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Utilization of Agro-Industrial Waste Residues for the Production of Amylase from *Aspergillus oryzae* IIB-6

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Authors' contributions

This work was carried out in collaboration between all authors. Author BF performed bench work and statistical analysis. Author ZH conceived the work. Author MAK wrote the first draft of the manuscript. All authors equally participated in the preparation of the manuscript, read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to evaluate different solid substrates and moistening agents for the production of an extracellular 1, 4-α-D-glucan glucohydrolase from a newly isolated *Aspergillus oryzae* IIB-6 by solid state fermentation and optimization of culture conditions for the maximal production of enzyme.
Study Design: Six different agro-industrial waste residues (rice straw, rice bran, corn flakes, wheat bran, wheat flakes, and grinded wheat kernel) were procured from the local market. These substrates (10 g) were moistened (1:1) with different moistening agents (distilled water, tap water, mineral salts solution ($FeSO_4.7H_2O$ 0.02, $MgSO_4.7H_2O$ 1.0, $(NH_4)_2SO_4$ 4.0, KH_2PO_4 0.6, K_2HPO_4 1.4 mg/gds at pH 5), 0.1 N HCl, sodium acetate buffer (pH 5.5), sodium phosphate buffer pH 7.5) and screened for the production of 1,4-α-D-glucan glucohydrolase for 96 hours in static cultures. The substrate and moistening agent that gave maximum enzyme production were selected and their fermentation conditions were further optimized.
Place and Duration of Study: Institute of Industrial Biotechnology (IIB), GC University Lahore, Kachery Road Lahore, Pakistan, between February 2011 and March 2012.
Methodology: The levels of selected solid substrate, moistening agent and fermentation conditions such as pH, temperature, time of incubation, inoculum size etc. were optimized

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by one variable at a time method.
Results: Wheat bran at the level of 5 g and mineral salts solution containing $FeSO_4.7H_2O$ 0.016, $MgSO_4.7H_2O$ 0.8, $(NH_4)_2SO_4$ 3.5, KH_2PO_4 0.48, K_2HPO_4 1.12 mg/gds gave relatively best enzyme production. The maximum enzyme activity 7800 U/gds (407 Umg^{-1} protein) were achieved when wheat bran with 80% moisture content was incubated at 30°C, pH 5, after inoculating with 10% spore suspension (1.2×10^6 CFU/gds) for 72 h.
Conclusion: *Aspergillus oryzae* IIB-6 was a good producer of 1,4-α-D-glucan glucohydrolase in wheat bran medium containing mineral salts as an additional trace elements so that it can be used for biotechnological purposes.

Keywords: Agricultural by-products; Aspergillus oryzae; 1,4-α-D-glucan glucohydrolas; solid-state fermentation.

1. INTRODUCTION

Exo-1,4-α-D-glucan glucohydrolase (GGH, EC. 3.2.1.3) is an industrially important enzyme that releases glucose from non-reducing end of starch and related oligosaccharides, involved in the hydrolysis of α-1,4 faster than α-1,6 and α-1,3 linkages. GGH is second to the proteases in worldwide distribution and sales among industrial enzymes [1]. Currently, amylases have a great importance in biotechnology with a wide spectrum of applications, such as in textile, leather, detergents, food, brewery, starch processing and in various strategies in the pharmaceutical and chemical industries for the synthesis of optically pure drugs and agrochemicals [2,3]. GGH can be produced under both submerged fermentation (SmF) and solid state fermentation (SSF) conditions. However, the global demand of enzyme is growing faster than its production implying that more economical process is required. SSF has numerous advantages over SmF like simple technique, low capital investment, low energy requirement, less waste water output and better product recovery [4]. GGH is found in animals (saliva, pancreas), plants (malt), bacteria and filamentous fungi (mold). However, for commercial purposes, molds have been employed, because the enzymes of fungal origin were found to be more stable than bacterial. *Aspergillus oryzae* has GRAS (generally regarded as safe) status been employed in the traditional Japanese fermented foods production for more than 1000 years. These included soy sauce (shoyu), rice wine (sake) and soybean paste (miso). The hyphal mode of fungal growth and their good tolerance to low water activity (a_w) and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates. Again, the hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of SmF, what makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. Penetration increases the accessibility of all available nutrients within particles which make fungi more suitable for the production of hydrolases by SSF [5,6,7]. SSF involves the growth of microorganisms on moist substrates in the absence or near-absence of free flowing water and the solid substrate acts as a source of carbon, nitrogen, minerals and carrier necessary for microbial growth. Moreover, as the fungi in a solid substrate growing under similar to their natural habitat; they can produce certain enzymes, metabolites, proteins and spores more efficiently than in SmF [5].

