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Exopolygalacturonase Production from Jojoba mill Solid Waste by *Aspergillus oryzae* FK-923 under Solid State Fermentation.

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ABSTRACT

Among all ten tested fungi, *Aspergillus oryzae* FK-923 was the most favorable strain for producing exopolygalacturonase (PGase) enzyme when cultivating on jojoba mill solid waste (JMSW) under solid state fermentation. Culture conditions, i.e., moisture content, initial pH value, temperature, incubation time were optimized to be 70 % (v/w), 5.0, 30^{o}C and 72 hrs respectively. The enzyme activity production was enhanced when urea was added as a nitrogen source to fermentation medium at level of 5.5 mg/g substrate. Under the above conditions, 656.6 U/g original JMSW was attained. Enzyme has optimum activity in range from pH 4.5-5.5 and optimum temperature was 50^{o}C. Eighty percent ammonium sulfate (w/v) was carried out to precipitate enzyme protein, as 32% of total protein contained 81 % of total (PGase) activity was attained with specific activity 40.97 U/mg protein against 16.8 U/mg protein in the crude culture extract. The remainder fermented substrate after the enzyme extraction corresponded to 54% (w/w) from the original substrate contained 16% crude protein compared to 11.2% in unfermented substrate, which can be used as fortification and concentrated rations in both poultry and ruminant feed.

Keywords: *Aspergillus* oryzae, exopolygalacturonase production, jojoba mill solid waste, solid state fermentation

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INTRDUCTION

Preparations containing pectin-degrading enzymes used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3.0 to 5.5 [1]. The pectinolytic enzymes degrade the pectic substances found in plant tissues, thereby, having numerous applications in various types of industries such as juice and food industries. [2], for example, increase in the yield of fruit juice from pulp, removal of haze from juices to get a clear product [3]. Due to the potential and wide applications of pectinases, their need to highlight recent developments on several aspects related to their production. Higher cost of the production is perhaps the major constraint in commercialization of new sources of enzymes. In addition, technical constraint includes supply of cheap and pure raw materials. Utilization of low-cost agro-industrial residues offers potential benefits for SSF, which is attractive for implementation of sustainable bioprocesses. Further advantages of SSF processes are lower energy requirement associated with higher product yields and less wastewater production with lesser risk of bacterial contamination. Nevertheless, there are many factors which have a critical influence on the process development in SSF, such as selection of microorganism and substrate or optimum physical chemical and biological process parameters [4]. Filamentous fungi are known to produce pectinolytic enzymes in submerged fermentation (SmF), as well as in solid-state fermentation (SSF) processes. They are capable of synthesizing and secreting large quantities of certain proteins into the extracellular medium. Degradation and utilization of diverse biopolymers enables cultivation of *Aspergillus* species on agricultural and agro-industrial residues, which can be used as low-cost substrates for microbial enzyme production in SSF processes [5]. Several agro-industrial waste and by-products such as orange bagasse [6], sugar cane bagasse [7], wheat bran [8], and other food processing waste [9] are effective substrates for depolymerizing enzyme production by solid-state fermentation. No data obtained in literature about using JMSW for enzymes production, so the aim of this study is to using *Aspergillus oryzae FK-923* for production of exopolygalacturonase under SSF technique using JMSW.

MATERIALS AND METHODS

Ten fungal strains namely *Aspergillus niger* F-119, *A. niger* F-93*, A. oryzae* FK-923, *A. fumigatus* F-993, *Trichoderma harezinum* F-416*, T. reesei* F-418*, T. vitide* F-127*), T. viride* F-321*, T. koningii* Fk-77) *and penicellium finculosium* NRCE -629*)* obtained from Microbial Chemistry Lab. National Research Center, Dokki, Giza. Egypt, were maintained on potato dextrose agar (PDA) slants at 30^{o}C for 72 hrs.

Inoculum preparation

Inoculum was made from three days old PDA cultures. The inoculum (containing 10^{6}-10^{7} spores) was suspended in 0.05 M phosphate buffer. The suspension, when necessary, was diluted with sterile 0.05 M phosphate buffer (pH 5.0) to give a spore count within the predetermined range. One ml of inoculums was added to each 5 g of JMSW in the container. The remaining liquid needed to obtain the desired moisture content in the JMSW was tap water (pH 5.0) which was mixed with the substrate before sterilization. This procedure was repeated for each container as requested.

The spore suspensions were prepared by adding 10 ml of sterilized water to the three days old PDA cultures and the surface was gently rubbed with a sterilized wire loop.

