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Research Article

Solid State Fermentation of Agro-industrial Residues for Glucoamylase Production from Endophytic Fungi *Penicillium javanicum* of *Solanum tuberosum* L.

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ABSTRACT

Glucoamylase production has been evaluated under solid-state fermentation of agro-industrial residues including groundnut shell, corncob, corn stover, sugarcane bagasse, wheat straw, barely straw and rice straw as renewable cheap substrates by different 14 endophytic fungal species. Among them the endophytic fungi *Penicillium javanicum* obtained from the root of *Solanum tuberosum* L. showed the maximum yield of glucoamylase using groundnut shell as solid substrate (289.23 ± 0.80 U/gds). Under the optimized production parameters in solid state fermentation process (250 mL Erlenmeyer flask containing 20 grams groundnut shell supplemented with 30% soya waste as an inexpensive, eco-friendly way of enzyme production sieved to 1mm, moistened to 55% initial moisture content with potato process wastewater, pH 5.0, inoculum intense 2 × 10^8 spore and incubated at 30°C for 5 days fermentation period), a fourfold increase (4.19-fold) in glucoamylase production was occurred. In our study there was a strong relation between the enzyme secretion and the trophophase. The purified enzyme exhibited specific activity 81.60 and 237. 24 U/mg with enzyme recovery equal to 51.11 and 22.14% and purification fold 2.2 and 6.39-fold after the precipitation with $(NH_4)_2SO_4$ and gel fractionation on sephadex G-100, respectively with maximum activity at 40-50°C and pH 5 and it was stable and retained 100% of its activity at temperature up to 60°C along with pH 5–7. The enzyme was not metallo enzyme due to EDTA and EGTA at 50 mM had no effect on glucoamylase activity but it was considered as a serine protease due to it lost 68 and 92% of its activity the serine protease inhibitor paramethyl sulfonyl fluoride (PMSF) at 10 and 50 mM, respectively.

Keywords: Fungi; Glucoamylase; Endophytic *Penicillium Javanicum*; Solid state fermentation (SSF); Optimization

INTRODUCTION

Filamentous fungi are being selected for the production of industrial enzymes as they have enormous capacity of production on low cost solid substrates [1]. Glucoamylases (E.C. 3.2.1.3) breakdown the glucose units from the non-reducing side of amylose, glycogen and amylopectin by hydrolyzing of α (1-4) and to a lesser extent, it also has the ability to hydrolyze a-1,6 linkages then D-glucose produced in consecutive way [2]. Glucoamylase enzyme has many applications in bakery products improvement, paper making, fabric industries, bioethanol, confectionary, pharmaceuticals and different food processing industry, then the use of amylase in many industries has made it very important to optimize production process to achieve maximum yield [3,4]. The microbial origin of starch hydrolysis enzymes in glucamylase solid cultures reveals numerous applications in all categories of industries by a narrow range of fungi but Aspergillus and penicillium have been considered the chief producer for industrial application [1]. The enzyme can be produced from various substrates by different methods, including submerged, semi-solid and solid-state fermentation processes [5]. Solid-state fermentation is a potent protocol for producing enzymes particularly glucoamylase on large scale [5].

Endophytic microorganisms are those that live in the interior of plants with no sign of disease or physical changes to the host. Endophytes are ubiquitous in the plant world and no report is known about plant species not associated with them. Endophytic fungi can produce therapeutics of biotechnological importance

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and they are increasingly recognized as a group of organisms that are likely to be valuable sources of new substances useful to mankind [6]. El-Gendy et al. [7] showed that forty four tested fungal endophytes of sponge exhibited a wide range of inhibitory activities against diverse pathogenic bacteria and fungi, antitumor activity, antioxidant activity along with they were prolific producers of industrial hydrolytic enzymes including cellulase, L-asparaginase, L-glutaminase, xylanase, alkaline protease, laccase, and lipase enzymes which are only a few instances of what was establish after isolating and culturing of such endophyte followed by purification and identification of some of their natural metabolites. Keeping the inner tissues of healthy plants is one of the little discovered niches of fungi and potato (*Solanum tuberosum* L.) is one of the highly important crops that widely cultivated in Egypt along with wider use of glucoamylase, the present study aimed to isolate and identify fungal endophytes from the roots of potato plant as such screening their ability to produce glucoamylase enzyme under solid state fermentation using different agro-industrial residues following by optimizing the production process parameters of glucoamylase from the hyper producer fungal strain.

MATERIALS AND METHODS

Chemicals

All the chemicals utilized in the current work were of purified grade and purchased from Sigma Aldrich.

Isolation and characterization of endophytic fungi

The isolation of fungal endophytes from plant roots were done according to El-Gendy et al. [6] and the obtained fungal isolates were identified based on their macroscopic and microscopic features for determination of the genera and species using standard manuals [8-10]. The fungal pure cultures were maintained in Czapeck Dox Agar media and reserved at 4°C until used.

Qualitative screening of fungal isolates for glucohydrolase production

Isolated strains were screened quantitatively for glucoamylase production as described by Abe et al. [11] using starch hydrolysis test on starch agar medium. Hydrolytic zones indicated the positive reaction was visualized using Lugol's iodine solution. Isolates were screened on the basis of the ratio of clearing zone diameter and colony diameter. Isolates producing excessive amylase were selected to secondary screening under SSF using different agro-industrial wastes.

Inoculum preparation

Inoculum preparation was done by suspending the spores in sterile Tween-80 (0.8%) from 5 days-old culture and each 250 mL Erlenmeyer flask was inoculated with one mL of spore suspension having a concentration of 2×10^7 spores/mL for SSF.