Generally, agro-industrial by-products such as wheat bran, green gram bran, black gram bran, corn flour, barley flour, jowar flour, maize bran, rice bran, wheat rawa, molasses bran, maize meal, millet cereals, wheat flakes, barley bran, crushed maize, corn cobes and crushed wheat have been used as solid substrates for SSF. Different combinations of different solid substrates with many different fungi have been studied for the production of

GGH. Microbial degradation of these residues by GRAS strain may improve the substrate value as animal feed [8,9,10]. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new areas have opened for their utilization as raw materials for the production of value added fine products [11]. Recently, Ramachandran et al. [9] reported the use of coconut oil cake (COC) as a substrate for the production of amylase by *A. oryzae* under SSF conditions. Raw COC supported the growth of the culture, resulting in the production of 1372 U/gds amylase in 24 h. Moisture level of the solid substrate depleted with time course of fermentation due to evaporation and metabolic activities. Hence, the establishment of the optima of moisture contents is very essential for the production of enzyme. Temperature and pH of the growth medium play an important role by inducing morphological changes in microbes, cell growth, protein denaturation, enzyme inhibition and enzyme secretion. Similarly, inoculum density has a significant role in the development of biological process [5,12]. GGH production from *A. niger* was extensively studied using wheat bran in both SmF and SSF. Since utility of *A. oryzae* has not been investigated for the economical commercial production of enzyme, the present study is undertaken to evaluate the use of agro-industrial residues as solid substrate to cultivate *A. oryzae* IIB-6 for the production of GGH under optimized SSF conditions for biotechnological purposes.

2. MATERIALS AND METHODS

2.1 Chemicals and Microorganism

The chemicals used in the present study, were all of analytical grade, purchased directly from Sigma (USA), E-Merck (Germany), Acros (Belgium), Fluka (Switzerland) and BDH (UK). *Aspergillus oryzae* IIB-6 was isolated from soil samples collected from various localities of Doha (Qatar) as described previously [13]. It was propagated on potato dextrose agar (4%, pH 4.8) slants, stored in cold cabinet (P342; Griffin) at 4±2°C and rehabilitated twice a month.

2.2 Inoculum Preparation

Ten milliliters of sterilized 0.005% Monoxal-OT (Di-octylester of sodium sulpho succinic acid) were aseptically transferred to a 3-5 days old culture having profused growth. The clumps of spores were broken with the help of a sterilized inoculating wire loop. The homogeneous suspension was made by gently shaking the tube.

2.3 Measurement of Viable Spore Density

Equal volumes (100 µl) of spore suspension and 0.4% trypan blue stain were mixed. Affixed a glass cover-slip to the haemocytometer (Marienfeld, Germany) then pipette 10 µl trypan blue/spore mixture at the edge of the cover-slip and allowed to run under the cover slip. Visualise the haemocytometer grid under the microscope. The density of the spores was measured in accordance to Sharma [14] and was found to be 1.2×10^7 CFU/ml.

2.4 Solid State Fermentation

Commercial quality different solid substrates i.e., rice straw, rice bran, corn flakes, wheat bran, wheat flakes and grinded wheat kernel were procured from local market. Static experiments were conducted in 250 ml Erlenmeyer flasks containing 10 g of solid substrate

moistened with mineral salts solution ($FeSO_4.7H_2O$ 0.02, $MgSO_4.7H_2O$ 1.0, $(NH_4)_2SO_4$ 4.0, KH_2PO_4 0.6, K_2HPO_4 1.4 mg/gds at pH 5) to 1:1 moisture level [15]. The flasks were cotton plugged and autoclaved (Model: KT-40l, ALP Co, Ltd 3-3-10, Midorigaoka, Hamara-shi Tokyo Japan) for 15 min at 121±2ºC, 15-lbs/in² pressure. After cooling at room temperature the flasks were inoculated with 1 ml of spore suspension. The fermentation was carried out at 30±2°C for 96 hours in an incubator (Model: MIR-153, SANYO, Japan).

2.5 Estimation of Moisture Contents

Moisture contents (%) of the substrates were estimated [10] by drying various amounts of wheat bran to constant weight at 80±2°C for 15 min in hot air oven (Model: 545804, Memmert 854 Schwabach Germany) and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrate was soaked with the appropriate quantity of mineral salts solution. The sample was then dried as described above and moisture content was calculated as follows:

Moisture contents (%) = (wt. of solid substrate - dry wt.) ×100/dry wt.

2.6 Extraction of Enzyme

At the end of fermentation, sodium acetate buffer (0.05 M, pH 5) in a 1:10 ratio was added to individual Erlenmeyer flasks and agitated thoroughly on rotary shaking (200 rpm) incubator (Model: 10×400 XX2.C SANYO Gallenkamp PLC, UK) for 60 min at 30±2°C [16]. The contents were filtered through muslin cloth. The filtrate was centrifuged at 4±2°C, 13,000 rpm (25,900×*g*) for 15 min and the clear supernatant was used for further analysis.

2.7 Analytical Methods

GGH was assayed according to the method of Caldwell et al. [17]. One milliliter of diluted enzyme was incubated with 1 ml of soluble starch solution (5%, w/v) in sodium acetate buffer (0.05 M, pH 5) at 60±2°C for 60 min with constant stirring at 100 rpm. The reaction was stopped by addition of 2 ml of 3, 5-dinitrosalicylic acid (DNS) reagent [18]. The amount of reducing sugar liberated was quantified by measuring color intensity at 546 nm on a UV/VIS double beam scanning spectrophotometer (Cecil CE 100-series, Aquarius Inc., London, UK) using D-glucose as standard. One unit of activity was the amount of enzyme that liberates 1 µmol of glucose from 5% soluble starch at 60±2°C, pH 5.0, per min and enzyme activity was expressed in terms of units per gram dry substrate (U/gds). The estimation of protein contents was carried out according to Bradford [19] at 595 nm, using crystalline bovine serum albumin as the standard. All experiments were conducted parallel in triplicate.

