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Hyper Production of Glucoamylase by *Aspergillus oryzae* FK-923 under Solid State Fermentation

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Abstract: Glucoamylase production has been investigated by solid-state fermentation of corn flour by *Aspergillus oryzae* FK-923. Highest glucoamylase production (5582.4 µmoles of glucose produced per minute per gram of dry fermented substrate) was observed on corn flour supplemented with 30% (w/w) wheat bran, 1% soluble starch, 0.1% (w/w) urea pH 5.5, 60 % (v/w) initial moisture and 30°C after incubation for 72 hrs using 0.2M phosphate buffer as eluting solvent. Ammonium sulfate 80% (w/w) saturation could recovered 76.4% from total enzyme activity of crude enzyme extract involved in 36.8% of total protein precipitated, rising the specific activity from126.30 U/mg protein present in crude enzyme solution to 262.2 U/mg protein present in ammonium sulfate precipitate. Optimum enzyme activity was observed at 55°C and pH 5.5. Enzyme was applied for hydrolyzing 25 % w/v slurry of potato starchy waste resulting 86% saccharification. Enzymatic hydrolyzate 20% glucose was utilized for bioethanol production by *Saccharomyces cerevisiae* F-727 yielded 11.4% v/v ethanol and 12gl^{-1} dry yeast biomass. *S. cerevisiae* F-707 was grown on enzymatic hydolyzate for baker's yeast production giving 0.42 g/g utilized glucose. The residual mixture contained unutilized medium and fungal biomass was 42% from original weight was analyzed, showed increase in organic matter (OM) crude protein(CP), ether extract (EE) and dietary crude fiber (CF) from 96.98, 9.81, 3.10 and 7.61% to 98.11, 18.45, 10.13 and 27.07% respectively. On the other hand a decrease was seen in both ash and hemicellulose from 4.01 and 17.54% to 1.89 and12.59%, respectively. The gross energy was increased from 454 to 485 Kcal/100g DM. Enzymes assay showed that the residual fermented substrate loaded with glucoamylase 556, alpha-amylase 216, xylanase 224 and celullases (FPase 36 and CMCase 28) U/g. and can be used as concentrates feed for cattle and poultry as a source of protein, energy and feed fortification.

Key words: *Aspergillus oryzae* · Corn flour · Glucoamylase · Solid state fermentation.

INTRODUCTION

Glucoamylase (EC 3.2.1.3, 1, 4-α-D-glucan glucohydrolase) is one of the most commercially important starch enzymes hydrolyzes both 1, 4-alpha- and 1, 6-alpha glucsidic linkages in polysaccharides yielding glucose only. Glucoamylases are industrially important hydrolytic enzymes of biotechnological significance and are currently used in food and pharmaceutical industries [1, 2]. Glucoamylases mainly used in the production of glucose syrup, high fructose corn syrup and alcohol [3]. The exclusive production of this enzyme is achieved by *Aspergillus niger* [4, 5], *A. oryzae* [6] and *A. terreus* [7] in enzyme industry. These strains are already reported to produce substantial amount of glucoamylase in submerged and solid-state fermentation [7, 8]. Traditionally, glucoamylases have been produced by submerged fermentation (SmF). A recent focus of the

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research and development effort is the application of glucoamylases in the enzymatic degradation of carbohydrate rich polysaccharides for production of energy syrup. The development of microbial strains, media composition and process control all have contributed to the achievement of high levels of extracellular glucoamylases. However, the glucoamylase costs are still too high for the establishment of a cost effective production of energy syrup. One approach to overcome this obstacle is to employ solid state fermentation (SSF). The SSF process has the potential to significantly reduce the enzyme production costs because of lower energy requirements, increased productivity, smaller effluent volumes and simpler fermentation equipment [9]. In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the substrate is the most important factor in enzyme production [10]. The production of amylolytic enzymes, particularly glucoamylase on solid substrate is more advantageous for the fermentation industry [11, 12]. Cereal bran and flours, potato residue and other starchy waste materials have been utilized as fermentation substrate for glucoamylase production by filamentous fungi [6, 11, 13, 14]. Microbial degradation of these residues by GRAS (generally regarded as safe) strain may improve the substrate value as animal feed [15]. Glucoamylase production by *A. niger* was extensively studied using wheat bran in SmF and SSF [16]. The aim of this study is to evaluate the use of corn flour to cultivate *A. oryzae* FK-923 for improved production of glucoamylase under solid state fermentation. Beside the higher glucoamylase activity, the new trend in our work is the utilization of fermented residue as safe valuable feed for cattle and poultry as source of protein, energy and enzymes.

MATERIALS AND METHODS

Substrate: Corn flour (CF) and wheat bran were purchased from local market.

Potato Starchy Waste: Potato starchy waste was obtained from Chipsy Trade Company Located at Industrial zone 6th October city, Giza, Egypt.

Microorganisms and Strain Maintenance: Both fungal and yeast strains used in this study were obtained from Microbial Chemistry lab. National Research Centre, Dokki, Giza, Egypt. The cultures were maintained on yeast peptone malt agar slants and subcultured at 15 days interval at 28 °C for 3 days.

Inoculum Preparation: The spore suspensions were prepared by adding 10 ml of sterilized water and 0.1% Tween-80 to 3days old slant cultures and the surface was gently rubbed with a sterilized wire loop. Spore count was determined by serial dilution and spread plating method.