Solid substrates

All agro residues developed as substrates for glucoamylase synthesis in SSF including wheat straw (WS), rice straw (RS), barely straw (BS), sugarcane bagasse (SCB), groundnut shell (GNS), corncob (CC), corn stover (CS), soybean meal (SBM), soya waste (SW), coarse waste (CW), wheat bran (WB) and corn steep solid (CSS) were collected from local supplier, dried, ground to powder and stored in plastic jar at room temperature. Agro residues (10 g) in a 250 mL Erlenmeyer flask were moistened with potato processing wastewater to 60% initial moisture contents, sterilized at 121°C for 30 min, cooled, inoculated with one mL of spore suspension (2×10^7 spores/mL), total contents mixed thoroughly and then incubated at pH 5, 30°C and 150 rpm for 5 days. All experiments were done in duplicate flasks.

Enzyme extraction

After fermentation, glucohydrolase was recovered by re-suspending the solid substrate in 50 mM citrate buffer (pH 5) (1:10) and stirring at room temperature for 2 hr. This suspension was filtered through Whatman filter paper number 1 and to obtain solid/cell-free filtrate, the filtrate was centrifuged at 5000 rpm for 10 min. Glucohydrolase activity was estimated in the cell-free filtrate.

Enzyme assay

Regarding glucoamylase activity, 0.1 mL of enzyme was mixed with 1.9 mL starch prepared in 50 mM sodium acetate buffer, followed by incubation at 50°C for 40 min and produced reducing sugars were estimated using 3, 5-dinitrosalicyclic acid (DNSA) reagent [12] using glucose as a standard. Glucoamylase activity units were calculated using standard curve for glucose. One unit of enzyme activity defined as the amount of enzyme needed to produce of one micromole of reducing sugar in one minute [12].

Optimization of *P. javanicum* glucoamylase production parameters under solid state fermentation

Glucoamylase production was optimized with respect to various nutritive and culture parameters including the effect of different agro-residues (10 g of WS, RS, BS, SCB, GNS, CC and CS/250 mL Erlenmeyer flask, individually), nutritional additives (inorganic nitrogen; $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$; organic nitrogen; yeast extract, peptone, tryptone, soybean meal, meat extract, malt extract and urea at a concentration of 0.1% as N base; natural raw materials; 10% w/w of soya waste, coarse waste, wheat bran and corn steep solid as well as carbon inducer; 1% w/w of mannitol, glycerol, raffinose, sucrose, lactose, glucose and fructose, individually), adding soya waste to groundnut shell medium at different ratio (10, 20, 30, 40 and 50%), substrate amount by varying the amount of solid substrate mixture (10, 20, 25 and 50 g of the substrate in 250 mL Erlenmeyer), concentrations of tween- 80 (0.2, 0.4, 0.6, 0.8 and 1.0%), inoculum size by inoculating each flask with one mL of spore suspension (2×10^4, 2×10^5, 2×10^6, 2×10^7, 2×10^8 and 2×10^9, individually), particle size (0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0 and 5.0 mm of groundnut shell and soya waste mixture), initial moisture contents (40, 50, 55, 60, 65, 70, 80 and 90% v/w), incubation temperatures (20-45°C), pH (3-10) and incubation periods (1 to 10 days). Then glucoamylase and biomass production were estimated in duplicate flasks and the data presented are mean value ± standard deviations (SD). Parameter optimized in one experiment was maintained in the following experiments.

Biomass estimation

Biomass estimation was done by N-acetyl glucosamine released after acid hydrolysis of chitin present in fungal cell wall and estimation of releases glucosamine from chitin at 535 nm as described by Shivaramakrishnam et al. [13]. Biomass was expressed as g per gram of dry substrate based on the calibration curve of glucosamine as standard.

Partial purification of *P. javanicum* glucoamylase

It was purified by fractional precipitation with $(NH_4)_2SO_4$ and then size exclusion chromatography using sephadex (G-100) according to Kumar et al. [14]. All purification processes were done at 4°C.

CHARACTERIZATION OF PARTIAL PURIFIED *P. javanicum* GLUCOAMYLASE

Glucoamylase activity and stability at different temperature and pH values

The enzyme activity and stability were performed by measuring the residual activity at pH 5.0 with varying temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C). Also the enzyme activity and stability of enzyme was measuring at 50 °C with various buffers (0.1 M) at different pH (pH 3-10) according to El-Gendy [15].

Effect of some chemicals on partial purified glucoamylase activity

Partial purified glucoamylase was pre-incubated for 1 hr at 30°C with diverse metals including Cu^{2+}, Na^{+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, Pb^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Hg^{2+}, at a final concentration of 10 mM. Also, the influence of different chemicals on purified enzyme was studied by pre-incubating the enzyme for 30 min at 30°C with a number of chemicals including detergent (Tween-20, tween-80 and Triton X-100 at 1%), sodium dodecyl sulphate (SDS), metalo-protease inhibitor (ethylenediaminetetraacetic acid EDTA and ethylene glycol tetraacetic acid EGTA at 10 and 50 mM) and serine protease inhibitor (paramethyl sulfonyl fluoride PMSF at 10 and 50 mM), separately. Residual activity after each treatment was then assessed in computation to the control in the absence of these reagents (100% was consigned to the control activity).

RESULTS AND DISCUSSION

Isolation and screening of glucoamylase endophytic fungal producers

In the primary screening out of 61 endophytic fungi obtained from the root of *Solanum tuberosum* L. plant using starch hydrolysis test, 14 species belonged to Penicillium and Aspergillus genera were excessive amylase producers on the basis of the ratio of clearing zone diameter and colony diameter produced. These fungal strains were subjected to secondary screening under SSF using diverse agro-industrial wastes (Table 1). Similarly, Rizk et al. [4] reported that amylolytic enzymes were restricted to a few types of mesophilic fungi mostly to Aspergillus species and Penicillium species.