2.8 Statistical Analysis

Treatment effects were compared by the protected least significant difference (LSD) method [20] using CoStat software (cs6204 w.exe.). Significant difference among the replicates has been presented as s Duncan´s multiple range in the form of probability (*P*=.05).

3. RESULTS AND DISCUSSION

Many attempts have been made to optimize culture conditions for GGH production from suitable strain of mold. In the present study these were investigated by traditional method involved varying one parameter at a time and maintaining pre-optimized SSF conditions, for the approximate optimization.

3.1 Evaluation of Different Agro-Industrial By-Products

Different solid substrates were used (Table 1), due to wide distribution of these by-products not only in Pakistan, but all over the world as agricultural residues; increases their availability to use as substrates on industrial scale for fungal GGH production. Among all the substrates tested, wheat bran with 2450±1.01 U/gds (257.35 U/mg protein, LSD~1.396) followed by wheat flakes with 2075±2.01 U/gds were the best substrates for enzyme production. Wheat bran is reported to give higher enzyme yield compared to rice bran and other agro-wastes and suitable for necessary manipulation [21,8,10,22]. Higher enzyme production using wheat bran and wheat flakes can be correlated with their starch content (75% and 73%, respectively). Moreover, naturally higher amount of the nitrogen also requires no or little addition of other nitrogen supplements in wheat bran containing medium. These elevated nitrogen contents of wheat bran makes it suitable for the production of enzymes such as proteases and amylases. Battestin and Macedo [23] reported that the presence of important mineral contents of wheat bran was essential for the mold growth resulting in an 8.6 fold increase in enzyme production. Similarly, Zambare [2] also observed wheat bran as the best substrate when compared with other solid substrates for the same enzyme production from *A. oryzae*. Rice bran and corn flakes with even higher starch contents (87% and 79.4%, respectively) gave (1.53 fold and 2.13 fold, respectively) less enzyme activity than wheat bran. This has been attributed to the carbon catabolite repression [24]. Similarly, other substrates did not support the best production. Hence, wheat bran was selected as the best substrate due to its unique nutrient composition for best enzyme production.

3.2 Evaluation of Different Levels of Wheat Bran

The level of solid substrate is vital in SSF, not only in tray processes but also in flask experiments. The level and nature of solid substrates influenced the porosity and aeration of the solid substrate [10]. Hence, diverse amounts (5-17.5 g/250 ml Erlenmeyer flask) of wheat bran were added to separate flasks and moistened with mineral salts solution (1:1) to study their effect on GGH production (Fig. 1). The highest enzyme activity 4700±2.30 U/gds (534 U/mg protein) was observed when 5 g wheat bran was used and varied significantly than others LSD~4.008. Protein contents were 8.8±0.18 mg/gds (LSD~0.152). Other scientists also reported 5 g wheat bran as an optimal for the production of enzyme [21,28,10,22]. As the level of wheat bran was increased beyond the optimal, the enzyme production was gradually decreased and insignificant enzyme activity 442±1.01 U/gds (138 U/mg protein) was observed when 17.5 g of wheat bran was used. Since, in large amount of substrate, the solid particles not only stick together to make clumps or mat, decreasing the porosity of the substrate but also occupy more space hence, reduces the air volume in the flask [2]. Consequently, 5 g was the sufficient amount of wheat bran for the best enzyme production.

Table 1. Evaluation of different agro-industrial by-products for GGH production from *A. oryzae* IIB-6 by SSF*

Solid Substrates	Botanical Name	Brand	Bioactive compounds (%)	Enzyme activity (U/gds)	Protein contents (mg/gds)	Specific activity (U/mg protein)
Wheat bran (coarse variety)	*Triticum aestivum*	-	Protein 16, Lipids 5, Total carbohydrates 75, Dietary fiber 53, Minerals (ash) 7.2, other components 2.8 [15,25].	2450±1.01	9.52±0.11	257.35
Wheat flakes†	*Triticum aestivum*	Faugi cereals	Total dietary fiber 12.8, Total carbohydrates 73, Protein 11.34, Lipids 2.41, Total sugar 8.7, Minerals (ash) 1.8 [10].	2075±2.01	8.61±0.01	240.99
Rice bran	*Oryza sativa*	-	Total dietary fiber 27, Total carbohydrates 87, Protein 16, Crude fat 30 (1.8 gum and 0.4 wax), Lipids 22.4 [7,26].	1600±2.08	7.33±0.06	218.28
Corn flakes†	*Zea mays*	Faugi cereals	Total fat 1.75, Total carbohydrates 79.4, total dietary fibers 3.58, total sugars 8, Protein 6.65, Minerals (ash) 0.8 [8].	1150±1.52	5.14±0.09	223.73
Rice straw	*Oryza sativa*	-	Cellulose 33, Hemicellulose 26, Lignin 7, Protein 4.2, Minerals (ash) 18.9 [2,27].	975±1.00	4.82±0.05	202.28
Grinded wheat kernel	*Triticum aestivum*	-	Protein 16, Lipids 2, Total carbohydrates 68, Dietary fiber 11, Minerals (ash) 1.8, other components 1.2 [10,25].	600±1.52	3.51±0.11	170.94
LSD	-	-	-	~1.396	~0.155	-

