Green polygalacturonase production by *Aspergillus awamori* NRC-F18 under solid-state fermentation

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Background and objective
Pectin-degrading enzymes applied in the food industries are preferred to be obtained from 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, ranging from pH 3.0 to 5. Pectinolytic enzymes, have several industrial applications in different industries such as food industries to concentrate fruit juices. They increased the yield of fruit juice from the pulp and removal of haze from juices to get a clear product. Research to find microorganisms to produce high-quality polygalacturonase (PGase) and technical constraint include supply of cheap and pure raw materials needed to produce inexpensive enzymes. Utilization of agroindustrial residues offers potential benefits for solid-state fermentation, which is attractive for the implementation of sustainable bioprocesses.
Materials and methods
Fungal strain screening for PGase production was studied in 250 ml Erlenmeyer flasks containing 5 g of tested substrate moistened to 50% (**v/w**, ml/g) with distilled water. One milliliter of spore suspension (10^6 spores) from each fungus was used as inoculum. The cultures were incubated at 30°C for 4 days. At the end of the incubation period, 100 ml of distilled water was added to each flask, blended by shaking at 150 rpm for 30 min, and harvested by filtration. The filtrates were saved as sources of crude enzyme. The selected fungal strain and substrate were incubated for 120 h at 30°C and culture was taken at 24 h intervals to detect the optimum incubation period. Sugar beet pulp was moistened to different moisture levels, that is, 1 : 1, 1 : 2, 1 : 3, and 1 : 4 (v/w) under the optimum incubation period to determine the more suitable moisture content for enzyme production. Sodium phosphate buffer at 0.1 M was used for adjusting the initial pH of fermentation medium to different values from 3.5 to 7.5 to study the effect of pH on enzyme secretion. The fungus was incubated under different temperatures, that s, 20, 25, 30, 35, and 40°C to study the temperature effect on enzyme production. Inorganic nitrogen sources (at level 0.92 mg N/g solid substrate) were applied in the fermentation medium to study their effect on enzyme yield.
Results and conclusion
Aspergillus awamori NRC-F18 showed promising PGase' production activity than other fungi screened. The study showed that the fungus gave promising results when the moisture content was adjusted to 70% (v/w), initial pH value 5.0, and incubation temperature at 35°C for 72 h. The enzyme activity was increased when urea was the sole nitrogen source at a level of 0.92 mg/g solid substrate supplemented to fermentation medium. Under the above conditions, 396.4 U/g original substrate was obtained. A study on the obtained PGase revealed that it has an optimum pH that ranged from 4.5 to 5.5, as well as it gave the highest activity when incubated at 50°C. Eighty percent ammonium sulfate (w/v) was applied to precipitate enzyme protein, as 24% of total protein involved 61% of total PGase activity obtained with a specific activity of 36.94 U/mg protein against 12.8 U/mg protein in the crude culture supernatant. After fermentation the mixture from unutilized raw material and fungal biomass after enzyme elution was 76% (w/w) from the original substrate dry weight involving 14.2% protein related to 9.2% before beet pulp fermentation which could be utilized as feedstuff component in rations for ruminant feed.

Keywords:
Aspergillus awamori NRC-F18, polygalacturonase production, solid-state fermentation, sugar beet pulp

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Introduction

Pectin-degrading enzymes applied in food industries are preferred to be obtained from 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 ranged from pH 3.0 to 5.5 [1]. Pectinolytic enzymes have several industrial applications in different industries such as fruit juice concentration and food industries [2]. They increased the yield of fruit juice from the pulp and removal of haze from juices to get a clear product [3] Research to find microorganisms to produce high-quality polygalacturonase (PGase) as well as technical constraint include supply of cheap and pure raw materials needed to produce inexpensive enzymes. Utilization of agroindustrial residues offers potential benefits for solid-state fermentation (SSF), which is attractive for the implementation of sustainable bioprocesses. Further advantages of SSF processes are lower energy requirement and higher product yields as well as less wastewater production and lesser risk of bacterial contamination. Many factors have a critical influence on the process development in SSF, such as selection of microorganism, substrate and optimum physicochemical and biological process parameters [4]. Filamentous fungi were found to be good producers for pectinolytic enzymes. They are capable of synthesizing and secreting large quantities of certain proteins into the extracellular medium. Agroindustrial wastes are used as a low-cost carbon source for the cultivation of *Aspergillus* species for microbial enzyme production in SSF processes [5]. Several agroindustrial wastes 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 SSF. Green production of enzymes or other microbial metabolites means that no wastes that cause pollution are released to the environment at the end of the production process.