Solid State Fermentation: Corn flour (5g) were kept separately in 250 ml Erlenmeyer flask, then moistened with 5ml of distilled water and sterilized at 121°C for 30 min. The fermentation process was started by adding one ml of spore suspension (2 x10^6 spores spores/ml) as prepared above. The whole content was mixed thoroughly and then incubated at 28 °C for 4 days in a stationary condition.

Enzyme Extraction: To the fermented CF 50 mM citrate buffer (pH 5) (1:10) was added and homogenized for 30 min. with a constant stirring at room temperature. These suspensions were filtered through Whatman filter paper number 1 and the filtrates were again centrifuged at 6000 rpm for 15 min. This solid-free supernatant was used as enzyme source for assaying glucoamylase activity.

Production Optimization: Glucoamylase production was optimized with respect to various nutritional and environmental parameters such as; initial moisture content (50-80 % v/w) pH (3-9) and temperature (25-34°C). supplementation with wheat bran (20-50%), carbon inducer (1-3% w/w) glucose, maltose, sucrose, potato starch and soluble starch, Various inorganic nitrogen i.e. ammonium sulfate, ammonium oxalate, ammonium phosphate, diammonium phosphate, triammonium phosphate, ammonium acetate, ammonium nitrate and sodium nitrate, as well as urea as inexpensive organic nitrogen source were supplemented at equivalent nitrogen on bases of nitrogen equivalent (0.01gN/g substrate) for studying its effect on enzyme production.

All the experiments were conducted independently in triplicate and the data presented here are mean value.

Enzymatic Assays: Glucoamylase activity was determined by measuring released reducing sugars by the dinitrosalicylic acid method [17] using glucose as a standard. The enzyme assay was carried out at 50°C,

using 1.0% (w/v) soluble starch in 50 mM phosphate buffer pH 5.0, as substrate. An enzyme unit (U) is the amount that releases 1 µ mole of reducing sugars per min under standard assay conditions and enzyme activity is expressed in terms of units per gram dry original substrate (Ug^{-1}). Alpha-amylase activity was determined in culture filtrates by measuring the amount of starch hydrolyzed in the reaction mixture by the iodine method [18]. One unit of enzyme activity has been defined as the amount of enzyme that hydrolyses 1 mg of starch/min under assay conditions. Xylanase activity was determined by measuring the released reducing sugar by the dinitrosalicylic acid method [17], using D-xylose as the standard. The enzyme assay was carried out at 50°C, using 1.0 % (w/v) birch wood xylan in 50 mM citrate buffer, pH 5.4, as substrate. An enzyme unit (U) is the amount that releases 1µ mol of reducing sugars per min under assay conditions. Filter paper (FPase) and carboxymethylcellulase (CMCase) activities were assayed according to the method described by Mandels *et al.* [19].

Partial Purification: Protein Enzyme Precipitation: Different concentrations of ammonium sulfate i.e. 20, 40, 60 and 80% (w/v) were used to precipitate the enzyme protein. Each ammonium sulfate treatments were added slowly to a 15 ml centrifuge tube containing 5 ml of the supernatant culture solution (pH 4.0). The tubes were agitated for 15 s in a vortex at room temperature and then refrigerated overnight. The protein precipitate was collected by centrifugation under cooling (6000 rpm, for 20 min at 4°C) and then dissolved in 0.05M phosphate buffer (pH 5.5) up to the initial volume (5ml). Similarly different cold ethanol or acetone concentrations were employed for protein enzyme precipitation. The pH of the supernatant was adjusted with 0.1M phosphate buffer to pH 4.0. The cold ethanol or acetone was slowly added to the culture supernatant under agitation (200 rpm) at 4°C. After the addition of ethanol or acetone, the agitation was stopped. After 30 min the mixture was centrifuged (2000 rpm) for 10 min at 4°C. The obtained pellets were re-suspended in 0.05 M phosphate buffer pH 5.5 at room temperature (~28°C). The total protein contents were separately determined in the supernatant, in the pellet and in the initial samples. Necessary enzymatic activities were then determined.

Protein Determination: The amount of total protein was determined according to the Coomassie blue method described by Ohnistti and Barry [20] using bovine serum albumin as a standard protein.

Determination of Enzyme Properties: Effect of pH on Glucoamylase Activity and Stability: The activity of the crude glucoamylase was measured in pH range of 2.5 to 9.0 at 50°C for 30 min using 0.05M phosphate buffer. The stability was tested by incubating the enzyme solution in appropriate buffer at 50°C for a period of 4 hrs. Aliquots were withdrawn at different times and cooled prior to activity measurements at pH 5.5 and 50°C.

Effect of Temperature: The crude glucoamylase precipitated by acetone (dissolved in 0.05M phosphate buffer, pH 5.5) was incubated with the substrate at different temperatures ranging from 30 to 80°C for a period of 30 min followed by the estimation of the enzyme activities. For determining thermo-stability, the enzyme dissolved in phosphate buffer solution pH 5.5 was incubated for 4 hrs at different temperatures and then cooled prior to measure the residual activity under optimum temperature resulted from the above determination (50°C).

Hydrolysis of Potato-Industry Wastes: Starchy waste hydrolysis was conducted according to the method [13], in 500 ml conical flasks containing, 20 % (w/v) slurry of starchy waste substrate in total volume 200 ml 0.05 M sodium phosphate buffer pH 5.5 containing 0.01% toluene. The flasks were incubated at 55 °C in shaking water bath. Samples were withdrawn at 4 hrs intervals, glucose was determined with glucose oxidase-peroxidase kits.