Optimization of glucoamylase production process parameters by *P. javanicum*

Effect of different agro-industrial residues: During screening the best agro-industrial residue for glucoamylase production in SSF, the groundnut shell supported the highest glucoamylase production 289.23 ± 0.80, 151.55 ± 0.65, 194.80 ± 0.48, 253.96 ± 0.74, 267.15 ± 0.98, and 213.80 ± 0.58 U/gds in 6 fungal species (*Penicillium javanicum*, Penicillium janthinellum, Penicillium chrysogenum, Penicillium brevicompactum, Aspergillus oryzae and Aspergillus terreus, respectively) and corncob stimulated the maximum yield of glucoamylase 170.50 ± 0.84, 224.32 ± 0.91, 115.29 ± 0.60, 233.75 ± 0.95, and 176.94 ± 0.39 U/gds from 5 species (Penicillium rubrum, Penicillium citrinum, Penicillium expansum, Penicillium ochrochloron and Aspergillus flavus) but sugarcane bagasse was favored as the best inducer for glucoamylase induction to 221.30 ± 0.73, 130.82 ± 0.35, and 210.33 ± 0.87 U/gds in three species (Aspergillus candidus, Aspergillus niveus and Aspergillus niger) (Table 1). The substrate suitability for glucoamylase production by the hyper glucoamylase producer endophytic *P. javanicum* was as per the following order: Groundnut shell (289.23 ± 0.80 U/gds) > corncob (267.31 ± 1.00 U/gds) > corn stover (250.20 ± 0.82 U/gds) > sugarcane bagasse (230.5 ± 1.00 U/gds) > wheat straw (180.5 ± 1.00 U/gds) > barely straw (177.12 ± 1.00 U/gds) > rice straw (160.40 ± 0.71 U/gds) (Table 1). Hence *P. javanicum* was selected for further studies as well as groundnut shell was selected as the solid support material for glucoamylase study in the current work. Based on these data in Table 1 we strongly suggested groundnut shell as a novel substrate for the production of fungal glucoamylase in solid state fermentation. The costs of glucamylase are still too expensive to produce cost-effective energy syrup. One way to overcome this hurdle is to use solid-state fermentation [1] because of lower energy necessities, improved productivity, lesser waste volumes, simpler tools beside and the solid substrate act as supplier for nutrients and assists as an anchorage for the fungal cells [6]. Ramesh and Murty [5] reported that different types of solid substrates like copra waste, pastry waste, waste bread, oat bran and bagasse of wheat bran and sugar cane are used as substrate for fungal glucoamylase production and the substrate suitability for Humicola grisea glucoamylase production was in the following

Table 1: Effect of different agro-industrial residues on glucoamylase production by different endophytic fungi in SSF (10 g dry substrate, pH5, 30°C, 60% initial moisture, inoculum 2 × 10^7 spore per flask, 5 days of incubation).

Fungal species isolated	Glucoamylase production U/gds from agro-industrial wastes*						
	Wheat straw	Rice straw	Barely straw	Sugarcane bagasse	Groundnut shell	Corncob	Corn stover
P. javanicum	180.50 ± 1.00	160.40 ± 0.71	177.12 ± 1.00	230.50 ± 1.00	289.23 ± 0.80	267.31 ± 1.00	250.20 ± 0.82
P. rubrum	113.63 ± 0.69	70.16 ± 0.65	78.41 ± 0.70	169.20 ± 0.90	125.92 ± 0.40	170.50 ± 0.84	64.00 ± 0.52
P. citrinum	119.42 ± 0.75	100.31± 0.40	105.00 ± 0.53	219.75 ± 1.00	200.45 ± 0.85	224.32 ± 0.91	192.00 ± 0.76
P. expansum	54.18 ± 0.39	50.18 ± 0.19	61.47 ± 0.22	100.50 ± 0.18	89.08 ± 0.51	115.29 ± 0.60	100.81 ± 0.47
P. janthinellum	85.90 ± 0.41	61.33 ± 0.15	42.12 ± 0.20	91.17 ± 0.22	151.55 ± 0.65	121.46 ± 0.83	116.10 ± 0.71
P. ochrochloron	119.48 ± 0.70	101.16 ± 0.66	149.08 ± 0.80	215.27 ± 0.87	225.39 ± 0.90	233.75 ± 0.95	200.27 ± 0.90
P. chrysogenum	61.28 ± 0.27	38.64 ± 0.19	40.83 ± 0.20	119.00 ± 0.40	194.80 ± 0.48	159.14 ± 0.44	100.19 ± 0.38
P. brevicompactum	130.00 ± 0.38	83.50 ± 0.26	119.02 ± 0.31	200.60 ± 0.66	253.96 ± 0.74	248.21 ± 0.85	213.50 ± 0.69
A. candidus	165.13 ± 0.51	145.98 ± 0.49	170.28 ± 0.61	221.30 ± 0.73	203.14 ± 0.63	162.30 ± 0.50	171.30 ± 0.52
A. niveus	93.45 ± 0.23	81.02 ± 0.20	74.50 ± 0.19	130.82 ± 0.35	115.20 ± 0.30	99.16 ± 0.23	101.18 ± 0.23
A. flavus	81.67 ± 0.22	64.71 ± 0.15	75.04 ± 0.20	112.40 ± 0.28	170.18 ± 0.39	176.94 ± 0.39	125.80 ± 0.30
A. oryzae	146.32 ± 0.61	129.89 ± 0.60	141.15 ± 0.61	203.06 ± 0.59	267.15 ± 0.98	250.36 ± 0.90	241.00 ± 0.90
A. niger	155.10 ± 0.42	131.57 ± 0.40	119.39 ± 0.34	210.33 ± 0.87	161.37 ± 0.60	200.51 ± 0.65	182.37 ± 0.58
A. terreus	177.16 ± 0.35	150.44 ± 0.29	165.31± 0.32	204.19 ± 0.53	213.80 ± 0.58	199.20 ± 0.52	200.13 ± 0.52

order: wheat bran > maize bran > rice bran > rice husk > black gram bran. Also, Tayyab et al. [2], El-Gendy [15], Bhatti et al. [16] and Zambare et al. [17] suggested wheat bran for the maximum production of fungal glucoamylase.