Materials and methods

Microorganisms

Six fungal strains namely *Aspergillus oryzae* FK-923, *Aspergillus niger* F-258, *Aspergillus awamori* NRC-F18, *A. fumigatus* F-993, *Tricoderma viride* F-107, and *T. viride* F-321 were obtained from the Microbial Chemistry Department, National Research Center, Dokki, Giza, Egypt, and were maintained on potato dextrose agar slants at 30°C for 72 h.

Inoculum preparation

Inoculum was made from 3-day-old potato dextrose agar cultures. The inoculum (containing 10^6–10^7 spores/ml) was suspended in 0.05 M phosphate buffer. The suspension, when necessary, was diluted with sterile 0.05 M sodium phosphate buffer (PBS) (pH 5.0) to give a spore count within the predetermined range. A volume of 1 ml of inoculum was added to each 5 g of sugar beet pulp (SBP) in 250 ml capacity conical flasks. The remaining liquid needed to obtain the desired moisture content in the SBP was tap water (pH 5), which was mixed with the substrate before sterilization. This procedure was repeated for each container as requested.

SSF and enzyme production by different fungi cultivated on various substrates: Fungal strain screening for PGase production was studied in 250 ml Erlenmeyer flasks containing 5 g of tested substrate moistened to 50% (v/w, ml/g) with distilled water. A volume of 1 ml of spore suspension (10^6 spores) from each fungal was used as an inoculum. The cultures were incubated at 30°C for 4 days. At the end of the incubation period, 100 ml of distilled water was added to each flask, blended by shaking at 150 rpm for 30 min, and harvested by filtration. The filtrates were saved as sources of crude enzyme. The selected fungal strain and substrate were incubated for 120 h at 30°C and culture was taken at 24 h intervals to detect the optimum incubation period. SBP was moistened to different moisture levels, that is, 1 : 1, 1 : 2, 1 : 3, and 1 : 4 (v/w) under the optimum incubation period to determine the more suitable moisture content for enzyme production. Sodium phosphate buffer at 0.1 M was used for adjusting the initial pH of fermentation medium to different values from 3.5 to 7.5 to study the effect of pH on enzyme secretion. The fungus was incubated under different temperatures, that is, 20, 25, 30, 35, and 40°C to study the temperature effect on enzyme production. Inorganic nitrogen sources (urea, ammonium sulfate, ammonium phosphate, ammonium nitrate, ammonium oxalate, diammonium hydrogen citrate, sodium nitrate, and potassium nitrate) at level 0.92 mg N/g solid substrate 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. Activity was measured by determining the amount of reducing groups released according to the DNS method described by Miller [10]. The substrate used for assay was 1% polygalacturonic acid 'PGA,' in 0.1 M citrate buffer pH 5.0. The assay mixture was prepared with the following components: 0.1 ml enzyme and 0.9 ml of 1% PGA solution. Blank was prepared for each sample by boiling the reaction mixture before addition of substrate. Tubes were incubated at 50°C for 30 min. The amount of reducing sugars released per ml per minute was calculated from the standard curve of galacturonic acid. One unit of enzyme activity is defined as the

enzyme that releases 1 µmol/ml/mingalacturonic acid 'PGA' under standard assay conditions.

Protein determination

Enzyme protein was determined by the method described by Lowry *et al.* [11] and crude protein in the residual fermented substrate was according to micro-Kjeldahl method of the AOAC [12].

Partial purification of enzyme

The crude enzyme filtrate was recovered (eluted) from the fungal SSF culture and centrifuged at 6000 rpm for 20 min under refrigerated condition. Solid ammonium sulfate salt was slowly added to the cold supernatant of crude enzyme preparation with continuous shaking on a rotary shaker (200 rpm) until it reached 60% w/v saturation. The shaking was continued until complete ammonium sulfate solubilization and then kept (left) overnight at 4°C in a refrigerator. The precipitated protein was 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) and subjected to further studies.