Fermentation for Ethanol Production: The starch enzymatic hydolyzate was concentrated and adjusted to 20% w/v glucose to be used as a sole carbon source in the ethanol production fermentation medium by *S. cerevisiae* F-727 according to the optimized condition described by Fadel [14]. Ethanol content of the fermented samples was measured with ebulliometer approved in distillation factories [21].

Fermentation Efficiency: Fermentation Efficiency was calculated as the ethanol and yeast biomass yield divided by the theoretical yield multiply by 100.

Fermentation for Baker's Yeast Production: The starch enzymatic hydolyzate was used as a sole carbon source in the baker's yeast production medium by *S. cerevisiae* F-707 according to the optimized condition described by Fadel [22]. Total viable cell count was determined using microscope with the help of haemocytometer; cell viability was checked by using methylene blue indicator. The dead

cells were stained with blue indicator while viable cells remained uncolored. Crude protein was measured by micro-Kjeldahl method [23]. Ash was carried out on dried sample at 105°C by ignition 3 samples each 50g in muffle furnace at 800°C for 5 hours and the residual ash was calculated as % from the dried initial weight [24]. Cell dry weight was determined using 20 ml samples of the yeast culture collected by centrifugation (10 min at 7500 x g, 4°C) in a pre-weighed dried tube and then washed with 20 ml of distilled water. The tube was dried overnight at 105°C and weighed again. Total carbohydrates was determined according to the method of Herbert *et al.* [25], total lipids according to Salton [26], nucleic acid was determined by the method described by Barton *et al.* [27].

Utilization Efficiency (UE): The dry yeast yield for one gram consumed sugar in fermentation medium [28].

Fermentative Power: Volume of CO_2 produced by 5g fresh yeast (28% total solids) in one hour was determined by SAJ apparatus approved in baker's yeast factories.

Analysis of a Residual Fermented Corn:

Chemical Analysis: Dry matter (DM) loss was determined by the difference between dry weight before and after fermentation and described as percentage of initial weight. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) by the detergent system [29] and acid detergent lignin (ADL) by method described by A.O.A.C. [30]. Hemicellulose contents were estimated as the difference between NDF and ADF, while cellulose content was the difference between ADF and ADL. Crude protein was measured by micro-Kjeldahl method described by A.O.A.C. [23]. Ash was carried out on dried sample at 105 ^{o}C by ignition 3 samples each 50 g in muffle furnace at 800°C for 5 hours and the residual ash was calculated as % from the dried initial weigh according to A.O.A.C. [24]. At the end of fermentation period and after the enzyme extraction, the remainder fermentation mass (Fungal biomass with the unutilized part of corn) was air dried till constant weight. Crude protein was measured by micro-Kjeldahl method described by A.O.A.C. [23], total carbohydrates [31] and the moisture % was determined after oven drying at 105°C to constant weight.

Preparation and Enzyme Assays: The air dried solid fermented substrate of *A. oryzae* F-923 was powdered. Dry powder (0.5) g was added into centrifugation tubes, added 10 ml of 1 % enzyme substrate for each then incubated at 50°C in shaker water bath for 30 min. centrifuged at 8.000× g for 5 min and the librated reducing sugars were determined for enzyme activities of xylanase, cellulase (FPase and CMCase), alpha amylase and glucoamylase.

RESULTS AND DISCUSSION

Selection of Fungal Strains for Glucoamylase Production: Selection of microorganisms comes on the top when need to produce enzymes with high activity and economic. Data presented in Table 1 show screening of five strains of *Asperigllus* spp. *A. oryzae* FK-923 have advantages than other tested fungal strains in glucoamylase activity as produces (2885.2 Ug^{-1}) under the same culture conditions after 96 hrs, so we chosen for further studies to optimize the culture conditions and characterization of glucoamylase enzyme. The exclusive production of glucoamylase enzyme is achieved by *Aspergillus niger* [32], *A. oryzae* [6, 33, 34] and *A. terreus* [7] in enzyme industry. These strains are already reported to produce substantial amount of glucoamylase in submerged and solid-state fermentation [7, 35].

Optimization of Culture Conditions: Effect of Incubation Period: Culture conditions were optimized for optimum production of glucoamylase by *A. oryzae* FK- 923. Maximum production of glucoamylase (2896.4 Ug^{-1}) was recorded after 72 h of growth at 28°C on a substrate having an initial moisture content 50% at a pH 5.0 (Table 2). Further increase in the incubation period resulted in a decrease in the production of glucoamylase. It may be due to the fact that after maximum production of amylase enzyme (maximum incubation time), the production of other by products resulted in the depletion of nutrients. These byproducts inhibited the growth of fungi and hence enzyme formation [36]. Maximum glucoamylase production (726 Ug^{-1} dry medium) from *A. niger* NCIM-548 was observed in 84 h on the solid surface of groundnut shell (0.5 mm particle size) supplemented with sucrose (1% w/v) and yeast extract (0.5% w/v) with 50% initial moisture content [35]. The highest glucoamylase enzyme obtained after 96 hrs by *Gliocladium* KE using Sago Hampas solid substrate, while the highest glucoamylase enzyme activity was obtained after 5 days by cultivating *A. oryzae* on rice bran under solid state fermentation [34, 37].

Effect of Initial pH Value on Glucoamylase Production: Optimal pH is very important for growth of the microorganism and its metabolic activities, as the metabolic activities of the microorganism are very sensitive to changes in pH, glucoamylase production by

Table 1: Selection of fungal strain for production of glucoamylase from corn flour by solid state fermentation.