Effect of different nutritional supplementations: Data in Table-2 clearly indicated that all inorganic nitrogen sources [$(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$] had diminishing effect on glucoamylase production by *P. javanicum* and they decreased glucoamylase productivity by 16.68, 5.43, 19.02, 4.68 and 7.30%, respectively. On the other hand, all organic nitrogen sources had positive effects on the enzyme production. Yeast extract, peptone, tryptone, soybean meal, beef extract, malt extract and urea increased the enzyme yield to 1.17, 1.24, 1.29, 1.60, 1.10, 1.23 and 1.03-fold. Interestingly using of the renewable raw materials soya waste, coarse waste, wheat bran and corn steep solid at (10% w/w) supported higher production levels of glucoamylase that estimated to be 451.70 ± 2.51, 400.15 ± 2.14, 432.24 ± 2.36 and 400.24 ± 2.19 U/gds (Table 2). Moreover both inorganic and organic nitrogen sources except ammonium sulphate and ammonium chloride increased the biomass of *P. javanicum* (Table 2). Similarly, the organic nitrogen supplementations like urea, peptone and yeast extract as a nitrogen source exhibited better glucoamylase production in SSF by fungi according to many previous studies [18]. Tayyab et al. [2] mentioned that glucoamylase from Asprgillus awamori was increase when wheat bran supplemented with urea or ammonium sulphate. Conversely to our results Zambare [17] mentioned that all organic nitrogen sources inhibited enzyme production by 30-35%.

Effect of different cultural conditions on *P. javanicum* glucoamylase: The impact of substrate contents and substrate amounts on glucoamylase production by *P. javanicum* in Table-3 supported a mixture composed of groundnut shell supplemented with 30% soya for the best production of glucoamylase due to this mixture increased the enzyme yield from 289.23 ± 0.80 U/gds in the groundnut shell medium alone to 576.11± 2.49 U/gds in the medium supplemented with 30% soya waste. Due to there is no significant increase in the enzymatic productivity in groundnut shell supplemented with soybean meal and GNS medium supplemented with soya waste, we used soya waste as supplemented material to GNS substrate to increase its nutrition values due to it is much cheaper than soybean meal. Moreover, when the effect of different substrate amount (10, 20, 25 and 50 g of GNS+30% SW per 250 mL of Erlenmeyer flask) were tested we find that 20 g of GNS+30% SW per flask (1:12.5) increased the glucoamylase formation in *P. javanicum* from 576.11 ± 2.49 with 10 g GNS+30% SW per flask (1:25) to 722.53 ± 2.51 U/gds and then it was decreased to 516.47

Table 2: Effect of nutritional supplementation on glucoamylase and biomass production by *P. javanicum* under solid state fermentation

*Nutritional supplementation	Glucoamylase production (U/gds)	Biomass (g/gds)
Control (GNS moistened with PPWW)	289.23 ± 0.80	0.22± 0.12
Inorganic nitrogen source		
Ammonium sulphate	241.00 ± 0.72	0.20 ± 0.12
Ammonium phosphate	273.52 ± 0.80	0.22 ± 0.13
Ammonium chloride	234.22 ± 2.82	0.20 ± 0.10
Ammonium nitrate	275.70 ± 4.94	0.21 ± 0.12
Sodium nitrate	268.13 ± 24.74	
Organic nitrogen source		
Yeast extract	336.91 ± 2.00	0.31 ± 0.14
Peptone	358.55 ± 2.00	0.32 ± 0.15
Tryptone	373.22 ± 2.15	0. 32 ± 0.20
Soybean meal	462.45 ± 2.56	0.29 ± 0.13
Beef extract	319.00 ± 2.52	0.30 ± 0.13
Malt extract	354.67 ± 2.10	0.41 ± 0.15
Urea	298.58 ± 1.92	0.25 ± 0.12
Raw materials (10%)		
Soya waste	451.70 ± 2.51	0.27 ± 0.11
Coarse waste	400.15 ± 2.14	0.27 ± 0.11
Wheat bran	432.24 ± 2.36	0.31 ± 0.20
Corn steep solid	400.24 ± 2.19	0.24 ± 0.14
Carbon sources supplementation (1%)		
Glycerol	205.17 ± 1.42	0.24 ± 0.05
Mannitol	295.10 ± 1.56	0.19 ± 0.06
Raffinose	189.60 ± 1.35	0.15 ± 0.02
Sucrose	350.74 ± 1.91	0.34 ± 0.18
Lactose	250.74 ± 1.60	0.17 ± 0.12
Glucose	176.58 ± 1.56	0.41 ± 0.22
Fructose	195.30 ± 1.63	0.40 ± 0.22

*Control (GNS moistened with PPWW)=groundnut shell supplemented with potato processing wastewater, Nitrogen sources supplementation equal to 0.1% as N-base.

Table 3: Effect of different substrate content, substrate mass, detergents and inoculum size on glucoamylase and biomass production by *P. javanicum* under SSF

Parameter	Glucoamylase production (U/gds)	Biomass (g/gds)
Substrate content (g/250 mL Erlenmeyer flask)		
GNS + 10% SW (Control)	451.70 ± 2.51	0.27 ± 0.11
GNS + 20% SW	490.18 ± 2.60	0,29 ± 0.13
GNS + 30% SW	576.11 ± 2.49	0.32 ± 0.12
GNS + 40% SW	489.60 ± 2.31	0.29 ± 0.10
GNS + 50% SW	396.10 ± 2.10	0.22 ± 0.09
Substrate amount per 250 mL of Erlenmeyer flasks		
10 g (GNS + 30% SW) (1:25)	576.11 ± 2.49	0.32 ± 0.12
20 g (GNS + 30% SW) (1:12.5)	722.53 ± 2.51	0.37 ± 0.12
25 g (GNS + 30% SW) (1:10)	516.47 ± 2.19	0.30 ± 0.12
50 g (GNS + 30% SW) (1:5)	395.30 ± 2.03	0.21 ± 0.11
Tween-80 (%)		
0.2	760.00 ± 3.49	0.34 ± 0.11
0.4	800.74 ± 3.51	0.37 ± 0.12
0.6	861.22 ± 3.56	0.37 ± 0.12
0.8	764.18 ± 3.50	0.34 ± 0.12
Inoculum size/flask		
2×10^4	434.79 ± 3.16	0.19 ± 0.00
2×10^5	600.11 ± 3.20	0.24 ± 0.00
2×10^6	700.86 ± 3.30	0.37 ± 0.09
2×10^7	861.22 ± 3.45	0.41 ± 0.15
2×10^8	970.50 ± 3.50	0.43 ± 0.12
2×10^9	614.15 ± 3.29	0.35 ± 0.14