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

Results

Screening and selection of fungal strain and suitable substrate for producing polygalacturonase

Selection of microorganism and suitable substrate are required to obtain inexpensive enzymes with high activity. Table 1 presents the data gained from the cultivation of five nontoxic fungal strains tested for producing PGase. *A. awamori* NRC-F18 produced the more promising enzyme activity at 258.6 U/g from original substrate SBP, so it was chosen for further studies to optimize the culture conditions and characterization of PGase enzyme.

Controlling temperature is an important factor for enzyme formation when SSF is applied. Maximum enzyme production by *A. awamori* NRC-F18 (232.4–264.8 U/g) when fungus was grown at temperature ranges of between 25 and 35°C Table 2 and is affected negatively when incubated at 40°C.

Table 3 shows the data obtained when the initial pH of fungus cultivation was adjusted between pH 3.5 and 7.5. The fungus produced promising enzyme production (286.4 U/g) and released protein (20.2 mg/g initial substrate and the specific activity reached 13.96 U/mg protein) when initial pH of fermentation was at pH 5.5. Enzyme synthesis was affected negatively after pH 6.0.

Moisture content is a limiting factor for the success of SSF technique and consequently enzyme production. The promising PGase activity was 368.2 U/g and specific activity was 16.78 U/mg protein/g substrate (23.2 mg protein excreted/g substrate was attained at an initial moisture content of 70%, v/w, adjusted before fermentation) (Table 4).

Table 5 shows that the maximum PGases activity (286.2 U/g) was achieved after 96 h, and the protein released was 23.5 mg/g substrate with a specific activity of 16.5 U/mg protein.

Table 2 Effect of temperature on exopolygalacturonase production from *Aspergillus awamori* NRC-F18 from sugar beet pulp under solid-state fermentation

Incubation temperature (°C)	Enzyme activity U/g	Protein mg/g substrate	Specific activity U/mg protein
20	112.3	11.4	9.59
25	232.6	19.2	12.04
30	258.6	20.2	12.93
35	264.4	20.2	13.11
40	93.6	12.0	7.80

Table 1 Screening of fungal strains for polygalacturonase production on different substrates at a moisture ratio of 1 : 1 v/w under solid-state fermentation technique

Fungal strains	Enzyme activity IU/g substrate			
	Corn flour	Sorghum flour	Sugar beet pulp	Wheat bran
Aspergillus awamori NRC-F18	206.4	198.5	258 .6	214.8
Aspergillus fumigatus F-993	201.4	188.9	216.8	222.4
Aspergillus niger F-258	205.6	201.0	226.0	226.8
Aspergillus oryzae FK-923	198.2	201.8	232.4	234.2
*Tricoderma viride*F-107	155.2	154.8	196.2	136.5
Tricoderma ricoderma viride F-321	170.9	126.0	188.6	116.6

Table 3 Production of polygalacturonase by *Aspergillus awamori* NRC-F18cultivated on sugar beet pulp under solid-state fermentation at different initial pH values

Initial pH value	Enzyme activity U/g	Protein mg/g substrate	Specific activity U/mg protein
3.5	167.4	16.4	10.32
4.0	196.6	17.2	11.18
4.5	224.3	18.8	12.05
5.0	264.5	20.2	13.11
5.5	286.4	20.6	13.96
6.0	227.4	18.4	12.49
6.5	186.8	17.1	11.97
7.0	120.4	15.2	9.00
7.5	64.5	14.2	4.51

Table 4 Polygalacturonase production by *Aspergillus awamori* NRC-F18 cultivated on sugar beet pulp adjusted to different moisture levels by solid-state fermentation technique

Moisture %, v/w	Enzyme activity U/g	Protein mg/g substrate	Specific activity U/mg protein
40	208.4	16.6	12.62
50	286.2	20.5	13.96
60	322.4	21.3	15.14
70	368.2	23.2	15.87
80	226.2	21.0.	10.77

Table 6 shows that the supplementation of nitrogen source in the fermentation medium resulted in enzyme enhancement compared with control, and enzyme activity (396.2 U/g) was seen by supplementation urea as sole nitrogen in fermentation medium followed by ammonium sulfate, diammonium phosphate, and ammonium nitrate (386.2, 362.2, and 342.8 U/g, respectively).