Fungal strains	Glucoamylase activity U/g	Proteinmg/g	Specific activity U /mg	Residual part %
A. niger F-93	2568.2	44.8	57.33	44.2
A.niger F-119	2167.5	42.6	50.88	46.2
A. oryzae FK-923	2855.2	38.2	74.74	48.6
A. oryzae FK-432	2242.8	40.2	55.79	48.8
A. fumigatus F-993	1968.6	45.5	55.79	42.4

Table 2: Effect of incubation time on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour.

Incubation Period (hrs)	Glucoamylase activity U/g	Protein mg/g	Specific activity U/mg	Residual part %
24	627.8	26.2	23.96	74.2
48	2102.6	38.8	72.22	74.2
72	2896.4	38.7	74.84	52.2
96	2855.2	38.2	74.74	48.6
120	2620.2	37.8	69.32	46.4

Table 3: Effect of initial pH value on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour for 48hrs at 28°C.

Initial pH value	Glucoamylase activity U/g	Protein mg/g	Specific activity U/mg	Residual Part %
3.0	1236.4	23.5	52.61	66.8
3.5	1624.6	25.3	64.21	62.4
4.0	1985.4	28.9	68.69	59.8
4.5	2280.0	34.6	69.32	54.5
5.0	2896.4	38.7	74.84	52.2
5.5	2984.0	39.2	76.12	52.2
6.0	2480.0	37.8	73.55	56.5
6.5	2480.0	38.5	64.42	57.8
7.0	2020.0	45.2	44.69	56.8

A. oryzae Fk-923 was affected by varying pH of the medium. Optimum pH was found to be 5.5 which gave maximum enzyme production 2984 Ug^{-1} after 96 h of incubation at 28°C (Table 3) and can produced in some low activities between pH 4.5-6.5. Our findings are comparable to previously reported results from literature; broad range for synthesis of glucoamylase was reported from *A. oryzae* [15, 33, 38]. Maximum glucoamylase production by *A. niger* NCIM-1245 reported at pH 4.7. In line with our work the culture conditions for glucoamylase produce maximum enzyme activity at pH 4.5 [9]. Maximum enzyme production of enzyme occurred at pH 4 to 6, while very little growth was observed without enzyme production in medium at initial pH 3 to 4 [39].

Effect of Temperature of Incubation on Glucoamylase Production: Growth temperature is a very critical parameter which affects enzyme production [40]. Table 4 illustrates the effect of temperature on glucoamylase production by *A. oryzae* FK-923. Maximum glucoamylase synthesis was observed in the medium incubated between 28-31°C with optimum glucoamylase yield of (2984Ug^{-1}). Increasing incubation temperatures resulted in decreasing enzyme synthesis by the fungus, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation [41]. It has previously been reported that *A. awamori* NRRL 3112 and *A. niger* NRRL 337 produced maximum glucoamylase at 35°C [42]. *Aspergillus* sp. A3 gave glucoamylase titers at 30 °C under optimum SSF process conditions [9]. A decrease in enzyme activities was observed in mesophilic temperature range. Similar results have been previously reported for glucoamylase production by *A. oryzae* [32, 43]. Effect of temperature on glucoamylase production was studied by various researchers on different fungal strains. It was found that A. *awamori, A. niger, A. tamari* and *A. terreus* showed maximum amylolytic enzyme production at 40 and 45°C. Similar results for production of glucoamylase by *Rhizopus* sp. at 45°C was reported [44]. Previous reports depict that 35°C temperature shows maximum enzyme activity by *R. delemar* and *A. niger* [36, 45].

Effect of Solid: Liquid Ratio on Glucoamylase Production: The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. An increase in moisture level is believed to reduce the porosity of the corn flour, thus limiting oxygen transfer [40]. Low moisture content causes reduction in the solubility of nutrients of the substrate and a low degree of

Table 4: Effect of temperature of incubation on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour for 48hrs.

Temperature °C	Glucoamylase activity U/g	Protein mg/g	Specific activity U /mg	Residual part %
25	2425.5	35.6	68.32	58.6
28	2984.0	39.2	76.12	52.2
31	2984.0	39.2	76.12	52.2
34	2345.4	34.8	67.40	56.4
37	1122.2	26.2	42.83	62.3

Table 5: Effect of solid: liquid ratio on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour for 48hrs at 28°C.

Solid : liquid ratio	Glucoamylase activity U/g	Protein mg/g	Specific activity U /mg	Residual part %
1:1	2984.0	39.7	76.12	52.2
1:1.5	3291.6	40.2	79.89	50.8
1:2	2745.6	38.6	71.13	54.6
1:2.5	2512.8	37.2	67.55	56.4
1:3	2224.2	35.6	62.48	56.8

Table 6: Effect of wheat bran supplementation on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour for 48hrs.

Wheat bran %	Glucoamylase activity U/g	Protein mg/g	Specific activity U /mg	Residual Part %
Control	3291.6	40.2	79.12	50.8
20	3444.0	41.6	82.79	50.2
30	3827.5	42.2	88.60	48.6
40	3273.6	39.4	83.09	52.2
50	2761.6	38.2	77.53	56.4

swelling [42]. A high enzyme titer (3291.6 Ug^{-1}) was attained when the substrate: moisture ratio was 1:1.5 (Table 5). Generally 40-70% of initial moisture content has been reported for fungal growth and substrate utilization. Fungal growth occurred at low moisture content but the enzyme yield was decreased significantly [46]. Different microorganisms show better performance at different moisture levels. *Aspergillus* sp. A3 produced maximum glucoamylase with 80 % moisture content [16], whereas *Rhizopus nigricans* PCSIR-18 gave optimum glucoamylase yield at 60 % w/v moisture [47]. Maximum glucoamylase production was reported with 90% (v/w) initial moisture content [48]. Reduction in enzyme activities was associated with early sporulation and also non-availability of nutrient due to low moisture or water activity. Low water activity affected the microbial activities because of limited nutrient solubilization and low degree of substrate swelling. Even high moisture content affected the microbial enzyme activities because of substrate stickiness, clumps, less porous nature of substrate and very limited oxygen transfer in vessel.