± 2.19 and 395.30 ± 2.03 U/gds with 25 and 50 g of GNS+30% SW per flask, respectively (Table 3). The level of substrate per unit area of working volume of the flask influenced the porosity and aeration of the substrate and hence affects the enzyme productivity. Previously, effect of different amounts (5, 10, 15, 20, 25 and 30 g/flask) of the mixed bran (wheat bran:rice bran, 1:2) on glucoamylase production in 500 mL Erlenmeyer flask was evaluated by El-Gendy [15] and found that maximum enzyme activity was observed in the flasks containing 20 g of this mixture per 500 mL flask (2:50). Bhatti et al. [16] and Ellaiah et al. [19] stated that 1:50 and 1.5:50 of substrate quantity to flask volume ratios were found optimum for glucoamylase production from Aspergillus sp. A3 and Fusarium moniliforme in SSF, respectively.

Higher amount glucoamylase was obtained from *P. javanicum* with the addition of tween-80. Adding of tween- 80 at a concentration of 0.2, 0.4, 0.6 and 0.8% increased enzyme productivity by 5.19, 10.83, 19.20 and 5.77%, respectively due to some surfactants can influence the production and secretion of raw starch degrading enzymes through changing the permeability of cell membrane (Table 3). These data are in line with that obtained for glucoamylase production by Aspergillus sp. JAN-25 which increased by 32% with adding tween-80 [15] but Bhatti et al. [16] reported a decrease in enzyme biosynthesis by Fusarium solani in SSF with the addition of surfactants. Moreover, The effect of different inoculum size in Table-3 indicated that the activity of glucoamylase enzyme was increased from 434.79 ± 3.16 U/gds at inoculum size of 2×10^4 to 600.11 ± 3.20, 700.86 ± 3.30, 861.22 ± 3.45, 970.50 ± 3.50 and 614.15 ± 3.29 U/gds with increasing the inoculum size to 2×10^5, 2×10^6, 2×10^7, 2×10^8 and 2×10^9 (spores/flask), respectively. Also the *P. javanicum* biomass was increased from 0.19 ± 0.0 to 0.24 ± 0.0, 0.37 ± 0.09, 0.41 ± 0.15, 0.43 ± 0.12 (g/gds) and then decreased to 0.35 ± 0.14 (g/gds) at these inoculum size, respectively. Inoculum intensity is a significant consideration for SSF because higher inoculum sizes are inhibitory factors for good growth and metabolites production whereas lesser inoculum sixe need additional time to ferment such substrate in SSF [16]. Maximum enzyme activity was observed from endophytic Aspergillus sp. JAN-25 with 107 spore/gds [15].

Appropriate particle size should provide enough surface area for mycelium growth of *P. javanicum* and satisfy the oxygen demand, data in Figure 1 indicated that particle size of substrate greatly affected the enzyme and biomass productivity, whereas fine particles of 1.0 mm supported the maximum yield of enzyme (970.51 U/gds) and the biomass (0.40 g/gds) and gradually decreases with increasing particle size as less surface area available to the microbial enzyme to be acted upon (Figure 1). Hanif et al. [1] reported that particle size 2 mm supported the highest production of glucoamylase from Aspergillus sp. CP then decreased at particle size of 3 mm-4 mm. However, The amount of moisture is a vital determinant parameter for synthesis of enzymes in SSF, the producing strain *P. javanicum* prefers 55% moisture content in the supported martial (GNS+30% SW) for the best glucoamylase production (1210.44 U/gds) and 60 to 65% moisture content for the highest growth yield (0.39 g/gds, respectively) and then fall off at higher moisture content as the substrate became waterlogged and decrease surface to volume ratio (Figure 2). Elevate moistness decreases absorbency of substrate and negatively influences oxygen transfer but little moistness diminishes water movement to levels not conducive to supporting growth and metabolism of the producing fungi. Maximum glucoamylase activity was obtained from Aspergillus sp.

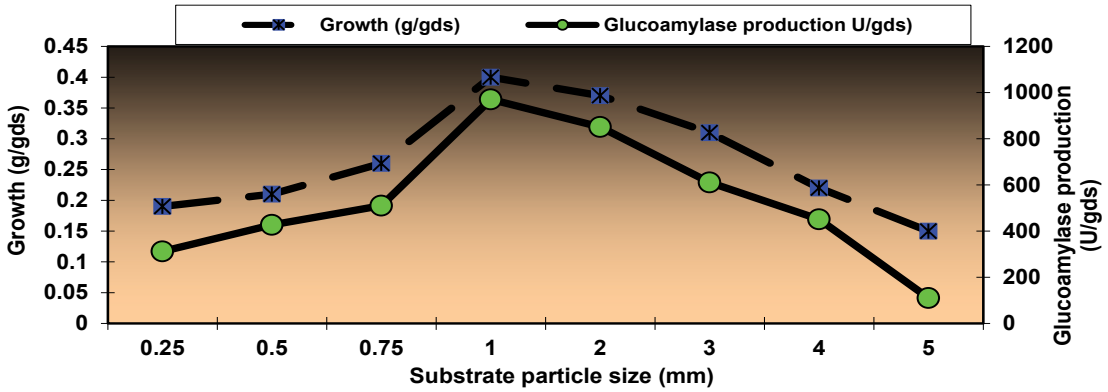

Figure 1: Effect of substrate particle size (mm) on glucoamylase (U/gds) and biomass (g/gds) production by *P. javanicum*