Partial purification of enzyme

Sixty percent ammonium sulfate (w/v) was applied to precipitate enzyme protein, as 24% of total protein involved 61% of total PGase activity, with a specific activity of 36.94 U/mg protein against 12.8 U/mg protein in the crude culture supernatant.

Characterization of enzyme

The enzyme was found with complete original activity when incubated at 50°C for 4 h, and have nearly 92 and 84% of its activity when incubated at 55 and 60°C, respectively, after the period (Fig. 1). On the other hand, the present study stated that enzyme was stable between pH 4.5 and 5.5 and at higher pH (over pH 6.5) it markedly decreased (Fig. 2).

Feed evaluation of fermented substrate after enzyme elution

The data shows (Table 7) upgrading in feed quality for SBP after the fermentation process for the production of

Table 5 Production of polygalacturonase by *Aspergillus awamori* NRC-F18 cultivated on sugar beet pulp under solid-state fermentation technique at different incubation periods

Incubation periods (h)	Enzyme activity U/g	Protein mg/g substrate	Specific activity U/mg protein
24	124.4	18.3	6.80
48	284.8	21.00	13.56
72	368.2	23.2	15.87
96	386.2	23.2	16.50
120	382.4	23.5	16.27
144	318.6	23.6	13.50
168	276.2	24.7	11.18

Table 6 Polygalacturonase production from *Aspergillus Awamori* NRC-F18 cultivated on sugar beet pulp with different nitrogen sources, under solid-state fermentation technique

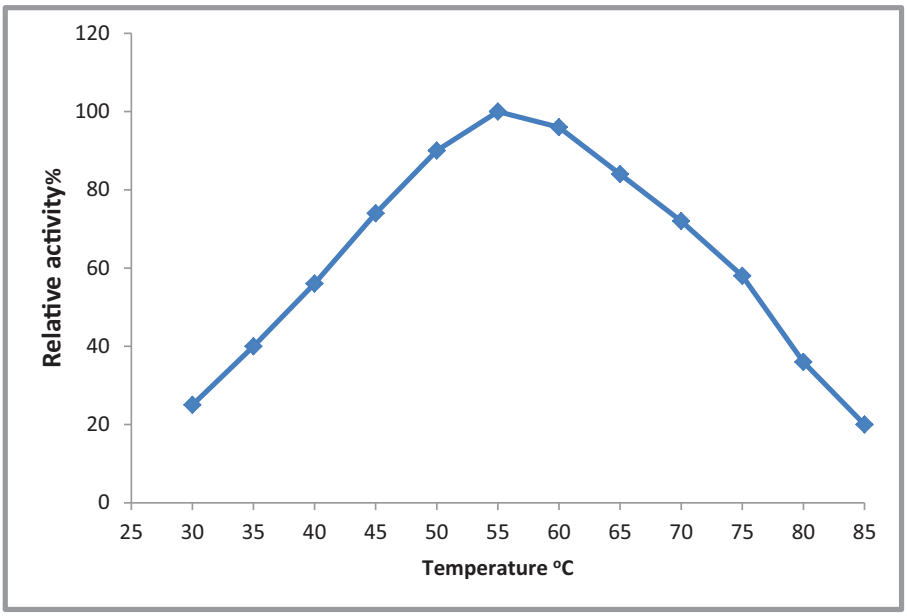
Source of nitrogen	Enzyme activity U/g	Protein mg/g substrate	Specific activity U/mg protein
Control	306.2	23.6	11.4
Ammonium phosphate $(NH_4)_2PHO_4$	362.8	25.2	14.4
Ammonium sulfate $(NH_4)_2SO$	386.2	23.8	16.5
Urea CO $(NH_2)_2$	396.6	20.3	17.2
Ammonium nitrate NH_4NO_3	342.2	19.6	15.9
Ammonium oxalate monohydrate $(NH_4)_2C_2O_4.H_2O$	324.8	17.2	13.2
Diammonium hydrogen citrate $C_6H_5O_7(NH_4)_2H$	318.2	18.3	12.3
Sodium nitrate Na_2NO_3	372.2	19.5	13.9
Potassium nitrate KNO_3	352.3	21.7	14.5

lignocellulose-degrading enzymes. The improvement is seen in increasing of crude protein from 9.4 to 13.7%, increasing in TDN from 67.0 to 69.0%, and performance in gross energy from 3995 to 4384 kcal/kg. On the other hand, there was a decrease in crude fiber, acid detergent fiber (ADF), and neutral detergent fiber (NDF).