Effect of Wheat Bran Supplementation: Data presented in Table 6 showed that supplementation of corn flour with 30% wheat bran was more suitable for enhancing enzyme production and this may be to enhance oxygen transfer and more suitable nutrients involved in wheat bran can simulate enzyme secretion (wheat bran 14% protein and corn flour 9% protein). Wheat bran was used extensively for glucoamylase production. Maximum enzyme activity 69 Ug^{-1} of dry wheat bran was achieved under optimum growth conditions in Solid-State Fermentation by *Fusarium solani* [49]. Wheat bran (WB), corn flour (CF), rye straw (RS), wheat straw (WS) and rice bran (RB) were used, wheat bran yield maximum production of glucoamylase giving maximum enzyme production 128 Ug^{-1} after 96 h by *Bacillus cereus* MTCC 1305 using solid state fermentation [50]. Optimum glucoamylase production 1986 µ moles of glucose produced per minute per gram of dry fermented wheat bran supplemented with 1% (w/w) starch, 0.25% (w/w) urea at pH 6, 50% (v/w) initial moisture and 30°C after incubation 120 hrs by *A. oryzae* [51].

Effect of Supplementation of Carbon Sources: Data presented in Table 7 showed that glucoamylase production was more increased with supplementation of maltose and soluble starch 1% (w/w) and less increase was seen with supplementation of potato starch and glucose, while sucrose not affected the activity of glucoamylase, This additional fact emphasizes the importance of having suitable sources of distinct nutrients to obtain good performance in such fermentations. The influence of supplementary carbon sources was studied by many investigators. Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides resulted in marginal increase in glucoamylase production

Table 7: Effect of supplementation of carbon sources on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour for 48hrs at 28°C.

Carbon sources	Glucoamylase activity U/g	Protein mg/g	Specific activity U/mg protein	Residual part %
Control	3827.5	42.4	88.60	48.6
Glucose 1%	3985.4	43.1	92.47	48.2
Glucose 2%	4284.5	44.6	96.07	46.4
Glucose 3 %	4284.5	45.8	93.55	45.8
Sucrose 1%	3826.6	42.1	88.78	49.2
Sucrose 2%	3761.2	42.6	88.29	50.6
Sucrose 3%	3648.7	41.7	87.08	51.2
Maltose 1%	4991.6	46.8	106.66	45.8
Maltose 2%	4494.8	46.2	108.11	46.2
Maltose 3%	4927.4	45.2	106.66	45.9
Soluble starch 1%	4982.2	44.8	111.21	44.2
Soluble starch 2%	4984.3	45.2	110.27	45.1
Soluble starch 3%	4982.4	45.6	109.26	45.3
Potato starch 10 %	4467.0	45.9	97.32	48.4
Potato starch 20 %	4179.8	45.4	92.07	48.9
Potato starch 30 %	4127.6	45.6	90.52	49.1

Table 8: Effect of different inorganic and organic nitrogen sources on the production of glucoamylase by *A. oryzae* FK- 923 grown on corn flour for 48hrs at 28°C.

Nitrogen sources	Glucoamylase activity U/g	Protein mg/g	Specific activity U/mg protein	Residual part %
Blank	4982.2	44.8	111.21	48.6
Ammonium sulfate	5445.8	45.6	119.43	47.5
Ammonium Oxalate	5388.4	46.4	115.05	47.9
Ammonium acetate	4856.8	46.2	105.13	48.8
Ammonium phosphate	5143.5	47.1	103.70	46.2
Diammonium phosphate	5466.8	47.6	114.85	46.2
Triammonium phosphate	4832.5	46.8	103.26	48.1
Ammonium chloride	4806.2	47.2	101.83	49.2
Ammonium nitrate	4365.4	46.1	94.46	48.6
Sodium nitrate	4725.5	48.2	98.04	52.2
Urea	5582.4	44.2	126.30	45.2

by *B. cereus* during solid-state fermentation using wheat bran. Highest production was observed with glucose. Earlier researchers reported soluble starch as the best carbon supplement for glucoamylase production in *M. thermophila* D14 and *Humicola Lanuginose* [52]. Among different carbon sources supplemented, glucose (0.04 g/g) showed enhanced enzyme production [42]. Starch and maltose gave the best enzyme production by *A. niger* [53].

Effect of Different Nitrogen Sources: Various inorganic nitrogen sources i.e. ammonium sulfate, ammonium oxalate, ammonium phosphate, diammonium phosphate, triammonium phosphate and ammonium acetate at equivalent nitrogen as well as urea as inexpensive organic nitrogen source were supplemented on bases of nitrogen equivalent for studying its effect on enzyme production. Data in Table 8 showed that urea, ammonium sulfate, ammonium oxalate, ammonium phosphate, diammonium phosphate, ammonium nitrate shown varying positive effects on production of glucoamylase and the highest activity was recorded by supplementation of urea which gave 5582.4 Ug^{-1} followed by ammonium sulfate and ammonium oxalate as it were 5445.5 and 5388.4 Ug^{-1} respectively. Low negative effect on the production of glucoamylase was achieved by triammonium phosphate, ammonium acetate, sodium nitrate and ammonium chloride. The obtained results confirmed that the effect of nitrogen source positively or negatively may be depend on two factors firstly organism and secondly the substrate. The effect of nitrogen sources on the glucoamylase enzyme production by some *Aspergillii* sp. was studied, They showed that urea have negative effect on the enzyme production by *Aspergillus tamari* and *A. awamori* while with *A. niger* and *A. terreus* it stimulate the production gave positive significant effect while ammonium sulfate also showed significantly positive effect on *A. tamari, A. niger, A. awamori* and specially *A. terreus* [54].