**± indicates the standard deviation (±S.D) of parallel replicates (n=3). Each mean value differ significantly at P = .05. Fermentation was carried out for 96 hours at moisture level 100%, temperature 30±2ºC and pH 5.0*

†Wheat/Corn flakes are produced from wheat/Corn, which is heated for a short time to about 135±2°C, after which the warm grain is rifle rolled. This process sterilizes the grain and makes the starch available

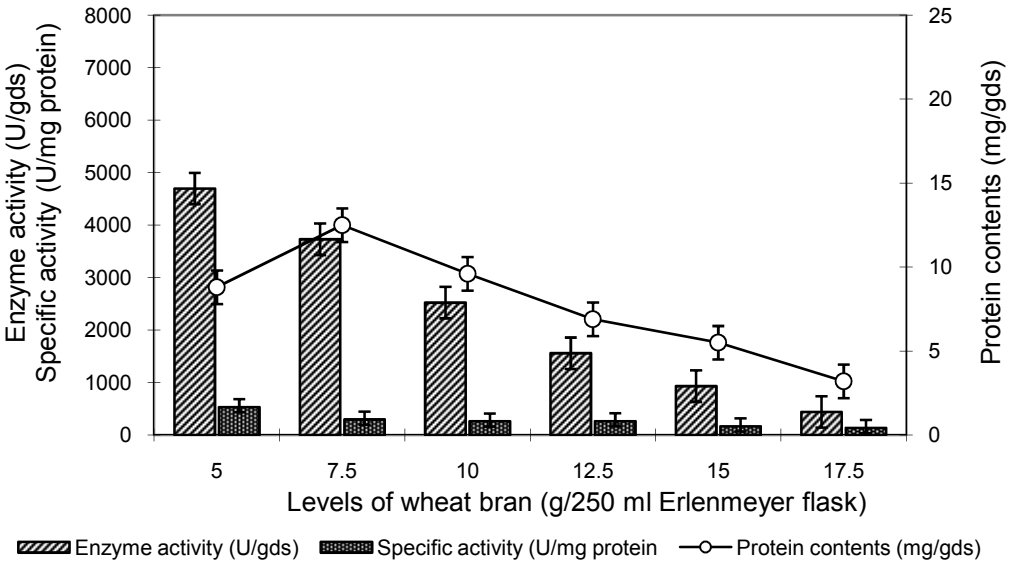

Fig. 1. Evaluation of different levels of wheat bran for GGH production from *A. oryzae* IIB-6 by SSF

Fermentation was carried out for 96 hours at moisture level 100%, temperature 30±2ºC and pH 5.0. Y-error bars show the standard deviation (± S.D) of parallel replicates (n = 3). Each mean value differ significantly at P = .05

3.3 Evaluation of Different Moistening Agents

Apart from the presence of important nutritional components, physical characteristics of wheat bran also play vital role in SSF process. Wheat bran has the ability to retain high moisture content in SSF. This ability of wheat bran promotes the mycelial growth just as in the natural environmental conditions. Thus wheat bran was moistened with different moistening agents viz., distilled water, tap water, mineral salts solution, sodium acetate buffer (pH 5.5), sodium phosphate buffer (pH 7.5), 0.1 N HCl, to 1:1 moisture level (Table 2). Distilled water was found to be (1950±2.64 U/gds) least effective for enzyme production with very low protein contents 3.5±0.02 mg/gds. Tap water gave 3100±1.73 U/gds as it promote growth of mycelia and accordingly protein contents were 7.01±0.02 mg/gds, but it was also not encouraging. Other workers obtained 2736 and 2833 U/gds from *A. oryzae* HS-3 when they used distilled water and tap water, respectively with wheat bran [21]. The maximum enzyme production 6900±1.01 U/gds (LSD ~4.590) was achieved when mineral salts solution was used. It promoted mycelial growth and the corresponding protein contents were raised to 15.41±0.03 mg/gds whih significantly varied than others LSD~0.0466. Similarly, other workers were also selected mineral salt solution as best diluent of wheat bran for optimum production of GGH from *Aspergillus sp.* [15]. Previously, Gosh et al. [29] also reported that in both liquid and solid cultivations, wheat bran was the most suitable substrate especially when supplemented with mineral salt solution for enzyme production from *A. terreus*. This is due to the fact that the type of nitrogen and phosphorus salts in the medium affected the enzyme production. Similarly, Selvakumar et al. [30] used mineral solution as the best moistening agent with tea waste for the enzyme production from *A. niger*. The enzyme activity was low down to 5500±2.64 U/gds; whereas specific activity was highest when 0.1 N HCl was used. It might be due to some acidic hydrolysis of substrate during

autoclaving as reported by Spets et al. [31] glucose as monosaccharide, can be produced by acid catalytic hydrolysis at temperature between 120 to 250°C from starch and cellulose as polysaccharides or it may be releases Ca ion chelated with phytic acid. Ca^{+2} ion is said to be important for the activity of amylase [32]. Sodium acetate and sodium phosphate buffers gave 4050±4.58 and 3300±2.01 U/gds, respectively. Sing and Soni [21] achieved 1480 U/gds using phosphate buffer (pH 7.5) with wheat bran from *A. oryzae* HS-3. Hence, mineral salts solution was selected as the best moistening agent.

