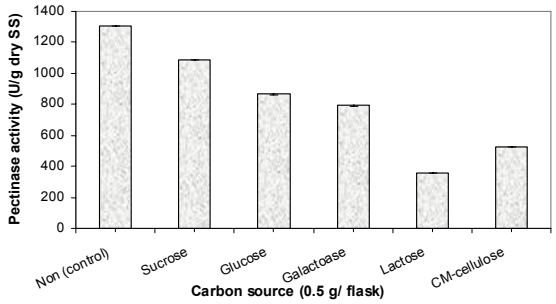
The production of PG was enhanced up to 2.12 times when one part of Ob and three parts of Ms was used as carbon source compared with the control (1Ob :1 Ms). Increasing the Ms quantity stimulated fungal growth and PG production, this result may be due to its composition (pectic substance, protein, mineral salts, vitamins, etc.). High levels of exo-PG, xylanase or CMC-ase activities can be obtained using a mixture of grape pomace and orange peels (1:1) in SSF (Díaz *et al*., 2012).

Effect of Supplementation with Synthetic Carbon Sources

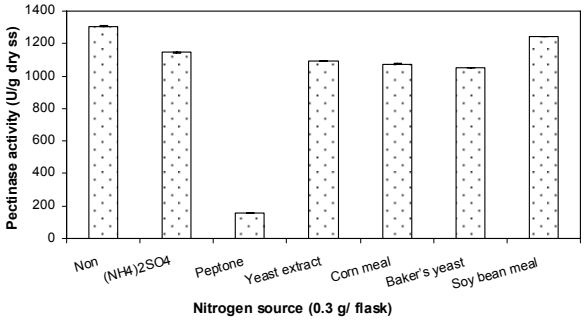
Addition of different carbon sources (sucrose, glucose, galactose, lactose and carboxymethyl cellulose (CM-cellulose) on an equal carbon basis was studied and highlighted in Figure 4(a). The enzyme production was strongly repressed in the presence of lactose, showing only 27.34% relative activity. Generally, all C-sources tested did not enhance enzyme production, this might be due to the effect of inducible substrate type (Ob and Ms) on the production of PG by the fungal strain. On the other hand, supplementation with other C-sources repressed the production of PG, which is induced and controlled by a feedback mechanism. The supplementation of the production medium with glucose or CM-cellulose resulted in a marked decrease in enzyme activity, reaching about 33.81 and 59.84%, respectively of PG as compared to the control. This result is in accordance with similar observations of Phutela *et al*. (2005), who supplemented SSF with different C-sources (glucose, CM-cellulose and galactose). They mentioned that PG was strongly repressed by the addition of these C-sources. However, this result is in contrast to that obtained by Solis-Pereira *et al*. (1993), who reported that supplementation of a culture medium containing pectin with glucose, sucrose or galacturonic acid (up to 10%) did not reduce PG production.

Effect of Supplementation with Nitrogen Sources

The results in Figure 4(b) showed that supplementation with nitrogen sources, on an equal nitrogen basis, did not enhance the fungal growth and hence the production of PG enzyme. The proteins in Ms and Ob (22.0 and 5.1% in dry samples, respectively) provide the production media with the nitrogen needed for fungal growth and enzyme production. On the other hand, enzyme production was strongly inhibited by 88.0% in the presence of peptone. Phutela *et al*. (2005) reported that the presence of yeast extract and ammonium sulfate supported maximal production of pectinase, followed by malt sprouts and ammonium sulfate. Kumar *et al*. (2011) showed that ammonium sulfate was the best inducer of pectinase in *Aspergillus niger* MCIM 548 using SSF process.



(a)



(b)

Figure 4: Effect of supplementation (a) with synthetic carbon sources on an equal carbon basis; (b) with different nitrogen sources on an equal nitrogen basis on the production of pectinases by *P. pinophilum*. The control culture in both cases was Orange bagasse and Molokhia stalks (ratio 1:3 w/w).

