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Screening and Molecular Identification of a Potential Fungal Strain for Polygalacturonase production under Solid State Fermentation

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Abstract

The microbial enzymes utilization has found broad application in technological processes of different industrial. The aim of this work was to evaluate polygalacturonase production by fungi isolated from soil. Screening of three hundred and thirty-six fungal isolates for polygalacturonase production were conducted. 206 were positive in assay plates for pectin depolymerization as evidenced by clear hydrolyzation halos. Among them, 11 presenting considerable pectinolytic activity were cultivated in solid-state fermentation for polygalacturonase production. The FSS62 isolate was the best polygalacturonase producer. The phylogenetic analysis based on 5,8S gene sequence similarities for the FSS62 isolate was *Penicillium chrysogenum* with 98 % homology. The cultivation of the FSS62 isolate on agricultural wastes resulted in high quantities of polygalacturonase (403 IU/g) using beet pulp after 72 hours of incubation. The enzyme was optimally active at pH 5.0 and 50 °C.

Keywords: Filamentous fungi, polygalacturonase, solid-state fermentation

1.Introduction

The pectic substances degradation by catalyzing of pectinolytic enzymes are of great importance in industry. Pectinolytic enzymes are a group of heterogeneous group enzymes including pectin lyase, pectin methyl esterase and polygalacturonase that hydrolyze the glycosidic bonds in pectic substances [24].

The production of pectinase occupies about 10% of the overall of enzyme preparations manufacturing. Today, the application of pectnolytic enzymes plays an important role in food technology for the fruits and vegetables maceration, as well as for clarification after extraction and concentration of their juices [10].

Polygalacturonases are the particular interest enzymes to industry because their action on pectin, hydrolyzing its internal and external bonds of glycosides, producing short molecular structures of pectin, decreasing the viscosity, increasing of the juices yield, and determining the appearance of crystalline for the final product [5].

Solid-state fermentation (SSF) can be used for the production of industrial microbial enzymes [17, 7]. Microbial growth and formation of the product usually occur on the surface and inside of solid substrate particles with low moisture content; hence SSF technique appears to be advantageous for the production of microbial enzyme. The advantages of SSF process over the submerged fermentation (SmF) process include higher yield of products [29], generation of less effluents and requirement of simpler equipment [2].

The microbial enzymes utilization have gained interest technological application in various industrial processes. Among the several enzymes commercialized, many are produced by the filamentous fungi fermentation [25, 11].

The objective of this work is to screening fungal strains isolated from soil for polygalacturonase production, improving the fermentation process conditions for the production of polygalacturonase by the best isolates selected.

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2.Materials and Methods

Fungus Isolation. Samples were collected from the soil covering diverse areas of Syria, forests, gardens, olive fields and cereal fields. One gram from each soil sample was dissolved and diluted to $1/10^4$ time with sterilized distilled water. Then, 0.1 ml volume of each dilution was pipetted onto plate of potato dextrose agar (PDA) and incubated at 30 ºC for three days. Fungi were isolated from each plate and subcultured on PDA. This process was continued until obtained pure isolates. Pure cultures were maintained on potato dextrose agar at 4 ºC.

Screening of polygalactoronase producing Fungi. A total of three hundred and thirty-six fungal isolates were screened for polygalactoronase activity. 25 µl of the prepared spores suspension from each fungal isolate was aliquoted onto pectin agar plates, incubated at 30°C for 5 days and screened for polygalactoronase detection. Positive polygalactoronase isolates were selected based on the clear zones of hydrolysis on the pectin. The best 11 isolates, having a larger clearing zone (more than 50 mm) were grown in solid-state fermentation and the produced amount of polygalactoronase was determined from the extract culture filtrate. Subsequently, the selected isolates were grown on PDA slants at 30 ºC and maintained at 4 ± 1 ºC and subcultured at every 15-day.