It has an inhibitory effect on glucamylase production by *Aspergillus awamori*, Sodium nitrate and ammonium chloride have shown varying positive effects on

production of glucoamylase on all *Aspergillus* species studied. Of highly significant effect are those of sodium nitrate on *A. tamarii, A. niger* and *A. awamori* and to a lesser extended *A. awamori and A. terreus*. Also of highly significant effects of ammonium chloride are those on *A. awamori*. Positive effects, also of ammonium chloride are those on *A. tamarii, A. niger* and *A. terreus*. Among the nitrogen sources used to increase production of glucoamylase enzyme by *Candida famata*, urea was the best nitrogen source followed by $(NH_4)_2PO_4$ [44]. The production of glucoamylase by *A. fumigatus* has increased by addition of ammonium nitrate to the basal medium [55]. Addition of ammonium sulfate, to the culture medium stimulated glucoamylase production by *A. terreus* [56]. An increase of glucoamylase production by *A. niger* was reported when the basal culture medium was supplemented with ammonium sulfate [55]. Ammonium sulfate and ammonium chloride reduced the final yield of glucoamylase enzyme produced by *A. foetidus*, while sodium nitrate stimulated the enzyme production [57]. reported increase of glucoamylase production by *A. awamori* NRRL by addition of urea to the culture medium, while other authors reported that very low levels of glucoamylase production was obtained when the nitrogen source was urea [9, 27, 58, 59].

Effect of Different Aqueous Solution for Glucoamylase Recovery from Fermented Substrate: The efficient solvent to leach enzyme from fermented substrate may depend mainly on three factors, i.e. type of enzyme, source of enzyme and substrate used as a medium [60]. Phosphate buffer 0.2M was found to be more efficient than other tested solvents in the extraction of glucoamylase from the fermented CF (Fig. 1) as the activity 5582.4 U/g^{-1} was achieved. About 6 and 8% increase in enzyme activity was attained compared to saline solvent and citrate buffers respectively. Distilled water was shown lower efficient than other tested solvents, this finding was agree with [61] who obtained optimum activity of alpha-amylase from wheat bran using phosphate buffer pH 7.0, on the other hand, pH 5.9 was found to be more suitable for alpha-amylase extraction [62].

Partial Purification of Glucoamylase Enzyme: Precipitation with Ammonium Sulfate: Ammonium sulfate applied in different saturation ratios like 20, 40, 60, or 80 % w/v (Fig. 2A).The highest enzyme activity recovered 76.4% was obtained with 80% w/v ammonium sulfate saturation involved in 36.8 % of total protein precipitated, rising the specific activity from126.30 U/mg protein present in crude enzyme solution to 262.2 U/mg protein present in ammonium sulfate precipitate. Previous studies on glucoamylase precipitation employed high levels of ammonium sulfate to salt out the enzyme from cultures supernatant, in different saturation ratios and found 80% ratio was the best ratio for precipitate the crude extract of enzyme [63].

Precipitation of Glucoamylase with Ethanol: Total proteins (46.8%) present in crude enzyme solution were precipitated by 80% (v/v) cold ethanol to give 68.6% glucoamylase activity present in crude enzyme solution (Fig. 2B) and enhance specific activity from 126.30 U/mg protein present in crude enzyme solution to 185.1 U/mg protein present in acetone precipitate.

Precipitation of Glucoamylase with Acetone: Precipitation by 80 % (v/v) cold acetone was more suitable than other applied concentrations, science it could precipitate 64.6 % of glucoamylase activity present in the crude enzyme extract found in 40.2% (Fig. 2C) and enhance the specific activity from 126.30 U/mg protein present in crude enzyme solution to 202.9 U/mg protein present in acetone precipitate. Crude extract was applied to acetone ratio of 1:2 which yielded optimum simultaneous precipitation of glucoamylase (35.3 %) [64].

Some Properties of Glucoamylase:

Effect of Temperature and pH on Glucoamylase Activity: The activity of enzymes is strongly affected by changes of temperature. Each enzyme works best at a certain temperature, its activity decreasing at values above or below that point. Fig. 3A showed an increase in glucoamylase activity with increasing in temperature; optimum activity reached a maximum between 50-65°C. The decrease in enzyme activity above 65°C is attributed to denature of enzyme protein. Over a period of time, enzymes will be deactivated at even optimum temperature. In an attempt to determine the heat stability, the enzyme solution was re-incubated in 0.1M citrate buffer pH 5.5 for 4 hrs at 50°C and 75°C to avoid the short incubation period and the protective effect of substrate. It was found that the enzyme expressed nearly its original activity after 4 hr incubation at 50°C and 52% of its original activity after 4hrs of incubation at 75°C. Similar enzyme stability at high temperatures has been reported for a few *Bacillus* strains [65- 67] and thermophilic fungi [68]. Extremely high or low pH values generally resulted in complete loss in activity for most enzymes. The pH is also a factor in the