Table 2. Evaluation of different moistening agents for GGH production from *A. oryzae* IIB-6 by SSF*

Moistening agents	pH	Enzyme activity (U/gds)	Protein contents (mg /gds)	Specific activity (U/mg protein)
Distilled water	6.8	1950±2.64	8.81±0.02	221.33
Tap water	7.8	3100±1.73	7.01±0.02	442.22
Mineral salts solution	5.0	6900±1.01	15.41±0.03	447.76
Sodium acetate buffer (0.05M)	5.5	4050±4.58	12.03±0.01	336.65
Sodium phosphate buffer (0.05M)	7.5	3300±2.01	6.61±0.02	499.24
0.1 N HCl	1.52	5500±2.64	3.35±0.01	1641.79
LSD	-	~4.590	~0.0466	-

**± indicates the standard deviation (±S.D) of parallel replicates (n=3). Each mean value differ significantly at P = .05. Fermentation was carried out for 96 hours at moisture level 100%, temperature 30±2°C and pH 5.0*

3.4 Evaluation of Different Levels of Moisture Contents

Reduction in enzyme activities is associated with early sporulation and also non-availability of nutrients because optimal moisture level or water activity is not available [2]. The critical importance of moisture level in SSF media, its influence on the bio-synthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles [2,10]. So, various initial moisture levels (20-120%, v/w, Fig. 2) and salts concentrations (Table 3) were also adjusted with mineral salts solution. The smallest amount (3500±2.64 U/gds) was observed when 20% of mineral salts solution was used. It is due to the fact that a low level of moisture content causes reduction in the solubility of nutrients of the substrate and a low degree of swelling which retarded the fungal growth and ultimately the enzyme production [2]. By increasing the level of the mineral salts solution to 60%, the enzyme production increased to 5600±2.64 U/gds which was 1.2 fold less than optimal. The optimum (6800±1.01 U/gds, LSD~4.108) enzyme activity was achieved when 80% mineral solution was used (protein contents 16.3±0.01 mg/gds, LSD~0.055). Similarly, Ellaiah et al. [8] also optimized 80% moisture level for the best GGH production from *Aspergillus* sp. The enzyme activities were decreased to 1.07 fold and 1.34 fold when moisture levels were increased to 100% and 120%, respectively. Because even high moisture contents affected the microbial enzyme activities because of substrate stickiness, clumps formation, less porous nature of substrate, higher soluble protein, enhanced aerial mycelial growth and very limited oxygen transfer [2]. Therefore, mineral salts solution at the level of 80% moisture content was optimized.

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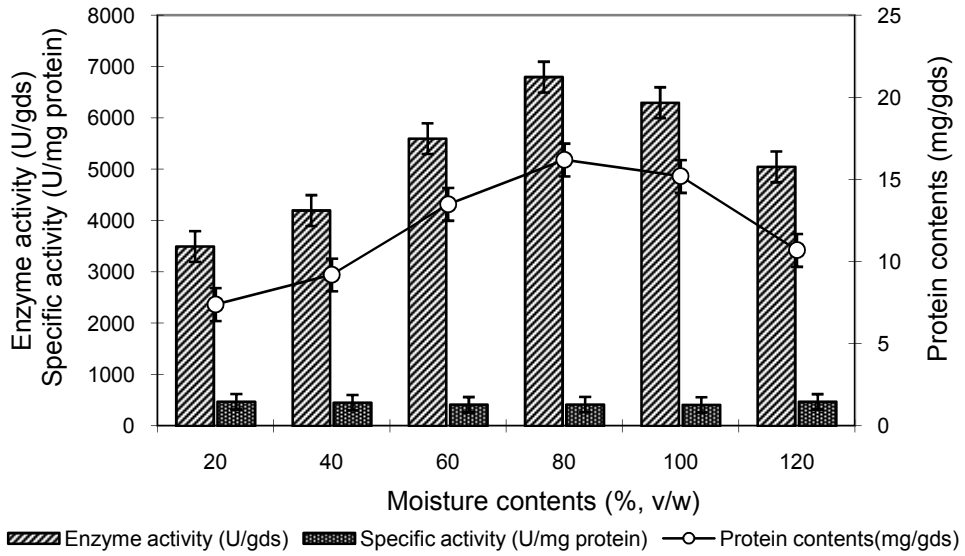

Fig. 2. Evaluation of different levels of moisture contents for GGH production from *A. oryzae* IIB-6 by SSF

Fermentation was carried out for 96 hours at temperature 30±2ºC and pH 5.0. Y-error bars show the standard deviation (± S.D) of parallel replicates (n = 3). Each mean value differ significantly at P = .05

Table 3. Mineral salts concentrations at different moisture levels in SSF

Moisture contents (%)	Mineral salts solution† (ml/5gds)	Mineral salts concentrations (mg/gds)
20	1	$FeSO_4.7H_2O$ 0.004, $MgSO_4.7H_2O$ 0.2, $(NH_4)_2SO_4$ 0.8, KH_2PO_4 0.12, K_2HPO_4 0.28
40	2	$FeSO_4.7H_2O$ 0.008, $MgSO_4.7H_2O$ 0.4, $(NH_4)_2SO_4$ 1.6, KH_2PO_4 0.24, K_2HPO_4 0.56
60	3	$FeSO_4.7H_2O$ 0.012, $MgSO_4.7H_2O$ 0.6, $(NH_4)_2SO_4$ 2.4, KH_2PO_4 0.36, K_2HPO_4 0.84
80	4	$FeSO_4.7H_2O$ 0.016, $MgSO_4.7H_2O$ 0.8, $(NH_4)_2SO_4$ 3.5, KH_2PO_4 0.48, K_2HPO_4 1.12
100	5	$FeSO_4.7H_2O$ 0.02, $MgSO_4.7H_2O$ 1.0, $(NH_4)_2SO_4$ 4.0, KH_2PO_4 0.6, K_2HPO_4 1.4
120	6	$FeSO_4.7H_2O$ 0.024, $MgSO_4.7H_2O$ 1.2, $(NH_4)_2SO_4$ 4.8, KH_2PO_4 0.72, K_2HPO_4 1.68