Solid-state fermentation. Selected isolates of fungi were checked for enzyme production in solid-state fermentation using wheat bran procured from local market. The selected isolates cultivation were carried out in 250 ml Erlenmeyer flasks containing 5 gm of wheat bran and nutrients plus distilled water to adjust the moisture to 75%. The fermentation medium consisted of: (gm/L) $Na_2HPO_4.7H_2O$ 9.06; NaCL 0.3; $MgSO_4.7H2O$ 0.09; and yeast extract 4.5 and peptone 3.5, as nitrogen source. 1 ml of the prepared spore suspension from each of the selected fungal isolates was transferred into the solid medium and placed in the incubator. After 5 days of cultivation, flasks were removed and the enzyme was extracted by adding 25 mL of solution consist of distilled water and 0.1% Triton X 100. The flasks contents were stirred for 1.5 hours on a magnetic stirrer. The clear supernatant was obtained by centrifugation (9800g for 15 min) used as enzyme source.

Enzymatic assays. Assay of polygalacturonase (PG) activity according to Marcia et al., [21]. PG activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent (DNS) assay [23]. The reaction mixture, containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture supernatant, was incubated at 40ºC for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid release per minute.

Effect of pH and temperature on polygalactoronase activity. The optimal enzyme activity pH was determined by changing the pH of assay reaction mixture using the following buffers (0.1 M): sodium acetate (pH 5.0), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 8), glycine–NaOH buffer (pH 9–10) and 1% soluble pectin as substrate. The enzyme activity was evaluated by measuring the polygalctoronase activity at different temperatures (40–80°C).

Selected isolates identification

Genomic DNA isolation. Total genomic DNA of selected isolates was extracted from 48 h - growing cultures in medium (2% glucose, 1 % yeast extract and 1 % peptone). 1.5 ml of cultured cells was collected by centrifugation at 17950 g for 5 minutes. Cells were washed with distilled water and digested by 750µl enzymatic lyses solution (10 µl of proteinase k 20 mg/ml, 2% SDS, 1% 2-mercaptoethanol, 1% CTAB and 10 mM EDTA in 50 mM Tris pH 8 buffer) and incubated at 60 °C for 30 minutes. The lysate was extracted with phenol: chloroform: isoamyl alcool (25:24:1) three times. The extract was purified by adding 1/10 of sodium acetate 3M volume and 1 ml ethanol. The mix was vortexed and placed on ice for 15 minutes. High molecular weight DNA sediment was obtained after 5 minutes of centrifugation at 17950 g was washed with 70% ethanol and air-dried. The final DNA pellet was dissolved in 50µl hydration solution and stored at -20°C. DNA concentration was estimated by measuring the absorbance at 260 nm. The quality of the isolated genomic DNA was calculated by the ratio OD_{260nm}/OD_{280nm}.

PCR amplification and sequencing of the 5, 8 S rDNA. Primers used for PCR and DNA sequencing are ITS1 (5'-TCC GTA GGT GAA CCTGCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TATGC-3').

PCR amplicons were purified using Microcon Y-100 filters (Millipore) and sequenced using ABI Prism® Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to manufacturer's instructions. The products of sequencing were purified by ethanol precipitation. Sequencing reactions were carried out on an ABI PRISMATM DNA Sequencer. The sequences obtained (Approximately 500 bp length) were assembled in silico (Vector NTI) using overlapping zones to form contiguous sequence.

Phylogenetic analysis. Phylogenetic analysis was realized by consensus sequences alignment of 5,8S genes collected in Gene Bank (An international database). Then, the resultants were expressed in homology percentage between the submitted sequence and the sequences of most relevant from the database.

3.Results and discussion

Screening of polygalactoronase producing fungus

The fungal isolates from soil samples taken from different sites of Syria, forests, cereal fields, olive fields, desert and gardens were evaluated for polygalactoronase activity on agar pectin plate. These strains were tested for pectin hydrolysis by plate assay, at pH 6.0. Two hundred and six were positive in assay plates for pectinase depolymerization as evidenced by clear hydrolyzation halos.

Table 1. Production of polygalacturonase by Fungal isolates under solid-state fermentation.