Discussion

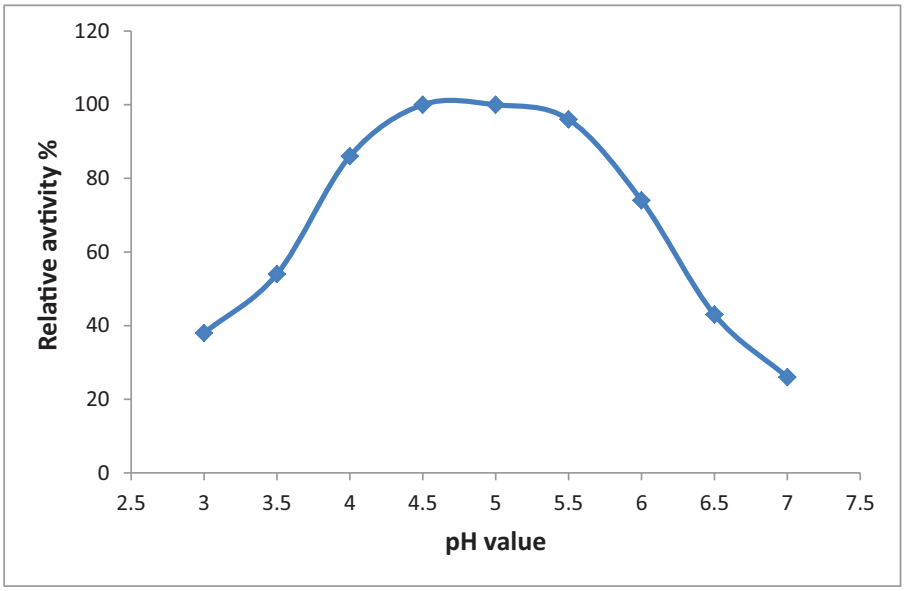
It has been reported that a large number of fungal strains and many substrates were reported for PGase production. Previous researches have reported that fungi such as *Penicillium* spp. and *Aspergillus* were utilized as explants for PGase production of microorganisms [13]. High PGase activity was produced by a thermophilic *Aspergillus fumigatus* isolated from decomposing orange peel [14]. In addition, high PGase yields were produced when agricultural and agro-industrial residues were utilized as a carbon source from solid-state culture of *Aspergillus sojae* [15], gamma-irradiated *Penicillium citrinum* [16], *Mucor circinelloides* ITCC 6025 [17], *Enterobacte raerogenes* NBO_2 [18], and *A. niger* CSTRF [19].

Figure 1



Activity of polygalacturonase produced by *Aspergillus awamori* NRC-F18 cultivated on sugar beet pulp, under solid-state fermentation method (70% w/v, moisture) at different degrees of temperature, after 96 h incubation period at 35°C and initial pH 5.5.

Figure 2



Activity of polygalacturonase produced by *Aspergillus awamori* NRC-F18 cultivated on sugar beet pulp, under solid-state fermentation method (70%, w/v, moisture content) at different pH values, after 96 h incubation period at 35°C.