†pH = 5.0

3.5 Time Course Profile of GGH Production

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on rate of mycelial growth and consequently the enzyme production. Hence, the time course profile of GGH production was also investigated (Fig. 3). Fermentation was conducted for 12-96 hours. The enzyme production was very low

(400±1.73 U/gds) at 12 hours. The protein contents were 2.31±0.02 mg/gds. It was increased by increasing the incubation period but up to a certain extent. Maximal enzyme production (7100±2.64 U/gds, 398 U/mg proteins) was achieved after 72 hours of inoculation. It was very significant LSD~5.361 and approximately 17.75 fold higher than the enzyme activity obtained at 12 hours. The protein contents were 17.81±0.01 mg/gds (LSD~0.0322). This behavior pattern of results is in agreement with those obtained by other workers for the production of enzyme from *Aspergillus* sp. [33]. The enzyme activity was decreased when incubation period was increased beyond the optimal and became low down to 6750±1.73 U/gds at 96 hours after the start of the fermentation. This might be due to the depletion of nutrients and production of other metabolites in culture [12]. Hence, 72 hours was optimized as an adequate incubation period for enzyme production.

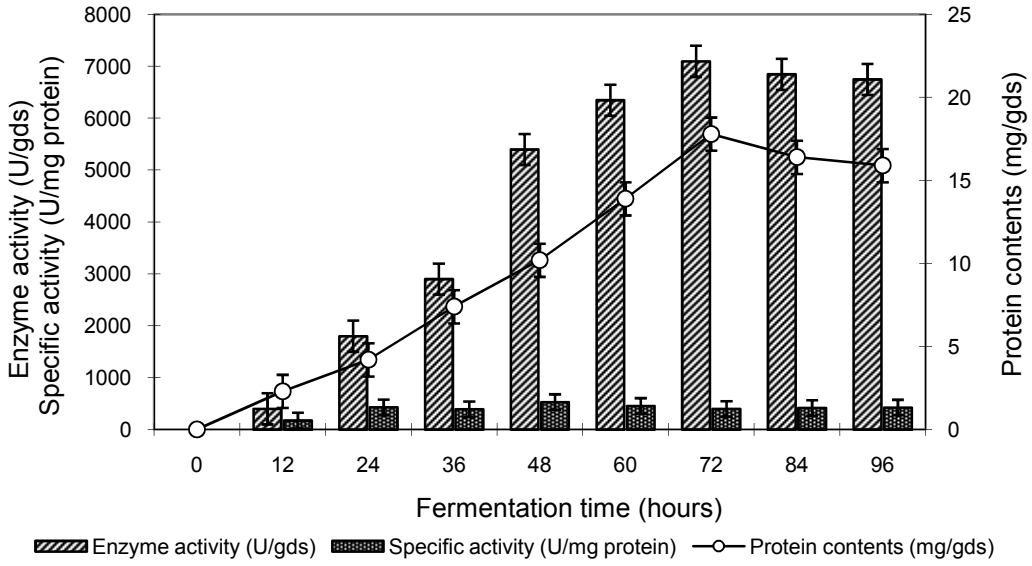

Fig. 3. Time course profile of GGH production from *A. oryzae* IIB-6 by SSF
Fermentation was carried out for 96 hours at moisture level 80%, temperature 30±2ºC and pH 5.0. Y-error bars show the standard deviation (± S.D) of parallel replicates (n = 3). Each mean value differ significantly at P = .05

3.6 Effect of Initial pH

Optimum pH is very important, the composition of cell wall and plasma membrane of microorganisms is known to be affected by the culture pH [34]. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor mycelial growth. The pH of the fermentation media may change since organic acids are produced during fermentation. The change in the initial pH of the medium may lead to change in the nature of the cell wall and cell membrane and hence affecting the growth of organism and GGH excretion just like its stability. These effects may be dependent on the ionic environment around the active site of the enzyme. Hence, the initial pH of the basal medium was varied (4.0-6.5) by maintaining the pH of the mineral salts solution with 1 N HCl and 1 N NaOH to study the diversity in the enzyme production at different pH (Fig. 4). At pH 4, the enzyme activity was 3700±1.73 U/gds (513 U/mg protein). It increased at pH 4.5 and found to be 5450±3.65 U/gds. The maximal (7000±2.01 U/gds) production of GGH was

achieved when the initial pH of the medium was adjusted to 5 and varied considerably (LSD~3.668) than other values. The protein contents were 17.5±0.01 mg/gds (LSD~0.069). Fungal cultures have been reported to give optimum enzyme production at pH 5 using various substrates [8]. Zambare [2] also reported optimum enzyme production from *A. oryzae* at pH 5. As relatively low values for enzyme production were recorded at neutral and alkaline conditions. So, when the pH was increased other than the optimal, the enzyme production was markedly decreased. Hence, the enzyme activities were 1.11, 1.34 and 1.56 fold less than the optimal, at pH 5.5, 6 and 6.5, respectively. Therefore, the initial pH 5 was optimized for the best production of enzyme.