Jojoba mill solid waste (JMSW) as complete medium for solid-state fermentation (SSF) and enzyme production by different fungi

Fungal strains screening for exopolygalacturonase production was studied in 250 ml Erlenmeyer flasks containing 5g of JMSW moistened to 50 % (v/w) with distilled water. One milliliter of spore suspension (10^{6} spores) was used as inoculums. The cultures were incubated at 30^{o}C for 4 days. At the end of incubation period 100 ml distilled water were added to each flask, blended and shacking at 150 rpm for 30 min, harvested by filtration. The filtrates were saved as sources of crude extracellular enzyme. The selected fungal strain was incubated for 120 hrs at 30^{o}C and culture was taken at interval 24 hrs to detect the optimum incubation period. JMSW was moistened to different moisture levels, i.e., 30, 40, 50, 60, 70, and 80% (v/w) under the optimum incubation period to determine the more suitable moisture content for enzyme production. 0.1 M sodium phosphate buffer were used for adjusting the initial pH of fermentation medium to different values

from 3.5 to 7.5 to study the effect to study pH on the enzyme secretion. The fungus was incubated under different temperatures, i.e.20, 25, 30, 35, and 40^{o}C to study the effect the temperature on the enzyme production. Four nitrogen sources (urea, ammonium sulfate, ammonium nitrate and ammonium oxalate at level 5.5 mg N/g JMSW were applied in the fermentation medium to study their effect on enzyme yield.

Enzyme assay

The PGase activity was assayed by estimating the amount of reducing sugar released under assay conditions. Exopolygalacturonase activity was measured by determining the amount of reducing groups released according to the DNS method described by [10]. The substrate used for assay was 1% PGA (polygalacturonic acid), of 0.1 M citrate buffer, pH 5.0. The assay mixture was prepared with the following components: 0.1 ml enzyme, 0.4 ml of 1% solution of polygalacturonic acid (PGA). Blank was prepared for each sample by boiling the reaction mixture before the addition of substrate. Tubes were incubated at 50°C for 10 min. The amount of galacturonic acid released per ml per minute was calculated from standard curve of galacturonic acid. One unit of enzyme activity is defined as the enzyme that releases 1 µmol ml^{-1} min^{-1} galacturonic acid under standard assay conditions.

Protein determination

Enzyme protein was determined by the method described by [11] and crude protein in the residual fermented substrate was according to micro kejldahel method [12].

Partial purification of enzyme

The filtrate recovered from SSF was centrifuged at 6000 rpm for 20 min at refrigerated condition. Solid ammonium sulfate was slowly added to the supernatant of crude enzyme preparation so as to reach 20% saturation. Addition of ammonium sulfate was carried out with continuous stirring in an ice bath, and then it was kept at 4°C overnight. The precipitated protein was removed by centrifugation at 6000 rpm for 30 min at 4°C. Ammonium sulfate was added to the supernatant to 80% saturation. The precipitated protein was again separated by centrifugation at 6000 rpm for 30 min at 4°C. The precipitated protein was dissolved in sodium acetate buffer (0.1 M pH 5.0).

Characterization of PGase

The temperature stability of enzyme was estimated by incubating the enzyme for 4 h at temperature from 35-80 °C in assay buffer and then measuring the remaining activity by standard assay determined the inactivation temperature. The pH stability of enzyme was evaluated by varying the pH of the reaction mixture between 3.0 and 8.0 at increment of 0.5. Activity was then assessed under standard conditions.

RESULTS AND DISCUSSION

Screening and selection of fungal strain for Exopolygalacturonase production from JMSW under SSF.

Selection of microorganism comes on the top when need to produce enzymes with high activity and economic. Table (1) shows that among ten strains tested for PGase production, *A. oryzae* FK-923 have advantages than other tested fungal strains in PGase activity production, grown on JMSW under SSF system as produces 448.2 U/g original substrate and specific activity 11.2/mg protein, so it was chosen for further studies to optimize the culture conditions and characterization of PGase enzyme. It has been reported that a large number of mesophilic species of *Penicillium sp.* and *Aspergillus sp.* have good prospect for PGase production microorganisms [14]. High polygalacturonase production by a thermophlic *Aspergillus fumigates* isolated from decomposting orange peel was obtained by [14]. Efficient polygalacturonase production from agricultural and agro-industrial residues by solid-state culture of *Aspergillus sojae* under optimized conditions have been reported by [15] polygalacturonase enzyme produced by gamma irradiated *Penicillium citrinum,* [13] production, of Polygalacturonase from *Mucor circinelloides* ITCC 6025 [17]. Polygalacturonase Production was obtained from *Enterobacter Aerogenes* NBO2 as well as from *Aspergillus niger* CSTRF [18, 19].