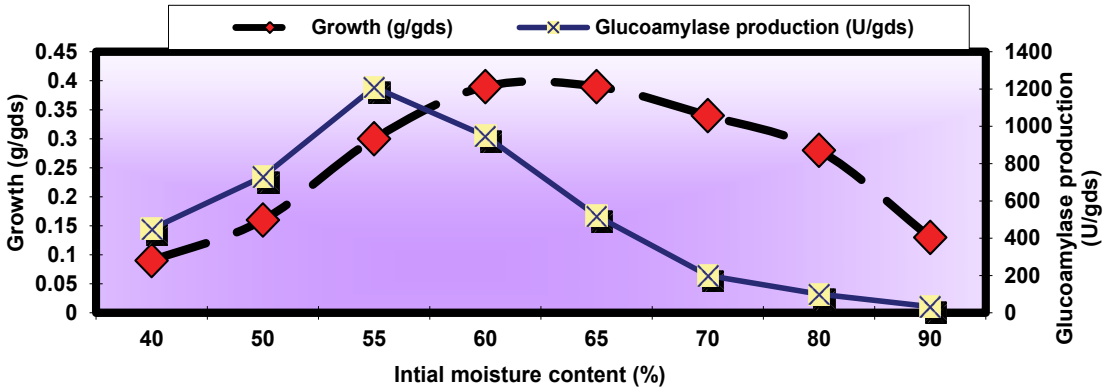

Figure 2: Effect of moisture contents (%) on glucoamylase (U/gds) and biomass (g/gds) production by *P. javanicum*

CP and H. grisea MTCC at 50% moisture content [1,5] as well as Aspergillus sp. JAN-25 at 50-60% [15] and Rhizopus sp. MKU 40 at 45% initial moisture level [20].

Glucoamylase synthesis was occurred between 20-40°C but the enzymatic yield was sensitive to the temperature of fermentation process, it was increased from 490.19 U/gds at 20°C to 1211.34 U/gds at 30°C and then sharply decreased to 310.18 and 140.0 U/gds at 35°C and 40°C, respectively with no enzyme secretion observed at 45°C (Figure 3). In addition maximum growth was obtained at 30 °C (0.35 g/gds) but it was decreased to 0.04 g/gds by increasing temperature to 45°C. Previously, glucoamylase from Aspergillus sp. CP, endophytic Aspergillus sp. JAN-25, Arachniotus sp., F. solani and A. fumigatus, was occurred at 20°C, 30°C, 32°C, 35°C, 40°C and 45°C [1,2,4,5,15,21]. Furthermore, the physical parameter, like pH has also significant effects to the growth and enzyme production. Most of fungi and yeasts are active at acidic environment (pH 4.0-6.5) for enzyme production [22]. In the present work, glucoamylase was secreted from *P. javanicum* in a wide pH range 3-9 with optimum productivity in acidic range (pH 5 of 50 mM sodium acetate buffer). The enzyme production was estimated to be 206.48, 714.64, 1210.14, 891.15, 500.74, 270, 98.12 and 33.16 (U/gds) with biomass yield equal to 0.13, 0.24, 0.36, 0.35, 0.29, 0.25, 0.14 and 0.07 (g/gds) at pH 3, 4, 5, 5.5, 6, 7, 8 and 9, respectively (Figure 4). Likewise, by varying the pH from pH 3 to 7 a linear increase in enzyme activity was recorded with a peak at pH 5 for glucoamylase from Aspergillus sp. CP and A. fumigatus [1,2] but the optimum glucoamylase production by C. gloeosporioides and A. oryzae was found at pH 5.4 and 5.8 [5] and the activity of α-amylase by A. niger increased by increasing pH value to 6.5 [4]. On the other hand, regarding to the typical time course of glucoamylase and biomass production, the incubation period is important factor that influences the enzyme production due to a difference in the lag and log phases of growth of different organisms, in Figure 5 maximum glucoamylase production by *P. javanicum* was occurred during the trophophase which completed during 6 day of fermentation. During ten days of solid state fermentation, the enzyme productivity was occurred after the first day (87.17 U/gds) of fermentation and reached its maximum yield (1210.29 U/gds) at the fifth day of fermentation and then decreased at the sixth day to 850.9 U/gds before the beginning of idiophase because of the trophophase was still occurred and the *P. javanicum* growth was increased at the sixth day to 0.4 g/gds (Figure 5). Many studies reported that maximum glucoamylase was produced on the 5th day of cultivation of different fungal species like A. oryzae, A. niger, Rhizopus oryzae, Thermomyces lanuginosus and A. flavus NSH9 [20] but in a few studies, it was observed after 3 days like glucoamylase produced by A. fumigatus which clearly indicates the fungal growth phase [2] and 4 days of cultivation like glucoamylase produced by F. solani, F. moniliforme and Aspergillus sp. JAN-25 [5,15].

Partial purification of *P. javanicum* glucoamylase: Crude glucoamylase was purified by consecutive stages including, $(NH_4)_2SO_4$ fractionation and size exclusion chromatography using sephadex (G-100) (Table 4). Glucoamylase specific activity reached 81.60 and 237. 24 U/mg with enzyme recovery equal to 51.11 and 22.14% and purification fold 2.2 and 6.39-fold after the precipitation with $(NH_4)_2SO_4$ and gel fractionation on sephadex G-100, respectively..