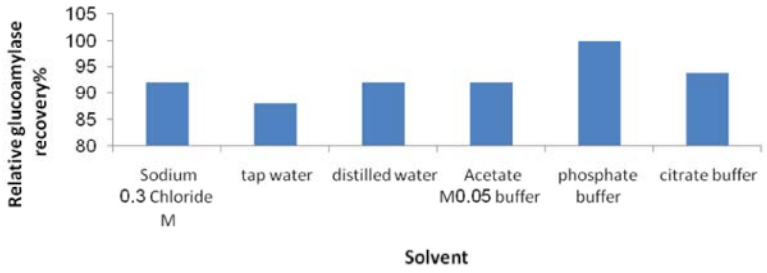

Fig. 1: Effect of different aqueous solution on glucoamylase recovery from fermented substrate by A. oryzae FK-923 grown on corn flour for 48hrs at 28°C.

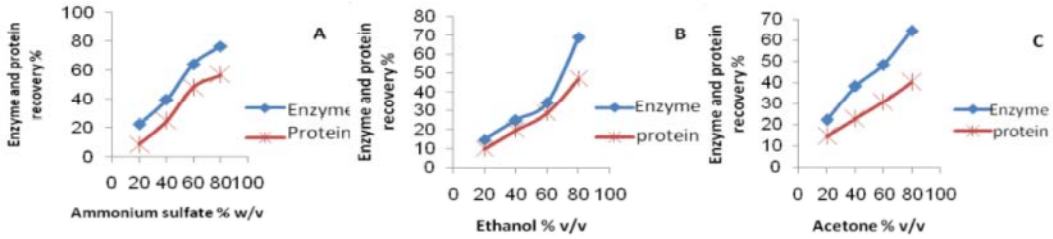

Fig. 2: Recovery of glucoamylase protein by ammonium sulfate (A), ethanol (B) and acetone (C).

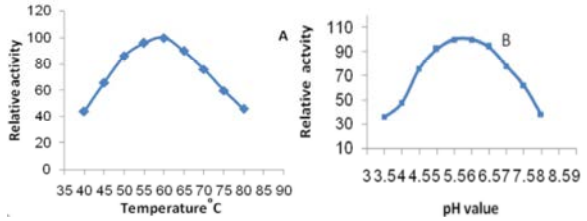

Fig. 3: Effect of temperature (A) and pH value (B) on glucoamylase activity produced by A. oryzae FK-923 after 72hrs of grown on CF at 28 °C by SSF 100% activity corresponded to 5582.5 U g-1 original CF

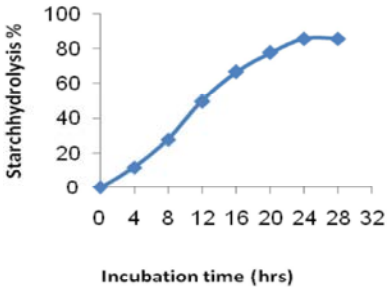

Fig. 4: Hydrolysis of starchy waste by crude glucoamylase extract

stability of enzymes. As with activity for each enzyme there is also a region of pH optimal stability. The enzyme exhibited maximum activity in a pH range of 5.0-6.5 and optimum was recorded at pH 5.5-6.0 (Fig. 3B).The enzyme also exhibited remarkable stability at pH values ranged from 5.0-6.5 for 4 hrs by retaining its original activity at these pH values. These results are in agreement with those reported previously by Polizeli *et al.* [69].

Hydrolysis of Starchy Waste: Fig. 4 illustrates that 86 % of starchy waste was hydrolyzed by 20% (v/v) crude extracted enzyme solution (extracted from one gm fermented corn flour by *A. oryzae* FK-923) in 25% slurry starch after 24 hrs of incubation at 55°C giving 18.6 % glucose, comparing with previous results, 64% hydrolysis was obtained when 25 % (v/v) culture filtrates of *A. niger* F-93 was applied for hydrolysis of 20% starch solution [13]. Glucose 204.5 gl^{-1} was obtained from enzymatic hydrolysis of 30% slurry of cassava starch [70]. Starch hydrolysis is accomplished by alpha-amylase for liquefaction and glucoamylase for saccharification. Different concentrations of commercial alpha- amylase and glucoamylase for used for hydrolyzing of cassava starch and found that 0.25 alpha- amylase and 0.15 glucoamylase were the best to give high glucose concentration, 204.5 g/l [71].

Fermentation of Enzymatic Hydrolyzate for Bioethanol Production. One of the greatest challenges for the 21 century society is to meet the growing demand for energy for transportation in a sustainable way. Petroleum based products are in critical state and alternatives is ethanol, which adds octane value to the gasoline blend and provides a cleaner burning. Fig. 5 illustrates the bioethanol fermentation of starch hydrolyzate obtained by

Table 8: Analysis of baker's yeast yield *S. cerevisiae* F-707 grown on glucoamylase starch hyrolyzate medium by shaking culture (250 rpm) at 34°C for 48 hrs.

Analysis	New baker's yeast	Egyptian Commercial Baker's yeast
Total viable cells /g 28 % total solids	1.9 x10^9	1.9 x 10^9
Total carbohydrates	39.4 %	38.8 %
Crude protein	49.6 %	51.4%
Nucleic acids	5.2 %	6.2%
UE	0.46	0.48
Total lipids	3.4%	2.8%
Ash content	7.6 %	7.6%
Fermentative power 1st h	850cc CO_2	850cc CO_2
Fermentative power 2nd h	1050cc CO_2	1050cc CO_2

Table 9: Chemical composition, cell wall constituents and gross energy for raw and fermented corn grains.