Fungi isolates	Clearing zone diameter (mm)	Polygalacturonase (IU/g)
FSS7	51	134
FSS 60	52	94
FSS 62	50	158
FSS 114	52	92
FSS 117	54	125
FSS216	50	100
FSS225	53	108
FSS253	55	127
FSS278	52	118
FSS283	53	110

Among them, 10 presented hydrolyzing zone diameter of more than 50 mm were selected and cultivated in solid-state fermentation for polygalacturonase production. Of those 10 isolates, eight were able to produce > 100 IU/g of wheat bran (table 1). Table 1 shows the diameter of hydrolyzing zone and the level of polygalactoronase production in solid-state fermentation for the eight isolates. From this group, the highest producing polygalactoronase isolate, FSS62 was selected for further studies.

Identification of the Selected Isolate

The methods of DNA sequence analysis are reproducible and rapid means of identification, therefore, they have been widely used [16]. Identification of FSS62 isolate was done using 5,8S DNA gene sequences. The search analysis of nucleotide BLAST similarity, based on 5,8S DNA gene sequence revealed that this isolate belongs to the genus *Penicillium*. The closest phylogenetic neighbour according to the 5,8S DNA gene sequence data for FSS62 was *Pencillium chrysogenum* with 98 % of homology.

Polygalacturonase production with different agro-wastes

Studies on polygalacturonase production by FSS62 was carried out using different agro-wastes including wheat straw, wheat bran, corn cobs hulls, beet pulp, soya been cake, cotton seed cake, orange pulp and orange peels. The results (Figure 1) show that beet pulp supported maximum polygalacturonase production (302 Ug-1) followed by wheat bran (170 Ug-1). The minimum activity of 22 Ug-1 of polygalacturonase and 51 Ug-1 of polygalacturonase was observed in wheat straw and cotton seed cake respectively. The results showed that beet pulp and wheat bran enhanced enzyme production as compared to other agricultural wastes. This might be due to the existence of essential nutrients in beet pulp and wheat bran that include vitamins, pectin, different proteins and other sugars contents [1]. It has been previously reported that wheat bran and beet pulp act as good substrates for production of polygalacturonase [14, 26]. Maller et al. [20] and Ketipally et al., [15] reported that orange peel as the best carbon source for polygalacturonase production activity on solid state fermentation.

Effect of incubation time on polygalacturonase production

Concerning enzymes production, it is important to find the best time to produce and extract the enzyme during the fermentation process [4]. To determine the period of optimum incubation for the high yield of enzyme production. *FSS62* was incubated up to 6 days. The maximum pectinase activity (444 U/g) was found at the 3th day of incubation (Fig. 2).

Similar results were reported for maximal production of polygalacturonase by *Aspergillus niger* [18] and by *Aspergillus fumigatus* ITCC 6915 [13]. The decrease in the activity after 72 h could be due to the depletion of nutrients in the medium, high fungal cells density and decreased in oxygen concentration. Low incubation time leads to low enzyme activity. It means that pectinase production activity is correlated with the incubation time, which was also found from other investigations. Earlier, Said *et al*. [27] have reported maximum pectinase activity in *Penicillium frequentans* after culturing for 48 h, whereas, Doughari and Onyebarachi [8] found the maximal production of polygalacturonase by *Aspergillus flavus* recorded on day 4 (96 h) of fermentation after which there was a decline.

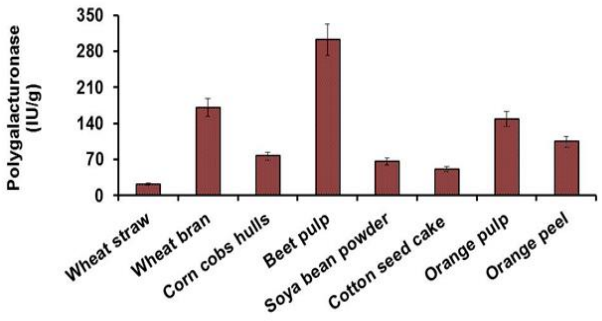

Figure 1. Effect of some agricultural wastes on polygalacturonase production from FSS62.