On the other hand, numerous low-cost substrates were utilized for PGase production such as fruit-processing wastes [9] (agricultural residues and agro-industrial by-product [7], orange wastes [20], and grape pomace [21]. Controlling temperature is an important factor for enzyme formation when SSF is applied [22]. Incubation temperature of microorganism has an effect on protein released in the cultivation medium as well as specific activity [23]. *A. fumigatus* can be produced maximally when cultivated on temperatures adjusted to 50°C [24]. Freitas *et al.* [25] reported that a temperature of 45°C was the best for PGases production by *Monascus* spp. N8 and *Aspergillus* spp. N12. Thakur *et al.* [17] studied the same for *M. circinelloides* and the optimum temperature was found to be 35°C for *A. niger* CSTRF[2].The pH value of the culture medium was reported as affecting the permeability of cells as well as stability of enzyme. Different fungi secrete PGase in acidic media from pH 4.0 to 5.0 [15–26]. *A. niger* CSTRF was reported to

Table 7 Chemical composition of sugar beet pulp after and before fermentation

Component (%)	Before fermentation	After fermentation
Dry matter	90.0	92.4
Aflatoxin	Nil	Nil
Crude protein	9.2	13.7
Crude fiber	20.3	16.2
ADF	27.0	22.5
NDF	43.0	34.2
Ash	4.9	5.4
Cellulose	38.6	32.4
Gross energy (kcal/kg)	3785.0	4396.0
Total digest nutrient	67.0	69.0

ADF, acid detergent fiber; NDF, neutral detergent fiber.

give high PGase activity at pH 4.5 [23]. The moisture causes swelling of the substrate and facilitates utilization of the nutrients by the organism. Previous studies have reported that if the substrate is too moistened, the substrate porosity decreases, which reduces oxygen diffusion and accessibility for microorganisms, whereas, at low moisture levels, growth of the microorganism affected negatively as the nutrient is difficult to release owing to fungus mycelium penetration through the substrate [21]. Moisture contents limit the availability of oxygen for fungus as well as feasibility to utilized nutrients from the substrate. A promising enzyme is produced at a certain point of moisture and then leveled off. *A. sojae* ATCC 20235 produced the best enzyme activity when applying SBP as an inducer substrate when fermented with wheat bran as the medium moistened to 80%, v/w with 0.2 M HCl. Previous studies on this aspect revealed various results for different strains. Maximum PGases activity was reported on the fifth day for *Penicillium* spp. [27] and on the fourth day for *Alternaria alternata* [28]. Generally, enzyme activity increases with an increase in incubation time and reached its maximum after 96 h, which agrees with the present study. Highest enzyme yield was achieved by *A. sojae* ATCC 20235 after 8 days. The present finding agreed with that previously reported by Gokhale *et al.* [29], which found that urea prevents the drop in pH in unbuffered fermentation medium. On the other hand, Kuhad *et al.* [30] gained optimal PGase production by *Streptomyces* sp. when urea was added to the basal medium devoid of yeast extract and peptone. Thakur *et al.* [17] reported that the enzyme was stable up to the 4th h at 42°C. de Andrade *et al.* [31] found that the enzyme gave about 82 and 63% of its original optimum activity when incubated at 60 and 70°C, respectively, for 2 h. Previous works agree and support the present finding as it has been reported that optimum activity for PGase produced by *Cylindrocarpon destructans* and *Thermoascus aurantiacus* was pH 5.0 [6,32,33], while the optimum enzyme activity was at pH 5.5 for that produced by *M. circinelloides* [17], and enzyme produced by *A. niger* CSTRF gave its optimum activity at pH 4.5 [23]. Biologically treated SBP by fungi was evaluated as feed mixture rations for growing sheep, goats, and large animals such as lactating cows, lambs, dairy, as buffalo ration and beef cattle [34–37]. Biologically treated SBP led to upgrading crude protein to about 19% [38]. They found that feed conversion and cost of feed/kg gain were the best for ration contained 25% biologically treated SBP. The present study results (Table 7) indicate that the introduction of biologically treated SBP at a rate of 25% of the concentrate feed mixture could improve feed conversion of growing lambs and was economical [38].