Effect of Initial Moisture Content on Enzyme Production

An optimum moisture level has to be maintained as lower moisture tends to reduce nutrient diffusion, microbial growth, enzyme stability and substrate swelling (Xiros *et al*., 2008). The existence of an optimum moisture content of the medium has profound effects on growth kinetics, and on the physicochemical properties of solids, which in turn affects productivities (Lonsane *et al*., 1992). The moisture level of 68.2% yielded the highest PG activity of 1307.37 U/g dry ss (data not shown). This result is near to that obtained by Kumar *et al*. (2011) for optimum production of *Aspergillus niger* NCIM 548 pectinase using SSF with 70% moisture content. A high moisture content has negative consequences for growth, as the porosity of the medium and oxygen diffusion were reduced when lignocellulosic substrates were used as the C-source in SSF (Silva *et al*., 2005). As reported by Xiros *et al*. (2008), moisture content affects heat and mass transfer inside the culture. Higher moisture content may influence O_2 transfer rates by affecting the bed depth and lead to particle agglomeration of the moist fermenting solids. However, low moisture levels are known to decrease the metabolic and enzymatic activity, probably due to reduced solubility of nutrients from the solid substrate, low substrate swelling and higher water tension (Uyar and Baysal, 2004, Arabi *et al*., 2011).

Effect of Solvent Type on Enzyme Extraction

The extraction efficiency is critical to the recovery of the enzyme from the fermented biomass, hence selection of a suitable solvent is necessary. Different solvents selected for this study were acetate buffer, Tween 80, Tween 40, glycerol and polyethylene glycol (PEG). It is clear that among all the extractants employed (Figure 5), acetate buffer (0.05 M; pH 5.0) gave the best result and was found to be efficient in recovering PG from fermented solid (about 2.44 times as compared with control). Our results are in agreement with the observation of Castilho *et al*. (2000), who also found that acetate buffer (pH 4.4) extracted pectinases from wheat bran by *Aspergillus niger* better than glycerol or distilled water. Adsorption of the enzyme to cells or solid substrates has been attributed to ionic bond, hydrogen bond and Van der Waal's forces (Agrawal *et al*., 2005). Extraction increased with buffer as compared to distilled water probably due to the salting-in effect of the salt. Some enzymes produced under SSF have been recovered from the solid phase by treatment with distilled water (Silva *et al*., 2005; Patil and Chaudhari, 2010), de-ionized water (Giese *et al*., 2008), surfactant Tween 80 (Rezende *et al*., 2002) and buffer (Rezende *et al*., 2002; Phutela *et al*., 2005; Linde *et al*., 2007).

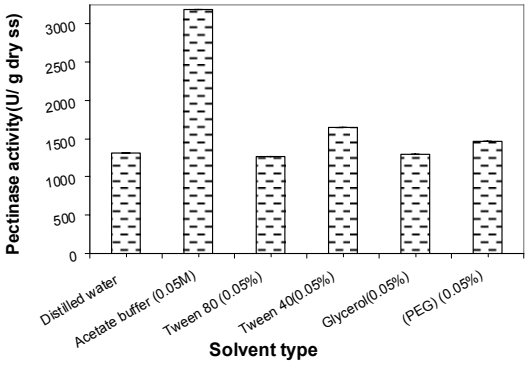


Figure 5: Effect of solvent type on the extraction of pectinase enzyme from fermented material (Ob and Ms).

Effect of Buffer Molarity on Enzyme Extraction

Extraction was performed with acetate buffer (pH 5.0) of varying molarity as extractant. The recovered activity increased with increasing molarity up to 0.05 M (data not shown). Above and below this value the enzyme yield was negatively affected due to the salting-in effect of the salt. Aikat and Bhattacharyya (2000) reported that the extraction of protease from fermented wheat bran by *Rhizopous oryzae* increased with potassium phosphate buffer molarity.