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Table 1: Screening of fungal strains for exopolygalactuornase production from JMSW

Fungal strains	PGase activity U/g	Protein mg/g substrate	Specific activity U/mg protein
A niger F-119	418.2	38.7	10.8
T. viride F-321	274.8	33.5	8.2
T. viride F-127	289.6	37.1	7.8
T. reesei F-418	416.6	40.8	10.2
T. harezinum F-416	396.2	46.1	8.6
A. fumigatus F-993	395.4	47.1	8.4
A niger F-93	411.4	38.1	10.8
A. oryzae F-923	448.2	40.0	11.2
T. konogii Fk-77	387.2	41.2	9.4
*P.finculosium*NRCE 629	366.6	35.9	10.2

Effect of initial temperature on exopolygalacturonase production by *A. oryzae* FK-923 from JMSW under SSF.

Under SSF, temperature is a controlling factor for enzyme formation, [20]. Maximum enzyme production by *A. oryzae FK-923* (436.4-442.2 U/g) when incubation was conducted at Initial temperatures between 25-30°C (Table 2) and decreased sharply above 35°C the temperature affected both protein released and specific activity. Optimum temperature reported for PGases production of *A. fumigatus* was 50°C [14]. The effect of temperature on PGases production by *Monascus* sp. N8 and *Aspergillus sp*. N12 and the optimum temperature was studied and found to be 45°C, [21]. Temperature was studied for *Mucor circinelloides* and for *Aspergillus niger* CSTRF the optimum temperature was found to be 35°C [17,19].

Table 2: Effect of initial temperature on exopolygalacturonase production from *A. oryzae* FK-923

Initial temperature °C	PGase activity U/g	Protein mg/g substrate	Specific activity U/mg protein
20	342.2	31.7	10.8
25	436.4	39.3	11.1
30	448.2	40.0	11.2
35	240.8	28.0	8.6
40	73.6	14.0	5.3

Effect of initial pH on exopolygalacturonase production by *A. oryzae* FK-923 from JMSW under SSF

Table 3: Effect of initial pH on exopolygalacturonase production from *A. oryzae* FK-923

initial pH	PGase activity U/g	Protein mg/g substrate	Specific activity U/mg protein
3.5	320.2	35.2	9.1
4.0	352.8	36.6	9.6
4.5	424.2	38.6	11.0
5.0	448.6	40.1	11.2
5.5	416.2	37.5	11.1
6.0	377.3	36.2	10.4
6.5	342.7	34.0	10.1
7.0	220.2	25.0	8.8
7.5	179.5	24.3	7.4

For production of high levels of any enzyme, optimizing the growth parameters is of prime importance in industrial enzymology. Greater attention is given for optimizing the initial pH value of the moist solid medium, [8]. Data presented in Table (3) show that, the fungus expressed the highest degree of enzyme production (448.6 U/g) and released high protein (40.1 mg /g substrate) with highest specific activity (11.2 U/mg protein) at an initial pH 5.0 the enzyme synthesis was reduced markedly before pH6.0. The pH value of the culture medium affected the permeability of cells as well as stability of enzyme. The effect of hydrogen ion

on enzyme may be due to the stability of the enzyme at particular pH and denaturations of the enzyme proteins occur at pH differ, the optimum pH of such enzyme was reported by [14]. Earlier reports on PGase production by several fungi showed marked dependence on the initial pH of the medium [17], [22]. In general, acidic pH treatment of media is advantage in SSF processes applying fungi, due to their tolerance towards low pH-values, which is minimizing the risk of contamination during cultivation. Moreover, many fungi secrete PGase in acidic media and this is also the pH range where majority of their PGase show optimum catalytic activity, [15,23]. *Aspergillus niger* CSTRF produced high PGase activity at pH 4.5 [22].

Effect of Moisture contents on exopolygalacturonase production from *A. oryzae* FK-923 from JMSW under SSF.