	Corn flour	
Item	Raw	Fermented
Chemical composition %		
Moisture Component, % on DM basis	10.07	11.96
OM	96.98	98.11
CP	9.82	18.45
CF	7.64	27.07
EE	3.10	10.13
NFE	76.42	42.46
Ash	3.02	1.89
Cell wall constituents, % on DM basis		
NDF	33.94	46.71
ADF	16.40	34.12
Hemicellulose	17.54	12.59
Gross energy, Kcal/ 100 g DM	434	484

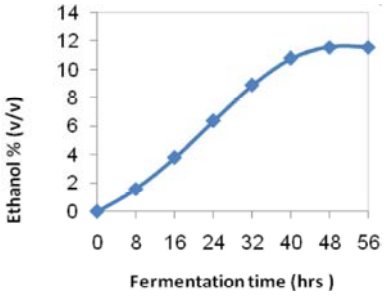

Fig. 5: Fermentation of enzymatic starch hydrolyzate for bio ethanol production.

crude enzymes extract eluted from culture *A. oryzae* FK-923 on CF by SSF inoculated by *S. cerevisiae* F-727. Maximum ethanol yield 11.5 v/v was obtained after 48 hrs. The final fermented mash involved 12 gl^{-1} yeast (on the basis of dry weight) and 0.5 % w/v remaining sugars, with fermentation efficiency 94.2 %. Ethanol 8.4% v/v was obtained from enzymatic starch hydolyzate by glucoamylase obtained from *A. niger* F-93 grown on starch waste by SSF [14]. Ethanol 10.9 % could produce from enzymatic starch hydrolyzate by alpha amylase and glucoamylase by *S. cerevisiae* [71]. However, the 10% inoculum was considered inadequate for the conversion of glucose to ethanol, ethanol 0.42 g/g sugar was obtained from enzymatic hydrolyzate of cassava waste using *S. cerevisiae* NCIM3640 [71], Many researchers used enzymatic starch hyrolyzates for ethanol production [73, 74].

Production of Baker's Yeast: Table 8 shows the data obtained from cultivating *S. cerevisiae* F-707 using enzymatic starch hydrolyzate as sole carbon source for production baker's yeast biomass and compared the obtained data with the commercial product from baker's yeast factory. Data reveals nearly the same total viable cells 1.9 x10^9 (CFU) and equal fermentative power for first and second hours.

Nutritive Value of Fermented Corn Flour Residue: The nutrient content of co-products classifies these feedstuffs as both high-protein and high-energy alternatives (Table 9). A large amount of research has been conducted studying the feeding value of these co-products. With respect to energy, grains have greater feeding value in comparison to corn. Also, the majority of research suggests that when used as a protein source it is equivalent to soybean meal and other sources of protein for beef cattle diets [75, 76, 77]. *Aspergillus oryzae* has apparently been an essential part of oriental food production for centuries and is now used in the production of many different oriental foods such as soy sauce, sake and miso. Potential uses include fermentations of numerous enzymes, e.g. amylase, protease, â-galactosidase, lipase and cellulase and organic compounds such as glutamic acid. While these products have a variety of potential commercial uses, some of them are mostly frequently used in food processing. The experience of safe commercial use of *A. oryzae* is extraordinarily well established. As a "koji" mold it has been used safely in the food industry for several hundred

years. *A. oryzae* is also used to produce livestock probiotic feed supplements. Chemical composition, cell wall constituents and gross energy for raw corn grains and fermented. (Table 9) show that protein was doubled since increase from 9.82 to18.45 % and EE doubled than three times from 3.1 to 10.13 % and this was due to the utilization of starch in CF by the fungus in amylolytic enzyme production. Data show also the reduction in hemicelluloses from 17.54 to 12.59 % content and this confirms and proves the ability of the fungus for producing hemicellulases and cellulases enzymes. The analysis clear that gross energy was increased from 434-485Kcal /100g DM. Enzymes assay showed that the residual fermented substrate loaded with glucoamylase 556, alpha-amylase 216, xylanase 224 and celullases (FPase 36 and CMCase 28) U/g.

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CONCLUSION

It has been demonstrated that *A. oryzae* FK-923 has the potential to utilize corn flour for production of glucoamylase enzyme. The glucoamylase activity of 5582.4 Ug^{-1}can obtained easily available substrate corn flour by *A. oryzae* FK-923 in SSF. Additional wheat bran, carbon (starch) and nitrogen (urea) supplements in corn flour under SSF increase glucoamylase yields. Our results obtained in this study is higher comparable to that obtained by other investigators using different substrates and microorganisms [42]. Wheat bran gave the highest enzyme production ($1602Ug^{-1}$) followed by rice bran ($1271Ug^{-1}$). (Wheat bran as the most promising substrate for glucoamylase production has been reported by Kaur *et al.* [16], Anto *et al.* [42] and Pandey *et al.* [46]. Production of starch-gel digesting amyloglucosidase under SSF using wheat bran, rice bran, other rice components and combination of these has been reported by Singh and Soni [84]. Rice husk and cotton seed powder yielded (875 Ug^{-1}). The glucoamylase activity of 1986 Ug^{-1} can be obtained on wheat bran under SSF. These findings can be very useful for enzyme industry. Beside the higher glucoamylase activity obtained, the new trend in our work is the utilization of fermented residue as safe valuable feed for cattle and poultry as source of protein, energy and enzymes [78-80, 85].

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