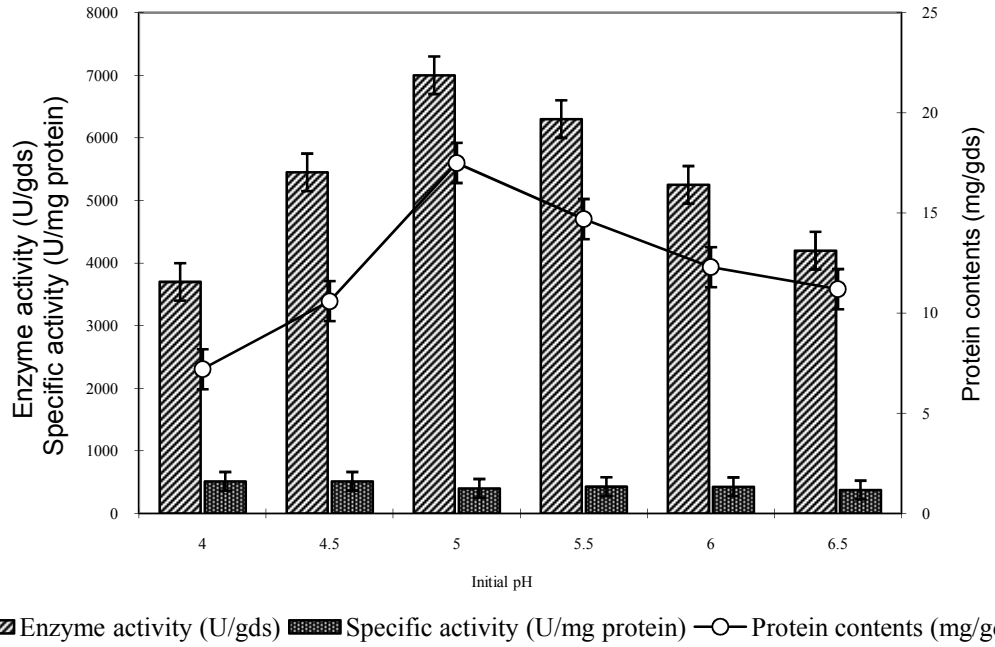

Fig. 4. Effect of initial pH on GGH production from *A. oryzae* IIB-6 by SSF

Fermentation was carried out for 72 hours at moisture level 80%, temperature 30±2ºC. Y-error bars show the standard deviation (± S.D) of parallel replicates (n = 3). Each mean value differ significantly at P = .05

3.7 Effect of Temperature

Growth temperature is a very critical parameter which varies from organism to organism depending on whether the culture is mesophilic or thermophilic and slight changes in growth temperature may affect GGH production. Because organisms have various mechanisms that allow them strictly to control secretion. Changes in the nature of cell envelop can affect the release of extracellular enzymes to the culture medium. Temperature is one of the factors that induces such changes in cell membrane and cell wall [35]. To ascertain the effect of temperature on enzyme production, the fermentation was carried out at various temperatures, ranging from 20 to 55°C (Fig. 5). Enzyme production was increased gradually with the increase in temperature from 20 to 30°C. Optimum enzyme production (7150±2.64 U/gds, LSD~4.086) was achieved at 30°C (protein contents 17.21±0.01 mg/gds, LSD~0.0318). It was approximately 1.27 fold higher than the enzyme activity obtained at

20°C. *A. oryzae* is a mesophilic fungus. At this temperature the membrane was highly permeable and the enzyme synthetic machinery of the organism was highly functional with the maximum conversion rate of the starch polymer into reducing sugars [12]. The present finding is in accordance with the work reported earlier with *A. oryzae* [2]. Almost same enzyme production was observed at the temperature ranging from 30 to 45°C. Somewhat, similar kind (30-40°C) of finding have also been reported by Singh and Soni [21] when they cultivated *A. oryzae* HS-3 on wheat bran. The GGH activities were decreased to 6050±1.73 U/gds (593 U/mg protein) and 4800±2.64 U/gds (500 U/mg protein) when temperature fell outside the mesophilic range, to 50 and 55°C, respectively, because of the reduction of the metabolic activity of the culture. Hence, 30°C was the best temperature for enzyme production.