Initial moisture content is one of the key factors influencing Enzyme production under SSF. The highest PGase activity (536 U/g) as well as released high protein (43.2 mg /g substrate) with highest specific activity (12.4 U/mg protein) was achieved when the moisture content was 70 % (v/w) (Table 4). It seems that the wet conditions cause swelling of the substrate and facilitates utilization of the medium by the organism. If the substrate is too moistened, the substrate porosity decreases which prevents oxygen penetration, while at very low moisture levels no growth of the organism will occur, [24]. Cell growth and oxygen consumption rate increased in conjunction with an increase in moisture content. However, the enhancement of enzyme production occurred up to a certain point and then leveled off. Many researchers have reported similar effect**.** The optimum moisture content (on a wet weight basis) was studied by many investigators. Highest enzyme yield was obtained by *A. sojae* ATCC 20235 applying 30% sugar beet pulp as inducer substrate in combination with wheat bran as medium wetted at 80% v/w with 0.2 M HCl [15].

Table 4: Effect of Moisture contents on exopolygalacturonase production from *A. oryzae* FK-923

Moisture % v/w	PGase activity U/g	Protein mg/g substrate	Specific activity U/mg protein
40	408.2	36.5	11.2
50	448.6	38.0	11.8
60	512.4	41.3	12.4
70	536.2	43.2	12.4
80	426.2	34.9	12.2

Effect of incubation time on exopolygalacturonase production from *A. oryzae* FK-923 from JMSW under SSF

Table 5: Effect of incubation time on exopolygalacturonase production from *A. oryzae* FK-923

incubation time(hrs)	PGase activity U/g	Protein mg/g substrate	Specific activity U/mg protein
24	124.4	18.3	6.8
48	384.8	32.1	12.0
72	542.8	43.1	12.6
96	536.2	43.2	12.4
120	522.4	43.5	12.0
144	518.6	44.7	11.6
168	476.2	46.7	10.2

Data presented in Table (5) show that PGases activity (542.8 U/g) was recorded maximum at the 3rd day of incubation. Previous studies on this aspect revealed different results for different strains for example maximum PGases activity was reported on the 5th day for *Penicillium* sp. [25] and on the 4th day for *Alternaria alternate* [26]. Generally enzyme activity increase with increase in incubation time and reached its maximum after 3rd day. This also supports the present study. Highest enzyme yield was obtained by *A. sojae* ATCC 20235 after 8th days [15].

Effect of nitrogen sources on exopolygalacturonase production from *A. oryzae* FK-923 under SSF

Data presented in Table (6) show that applied nitrogen source in the fermentation medium enhanced the enzyme production against control and the highest enzyme activity (656.6U/g) was observed with urea

which was followed by ammonium nitrate and diammonium phosphate (642.2 and 602.8 U/g respectively) ammonium sulfate, and ammonium oxalate were less stimulating enzyme production than other tested nitrogen sources. Gokhale et al., (1992)[27] also reported the capacity of urea to prevent the drop in pH in an unbuffered fermentation medium. [28] Reported optimal production from *Streptomyces* sp. when urea was added to the basal medium devoid of yeast extract and peptone.

Table 6: Effect of nitrogen sources on exopolygalacturonase production from *A. oryzae* FK-923

Nitrogen source	PGase activity U/g	Protein mg/g substrate	Specific activity U/mg protein
Control	542.8	43.8	12.4
Diammonium phosphate	602.8	45.0	13.4
Ammonium sulfate	598.8	37.9	15.8
Urea	656.6	4o.1	16.8
Ammonium nitrate	642.2	39.9	16.1
Ammonium oxalate	584.8	55.2	10.6

Partial purification of enzyme

The filtrate recovered from SSF was centrifuged at 6000 rpm for 20 min under refrigerated condition. Solid ammonium sulfate was slowly added to the supernatant of crude enzyme preparation so as to reach 20% saturation. Addition of ammonium sulfate was carried out with continuous stirring in an ice bath, and then it was kept at 4°C overnight. The precipitated protein was removed by centrifugation at 6000rpm for 30 min at 4°C. Ammonium sulfate was added to the supernatant to 80% saturation. The precipitated protein was again separated by centrifugation at 6000rpm for 30 min at 4°C. The precipitated protein was dissolved in sodium acetate buffer (0.1 M; pH 5.0).