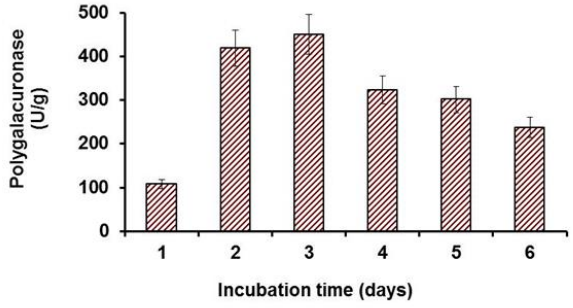

Figure 2. Effect of incubation period on polygalacturonase production from Penicillium FSS62

Effect of temperature and pH on polygalacturonase activity

Temperature and pH are two important indexes to influence the enzymatic activity. Crude polygalcturonases from *Penicillium* FSS62 were tested for the effect of temperature and pH for activity and stability. From the data shown in figures 3 and 4, Maximum polygalacturonase activity was found at pH 5.0 and 50 °C. Enzyme activity is clearly affected by pH [12, 13]**.** This is because catalysis bonding and substrates are often dependent on charge distribution on both, enzyme molecules and substrate. Previously, maximum polygalacturonase activity from *Penicillium* SPC-F20 has been reported with pH 5.5 [22]. Gupta and Lakhanpal [13] also reported that optimal polygalacturonase activity with pH 4.8. In another study, pH of 7.0 was reported to be optimum for maximum activity of polygalacturonase from *Penicillium ostreatus* [9]. An acidic pH of 4.0-5.0 was reported by Alana *et al.*,[3] to support high pectinase activity. Loudiere *et al.*, [19] reported, optimum activity for polygalacturonase at higher temperatures, 50-60ºC in *A. wentii* when grown on beet pulp.

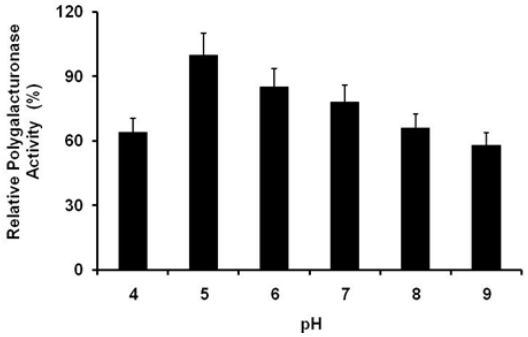

Figure 3. Effect of pH on the activity of polygalacturonase produced from Penicillium FSS62

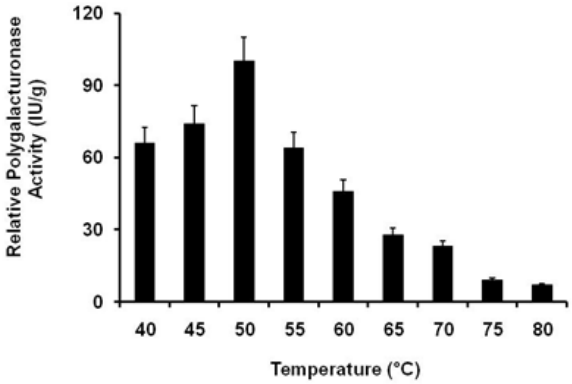

Figure 4. Effect of temperature on the activity of polygalacturonase produced from Penicillium FSS62

4. Conclusions

PG production by *Penicillium* FSS62 was optimized applying plant wastes, such as wheat bran and beet pulp which used in SSF. This study demonstrated a great potential for cost-efficient production of pectinolytic enzyme by Penicillium FSS62.

Production in laboratory scale under optimized conditions yielded high exo-PG activity and beet pulp was identified as significant pectinase inducer substrate.

Utilization of agro-industrial and agricultural by-products developed an attractive sustainable bioprocess for enzyme production. High enzyme yield obtained by *Penicillium* FSS62 under optimized conditions will be a promising starting point for scale-up and polygalacturonase purification studies.

Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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