Characterization of *P. javanicum* glucoamylase: In the current work, glucoamylase activity was maximal at 50-60°C and above this temperature activity decreased. Moreover, enzyme being stable up to 70°C and above this temperature stability gradually decreased (Figure 6). In line with our results purified glucoamylase activities from A. flavus HBF341, A. flavus and A. niger NRRL 3135 were

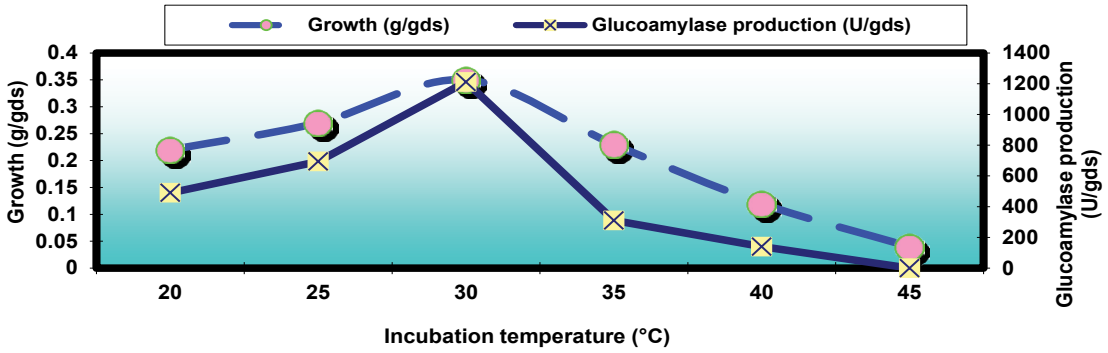

Figure 3: Effect of incubation temperature (°C) on glucoamylase (U/gds) and biomass (g/gds) production by *P. javanicum*

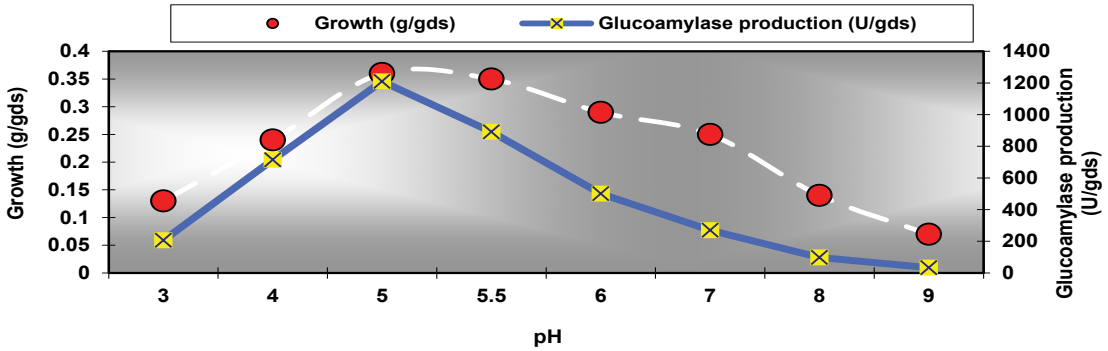

Figure 4: Effect of initial pH on glucoamylase (U/gds) and biomass (g/gds) production by *P. javanicum*

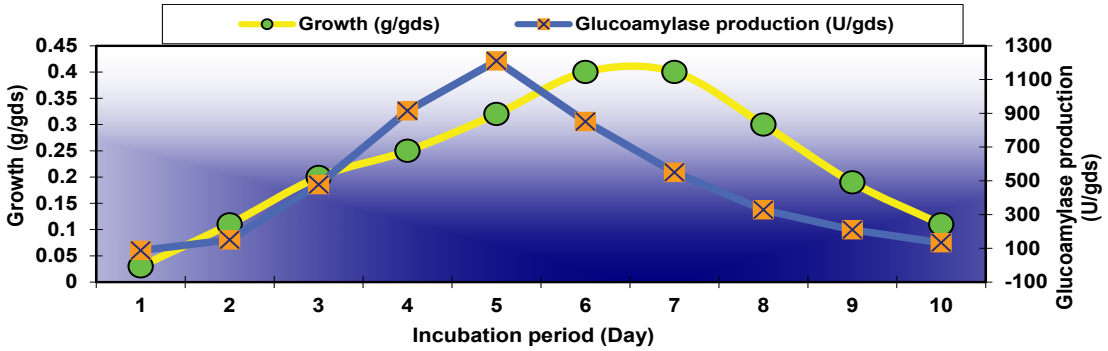

Figure 5: Effect of incubation period on glucoamylase (U/gds) and biomass (g/gds) production by *P. javanicum*

Table 4: Partial purification of *P. javanicum* glucoamylase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude extract	512.43	19000.92	37.08	100	1
80% $(NH_4)_2SO_4$ fraction	119	9710.45	81.6	51.11	2.2
Sephadex G-100	17.73	4206.18	237.24	22.14	6.39

reached a peak at 60°C [20,23] but an optimum temperature of 70°C was reported for T. lanuginosus A13.3798, A. flavus NSH936 and NSH9 [24]. However, few exceptional cases reported 40 to 45°C for maximum glucoamylase activity from Aureobasidium pulluan, F. solani and A. niger ATCC 1015 [16,25,26]. On the other hand, *P. javanicum* glucoamylase displayed maximal activity at pH 5.0 and then decreased with higher or lower pH values than the optimum one (Figure 7). It retained 33% of its activity at pH 3.0 while completely lost its activity at pH 10.0. The enzyme was stable and retained 100% of its activity in the pH ranged between pH 5 and 7 (Figure 7). Different authors reported that fungal glucoamylase is active at acidic pH, optimum pH was found to be at pH 4.0 for the glucoamylase activity of A. flavus [27] and Aspergillus tamari [28] as well as pH 4.0 to 5.0 in P. pastoris [20], pH 4.5-5.0 from Chaetomium thermophilum [29] and at pH 5 for A. flavus NSH9 glucoamylase [24]. Generally, almost fungal starch-hydrolyzing enzymes require pH range of 4.0-6.0 to be active [30,31].