Conclusion

SBP was used for PGase production through fermentation by *A. awamori* NRC-F18 under the SSF system. The study showed that fungus gave promising results when the moisture content was adjusted to 70% (v/w), initial pH value was 5.5, at an incubation temperature of 35°C for 96 h. The enzyme activity was increased when urea was the sole nitrogen source at a level of 0.92 mg/g solid substrate supplemented to the fermentation medium. Under the above conditions, 396.4 U/g original substrate was obtained. Study on the obtained PGase revealed that it has an optimum pH that ranged from 4.5 to 5.5, as well as it gave the highest activity when incubated at 55°C. Sixty percent ammonium sulfate (w/v) was applied to precipitate enzyme protein, as 24% of total protein involved 61% of total PGase activity was obtained at a specific activity of 36.94 U/mg protein against 12.8 U/mg protein in the crude culture supernatant. The mixture from unutilized raw material and fungal biomass after the enzyme elution was 76% (w/w) from the original substrate dry weight involved 14.2% protein related to 9.2% before beet pulp fermentation could be utilized as feedstuff component in rations for ruminant feed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Ueda S, Yusaku F, Lim JY. Production and some properties of pectic enzymes from *Aspergillus oryzae* A-3. J Appl Biochem 1982; 4:524–532.

2 Kashyap DRPK, Vohra S, Chopra R. Applications of pectinases in the commercial sector: a review. Bioresou Technol 2001; 77:215–227.

3 Kotzekidov P. Production of polygalacturonases by *Byssochlamys fulva*. J Indus Microbiol 1991; 7:53–56.

4 Thomas L, Larroche C, Pandey A. Current developments in solid-state fermentation. Biochem Eng J 2013; 81:146–161.

5 Ire FS, Vinking EG. Production, purification and characterization of polygalacturonase from *Aspergillus niger* in solid-state and submerged fermentation using banana peels production. J Advin Biol Biotechnol 2016; 10:1–15.

6 Martins ES, Silva D, Silva R, Gomes E Solid-state production of thermostable pectinases from thermophilic *Thermoascus urantiacus*. Process Biochem 2002; 37:949–954.

7 Silva D, Martins ES, Silva R, Gomes E. Pectinase production from *Penicilliumviridicatum* RFC3 by solid-state fermentation using agricultural residues and agro-industrial by-product. Braz J Microbiol 2002; 33:318–324.

8 Cavalitto SF, Arcas JA, Hours RA. Pectinase production profile of *Aspergillus foedidus* in solid state cultures at different acidities. Biotechnol Lett 1996; 18:251–256.

9 Leiden University. Using fungi to produce renewable energy. 2018. Available at: https://phys.orgnews/2018-06-fungi-renewable-energy.html

10 Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959; 31:426–428.

11 Lowry OH, Rosenbrough PJ, Fass AL, Randall RJ. Protein measurement with folin-phenol reagent. J Biol Chem 1951; 193:265–275.

12 AOAC. Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC). 11th ed. Washington, DC: 1970. 1:69–90.

13 Priya V, Sashi V. Pectinase enzyme producing microorganisms. Int J Sci Res Pub 2014; 4:1–4.

14 Phutela U, Dhuna V, Sandhu S, Chad BS. Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposting orange peels. Brazil J Microbiol 2005; 36:63–69.

15 Heerd D, Diercks-Horn S, Fernández-Lahore M. Efficient polygalacturonase production from agricultural and agro-industrial residues by solid-state culture of *Aspergillus sojae* under optimized conditions. Int J Sci Res Pub 2014; 4:2250–3153.

16 El-Batal AI, Osman EM, Ibrahim SAM. Optimization and characterization of polygalacturonase enzyme produced by gamma irradiated *Penicillium citrinum*. J Chem Pharm Res 2013; 5:336–347.

17 Thakur A, Pahwa R, Singh S, Gupta R. Production, purification, and characterization of polygalacturonase from *Mucor circinelloides* ITCC 6025. Enzyme Res 2010; 2:1–7.

18 Darah I, Nisha M, Lim SH. Enhancement of polygalacturonase production from *EnterobacterAerogenes* NBO_2 by submerged fermentation. Adv Stud Biol 2013; 5:173–189.

19 Juwon AD, Akinyosoye FA, Kayode OA. Purification, characterization and application of polygalacturonase from *Aspergillus niger* CSTRF. Mal J Microbiol 2012; 8:175–183.

20 Hachemi N, Nouani A, Benchabane A. Bioconversion of oranges wastes for pectinase production using *Aspergillus niger* under solid state fermentation. Int J Bioeng Life Sci 2015; 9:1004–1009.

21 Díaz AB, Caro I, Ory I, Blandino A. Evaluation of the conditions for the extraction of hydrolytic enzymes obtained by solid-state fermentation from grape pomace. Enz Microb Technol 2007; 41:302–306.