Effect of Buffer Volume on Enzyme Extraction

The volume of buffer (0.05 M; pH 5.0) was varied from 25 to 200 mL for 10 g of fermented material (data not shown). It was found that the amount of extracted enzyme increased with the increase of buffer volume. Enzyme activity increased up to 100 mL/ 10 g fermented material, where the PG recovery was about 1.03 times that using 50 mL buffer). More buffer released more enzyme from the fermented material. The solvent volume must be sufficient for complete enzyme extraction. Excessively large volume of buffer used for greater extraction would also yield an enzyme solution too dilute to be profitably utilized (Aikat and Bhattacharyya, 2000). There was a decrease in total activity when a lower volume of buffer was used for extraction. This might be due to insufficient solvent volume to penetrate the solid fermented mass.

Effect of Soaking Time on Enzyme Extraction

Soaking of the fermented substrate with acetate buffer (10 g / 100 mL) was done for different periods, varying from 30 to 150 min. The maximum amount of PG was extracted after 60 min (Figure 6). A longer soaking period did not result in a significant gain in recovery. Other studies reported that the time required to remove enzymes from a solid substrate was ranged from 30 min (Castilho *et al*., 2000) to 48 h under shaking conditions (Shata, 2005).

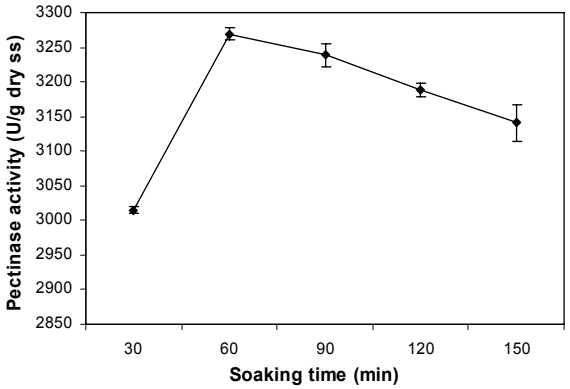


Figure 6: Effect of soaking time on the extraction of pectinase enzyme from fermented material (Ob and Ms).

Repeated Extractions of PG Enzyme

Repeated extractions were carried out more as a confirmatory test in order to determine whether most of the PG could be recovered in one extraction. This parameter was optimized by adding a fresh aqueous extractant after the extraction for each wash. Four consecutive extractions were performed and the results are presented in Table 1. About 68.93% (326.96 U/ mL) of total activity was obtained from the first extraction. Considering that 100% of the enzyme could be extracted with only four extractions, 97.79% (463.86 U/ mL) of PG was recovered from fermented material during three extractions. Shata (2005) reported that four repeated extractions are sufficient for maximum enzyme recovery from solid fermented medium.

Table 1: Repeated extraction of pectinase enzyme from fermented material (1: 10 w/v) ratio.

Number of recovery stage	Pectinase activity (U/ mL)	Pectinase activity (U/ g ss)	Pectinase activity (U/ g ss) %	Cumulative enzyme activity (U/ mL) %	Cumulative enzyme activity (U/ g dry ss)	Cumulative enzyme activity (U/ mL) %
First washing	326.96	3269.56	68.93	326.96	3269.56	68.93
Second washing	85.09	850.89	17.94	412.05	4120.45	86.86
Third washing	51.81	518.07	10.92	463.86	4638.52	97.79
Fourth washing	10.50	105.00	2.21	474.36	4743.52	100.00

CONCLUSIONS

In conclusion, we report the successful utilization of the agro industrial wastes Orange bagasse (Ob) and Molokhia stalks (Ms) for the production of polygalacturonase (PG) from *P. pinophlilum* Hedg 3503 NRRL under solid state fermentation technique in the absence of supplemented nutrients, only with distilled water as moistening agent. To the best of our knowledge, this is the first report on using molokhia stalks as a solid substrate for PG production. A mixture of Ob and Ms (1:3 ratio) is the most suitable substrate for fungal growth and PG induction due to its composition of pectic substance. Optimization of the production and extraction parameters increased the enzyme yield from 82.23 to 3269.60 U/ g dry ss. Most of the enzyme (about 98%) was recovered by three repeated extractions. Moreover, 68.9% of the total activity (326.9 U/ mL) was obtained in the first wash. It is therefore important to use agro industrial wastes like Ob and Ms as low-cost fermentable substrates in the biotechnological sector.

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