The influence of metals on glucoamylase activity in Table-5 can be classified into three categories as follows: 1) metals with positive effect the enzyme activity, this group include Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+} and Ba^{2+} that increased glucoamylase activity by 18, 30, 24, 38 and 14%, respectively; 2) metals with neutral effect on the enzyme activity comprise Co^{2+}, Cu^{2+}, Na^{+} and Ca^{2+}; and 3) metals with negative effect on the enzyme activity, this group include Hg^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+} that negatively affected the enzyme activity by 49, 30, 6 and 32%, respectively. These effects of metal ions can be attributed to changes in the solubility, behavior of ionizing nutrients and changes in the catalytic properties of the enzyme [15]. Moreover SDS decreased the enzyme activity to 58% but tween-20, tween-80 and triton X-100 (1%) increased it to 119, 123 and 110%,

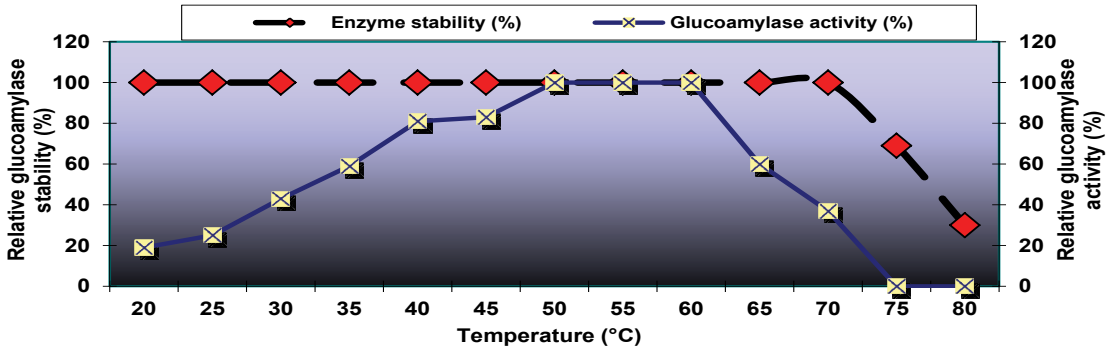

Figure 6: Residual activity and stability (%) of *P. javanicum* glucoamylase at different temperatures

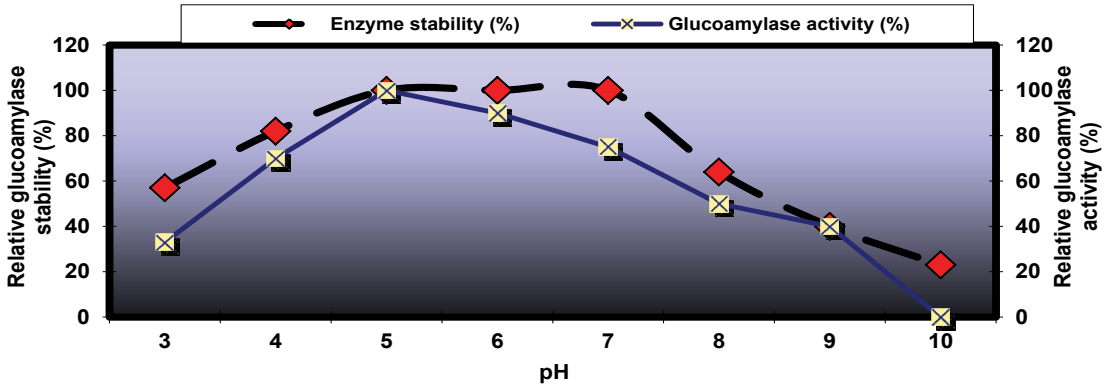

Figure 7: Residual activity and stability (%) of *P. javanicum* glucoamylase at different pHs

Table 5: Effect of some chemicals and inhibitors on *P. javanicum* glucoamylase activity

Metal additives (10 mM)	Residual activity (%)*	Detergents (1%)/protease inhibitors (mM)	Residual activity (%)*
Control (without additives)		Detergent (1%)	
Co^{2+}	100	Tween-20	119
Mn^{2+}	118	Tween-80	123
Cu^{2+}	100	Triton X-100	110
Mg^{2+}	130	Sodium dodecyl sulphate (SDS)	58
Zn^{2+}	124	Paramethyl sulfonyl fluoride (PMSF)	-
Fe^{3+}	138	10	32
Na^{+}	100	50	8
Ba^{2+}	114	EDTA	-
Hg^{2+}	51	10	100
Cd^{2+}	70	50	100
Ni^{+}	94	EGTA	-
Pb^{2+}	68	10	100
Ca^{2+}	100	50	100

respectively (Table 5). Interestingly the enzyme was considered as a serine protease due to it lost 68 and 92% of its activity after treatment with the serine protease inhibitor paramethyl sulfonyl fluoride (PMSF) at 10 and 50 mM, respectively but inhibitors of metalloprotease (EDTA and EGTA) at 10 and 50 mM showed no effect on glucoanylase under study (Table 5). These data are in line with the previous published data for fungal glucoamylase [15,18,20,24].

CONCLUSION

We can concluded that after optimizing all solid state fermentation process parameters (250 mL Erlenmeyer flask containing 20 gram groundnut shell supplemented with 30% soya waste as an inexpensive, eco-friendly way of enzyme production through solid-state fermentation, sieved to 1 mm, moistened to 55% initial moisture content with potato process wastewater, pH 5.0, inoculated with spore suspension 2×10^8 and incubated at 30°C for 5 days fermentation period), a fourfold increase (4.19-fold) in glucohydrolase production was occurred. In our study there was a strong relation between the enzyme secretion and the trophophase (Primary metabolism pathway occurs at the growth phase) due to the enzyme productivity in all experiments was reached its maximum yield before the producing organism begin its idiophase (secondary metabolism pathway occurs at the stationary) but overlap between trophophase and idiophase was also observed. Partial purified enzyme exhibited specific activity 81.60 and 237. 24 U/mg with enzyme recovery equal to 51.11 and 22.14% and purification fold 2.2 and 6.39-fold after the precipitation with $(NH_4)_2SO_4$ and gel fractionation on sephadex G-100. It was considered as a serine protease due to it lost 68 and 92% of its activity the serine protease

inhibitor Paramethyl sulfonyl fluoride (PMSF) at 10 and 50 mM, respectively.

AUTHOR'S CONTRIBUTIONS

Mervat M. A. A. El-Gendy and Nourah Hassan performed the laboratory work, analyzed the results and wrote the paper. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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