22 Castilho LR, Alves TLM, Medronho RA. Production and extraction of pectinases obtained by solid-state fermentation of agro-industrial residues with *Aspergillus niger*. Bioresou Technol 2000; 71:45–50.

23 Arotupin DJ, Akinyosoye FA, Onifade AK. Purification and characterization of pectinmethylesterase from *Aspergillus repens* isolated from cultivated soil. Afr J Biotechnol 2008; 7:1991–1998.

24 Phutela U, Dhuna V, Sandhu S, Chad BS. Pectinase and polygalacturonase production by a thermophilic Aspergillus fumigatus isolated from decomposting orange peels. Braz J Microbiol 2005; 36:63–69.

25 Freitas PM, Martin N, Silva D, Silva R, Gomes E. Production and partial characterization of polygalacturonases produced by thermophilic *Monascu* ssp. n8 and by thermotolerant *Aspergillus* sp. n12 on solid state fermentation. Braz J Microbiol 2006; 37:302–306.

26 Niture SK. Comparative biochemical and structural characterizations of fungal polygalacturonases. Biologia 2008; 63:1–19.

27 Gupta P, Dhillon S, Choudhary K, Singh R. Purification and characterization of an extracellular polygalacturonase from *Penicillium* sp. Ind J Microbiol 1997; 37:189–192.

28 Kunte S, Shastri NV. Studies on extracellular production of pectolytic enzymes by a strain of *Alternaria alternata*. Ind J Microbiol 1980; 20:211–214.

29 Gokhale DV, Patil SG, Bastawde KB. Protection of *Aspergillus* nigercellulases by urea during growth on glucose or glycerol supplemented media. Appl Biochem Biotechnol 1992; 37:11–17.

30 Kuhad RC, Kapoor M, Rustagi R. Enhanced production of an alkaline pectinase from *Streptomyces* sp. RCK-SC by whole-cell immobilization and solid-state cultivation. World J Microbiol Biotechnol 2004; 20:257–263.

31 de Andrade MVV, Delatorre AB, Ladeira SA, Martins MLL. Production and partial characterization of alkaline polygalacturonase secreted by thermophilic *Bacillus* sp. SMIA-2 under submerged culture using pectin and corn steep liquor. Ciencia Tecnol Alimentos 2011; 31:204–208.

32 Tari C, Gögus N, Tokatli F. Optimization of biomass, pellet size and polygalacturonase production by *Aspergillus sojae* ATCC 20235 using response surface methodology. Enzyme Microb Technol 2007; 40:1108–1116.

33 Sathiyaraj G, Srinivasan S, Kim HB, Subramaniyam S, Lee OR, Kim YJ, Yang DC. Screening and optimization of pectin lyase and polygalacturonase activity from ginseng pathogen *Cylindrocarpondes tructans*. Braz J Microbiol 2011; 42:794–806.

34 Abedo AA, El-Badawi AY, El-Ashry MA, Fadel M, Helal FIS. Microbial protein enrichment of sugar beet pulp by aerobic fermentation. Egypt Nutr Feed 2007; 10:553–568.

35 El-Sayed HM, El-Ashry MA, Metwally ELM, Fadel M, Khorshed MM. Effect of chemical and biological treatments of some crop-residues on their nutritive value. I. Composition and *in-vitro* disappearance. Egypt J Nutr Feed 2001; 4:387–398.

36 Fadel M, Ghoneem WMA. Effect of diets containing some biologically treated crop residues on performance of growing sheep. Egypt J Nutr Feed 2010; 13:21–36.

37 E1-Ashry MA, El-Sayed HM, Fadel M, Metwally ELM, Khorshed MM. Effect of chemical and biological treatments of some crop-residues on their nutritive value. 2-Effect of biological treatments on chemical composition and *in-vitro* disappearance. Egypt J Nutr Feed 2002; 5:43–54.

38 Abedo AA, El-Ashry MA, El-Badawi AY, Helal FIS, Fadel M. Effect of feeding biological treated sugar beet pulp on growth performance of sheep. Egypt J Nutr Feed 2005; 8:579–590.