Characterization of PGases

The temperature stability of enzyme was estimated by incubating the enzyme for 4 h at temperature from 40-80 °C in assay buffer and then measuring the remaining activity by standard assay determined the inactivation temperature. The pH stability of enzyme was evaluated by varying the pH of the reaction mixture between 3.5 and 7.5 at increments of 0.5. Activity was then assessed under standard conditions. The enzyme was stable at 50°C for 4 h and retained about 92 and 84% of its activity at 55 and 60°C respectively, after 4 h of incubation (Fig.1). [17] Reported that the enzyme was stable up to the 4th h at 42°C. [29] Reported that enzyme retained about 82 and 63% of its activity at 60 and 70°C respectively, after 2 h of incubation. In the present study, it was indicated that the maximum stability of the enzyme was at pH 5.0 followed by a fall in stability at lower or higher pH (Fig.2). Previous works supporting the present study are reported for *Aspergillus sojae* -5.0 [30], Mucor *circinelloides*-5.5 [17], *Cylindrocarpon destructans*-5.0 [31]. *Thermoascus aurantiacus* maximum activity was observed at pH 5.0 [6] and *Aspergillus niger* CSTRF was 4.5 [22].

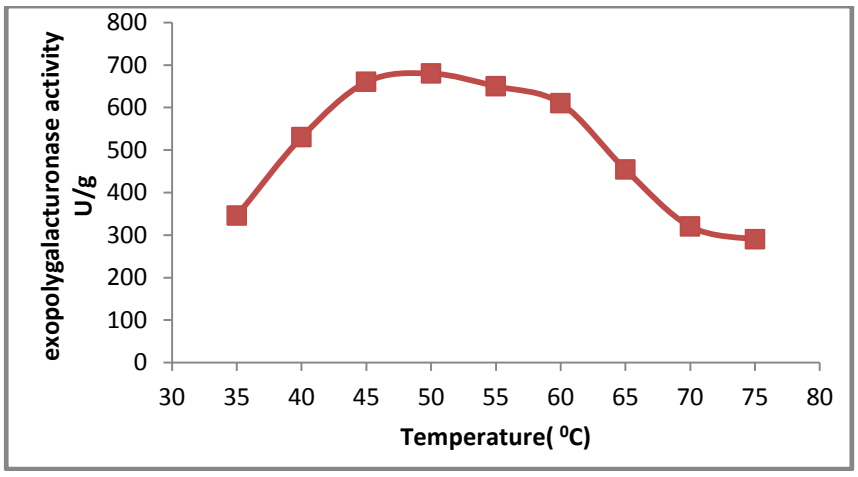

Figure 1: Effect of temperature on the activity of exopolygalacturonase produced by *A. oryzae* Fk-923

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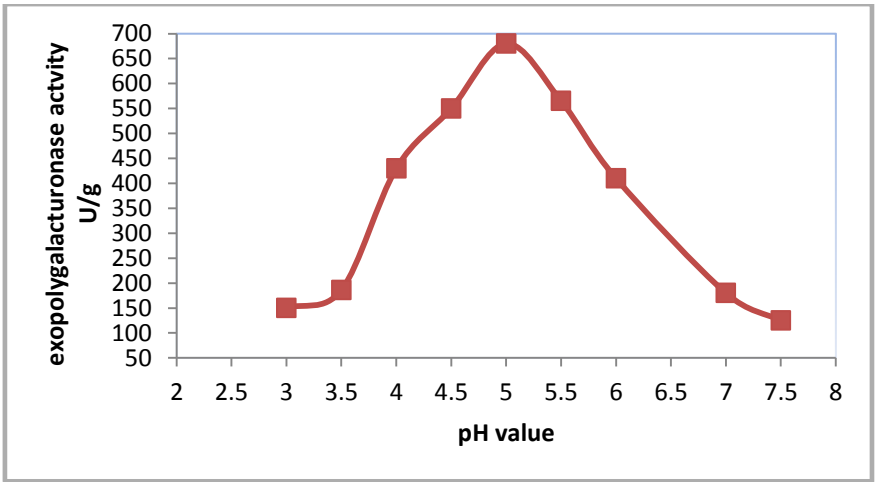

Figure 2: Effect of pH value on the activity of exopolygalacturonase produced by *A. oryzae* Fk-923

CONCLUSION

From the above data we can conclude that among all ten tested fungi *Aspergillus oryzae* FK-923 was the most favorable strain for producing exopolygalacturonase (PGase) enzyme when cultivating on jojoba mill solid waste (JMSW) under solid state fermentation. The enzyme activity production was enhanced when urea was added as a nitrogen source to fermentation medium at level 5.5 mg/g substrate. The remainder fermented substrate after the enzyme extraction contained 16% crude protein which can be used as fortification and concentrated rations in both poultry and ruminant feed.

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