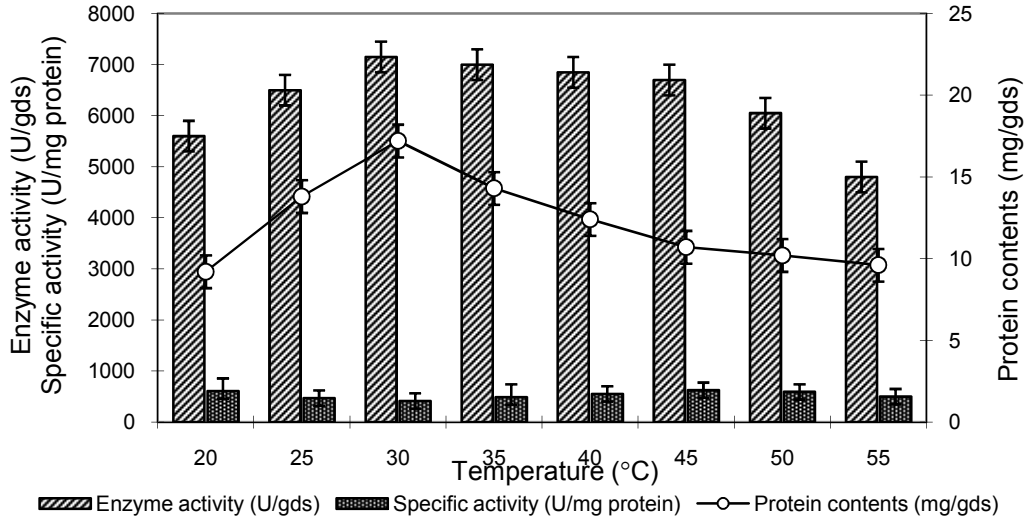

Fig. 5. Effect of temperature on GGH production from *A. oryzae* IIB-6 by SSF
Fermentation was carried out for 72 hours at moisture level 80% and pH 5.0. Y-error bars show the standard deviation (± S.D) of parallel replicates (n = 3). Each mean value differ significantly at P = .05

3.8 Effect of Inoculum Size

Varied amounts of spore suspension with different concentrations of colony forming units (CFU/gds) or spore density (Table 4) were added to fermentation medium to study the effect of different inoculum sizes (5-30%, v/w) on production of enzyme (Fig. 6). The least enzyme production (4100±1.73 U/gds, protein contents 10.3±0.01 mg/gds) was obtained when 5% inoculum was used. Because, lower inoculum levels require more time for fermenting the substrates in SSF still cultures [4]. It was increased and became maximum (7800±1.73 U/gds, LSD~3.859) when inoculum size was raised to 10%. The protein contents were 19.2±0.01 mg/gds (LSD~0.0291). It was 1.9 fold higher than the enzyme production achieved with 5% inoculum. Similarly, other workers reported that 10% inoculum size to be the best for GGH production from *Aspergillus* sp. [8,10]. The enzyme production (7100±2.64 U/gds; 408 U/mg proteins) was 1.09 fold less when the fermentation medium was seeded with 15% inoculum level. Since, higher inoculum levels, besides increasing spore

concentration, also increase water content of the solid substrate, thereby inhibiting mycelial growth and enzyme induction. Hence, 10% inoculum size was sufficient for the best production.

Table 4. Spore densities in different inoculum sizes

Inoculum size (%)	Spore suspension (ml/5gds)	(CFU† /gds)
05	0.25	6.0×10^5
10	0.50	1.2×10^6
15	0.75	1.8×10^6
20	1.00	2.4×10^6
25	1.25	3.0×10^6
30	1.50	3.6×10^6

† *Colony Forming Units*

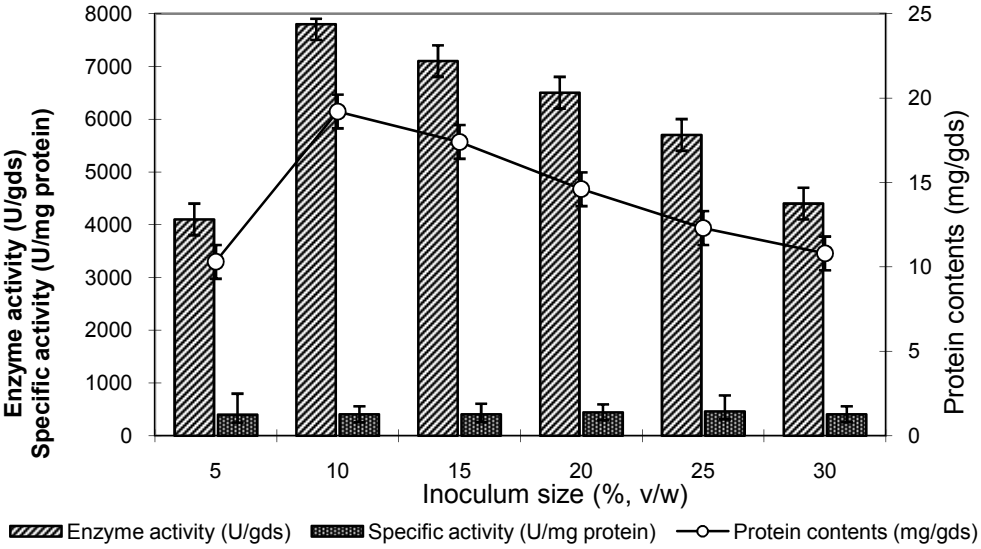

Fig. 6. Effect of inoculum size on GGH production from *A. oryzae* IIB-6 by SSF

Fermentation was carried out for 72 hours at moisture level 80%, temperature 30±2ºC and pH 5.0. Y-error bars show the standard deviation (± S.D) of parallel replicates (n = 3). Each mean value differ significantly at P = .05

4. CONCLUSION

From the above discussion, it was concluded that *A. oryzae* IIB-6 is a promising source of GGH as under optimized conditions a 3.18 fold higher amylase production was achieved. It can be employed for the economical production of enzyme for biotechnological and industrial applications, especially in food industries for glucose and high fructose corn syrup production and in starch industry for ethanol production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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