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SOLID STATE FERMENTATION FOR THE PRODUCTION OF B-GLUCOSIDASE BY CO-CULTURE OF *ASPERGILLUS NIGER* AND *A. ORYZAE*

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Abstract

Microbial consortium represents an important new frontier for biotechnology. It has been employed in many fields of biotechnology but its application for the production of β-glucosidase is yet to be explored. Therefore, present work has been aimed for the selection of co-culture of *Aspergillus* sp., and optimization of cultural conditions for the biosynthesis of β-glucosidase by solid state fermentation. For this purpose, mono and co-cultures of *Aspergillus niger*, *A. awamori* and *A. oryzae* were tested and co-culture of *A. niger* and *A. oryzae* gave comparatively better production of β-glucosidase. The enzyme production was optimal when agricultural by-product wheat bran (10 g) was used as solid substrate for fungal growth. Among 10 different diluents tested, M-2 containing (g/l, w/v) KH_2PO_4 2.0, $(NH_4)_2SO_4$ 1.4, urea 0.3, $MgSO_4.7H_2O$ 0.3, $ZnSO_4.7H_2O$ 0.0014, $FeSO_4.7H_2O$ 0.005, $MnSO_4$ 0.0016, $CoCl_2$ 0.002, $CaCl_2$ 0.002, polypeptone 1.0 and Tween-80 2.0 ml gave relatively higher enzyme production. The maximum production of β-glucosidase (2975±5.3 U/g/min) was obtained after optimization of cultural conditions such as incubation period (72 h), initial pH (5.5), substrate to diluent ratio (1:1) and inoculum size (2 ml of spore suspension i.e., 3:1 ratio of *A. niger* and *A. oryzae*).

Introduction

Beta-Glucosidase (EC 3.2.1.21) normally catalyzes the hydrolysis of β 1,4-glycosidic bond and releases D-glucose from the non-reducing ends of cellobiose and oligosaccharides (Harnpicharnchai *et al*., 2008; Cai *et al*., 1999). It is also considered to be the part of cellulase system, since it stimulates the rate and extent of cellulose hydrolysis by relieving cellobiose-induced inhibition of endo- and exo-β-glucanases (Wang *et al.*, 2009; Harnpicharnchai *et al*., 2008; Parry *et al*., 2001). This enzyme is of considerable industrial interest due to its extensive applications in the cosmetic, textile, detergents, grain wet milling, animal feed, tobacco and food industries, cassava detoxification, natural polymer modifications, organic chemical synthesis, diagnostics fields and deinking of printing ink from waste paper (Leite *et al*., 2007; Dhake & Patil, 2005). Microbial enzymes have the enormous advantage of being able to be produced in large quantities by solid state fermentation (Ray *et al*., 2007). Among large number of agro-industrial wastes (wheat bran, wheat straw, rice bran, rice husk, rice straw and bagasse etc.) are available as substrate for the production of enzyme, wheat bran contain plenty of cello-oligosaccharides that results in the increased activity of extracellular β-glucosidase (Sun *et al*., 2008).

Extracellular, intracellular and cell-wall-bound β-glucosidases has been reported in fungi, bacteria and yeast (Gunata & Vallier, 1999). Filamentous fungi are important in

industrial enzyme production, since they are able to synthesize and secrete large amounts of extracellular proteins (Oksanen *et al*., 2000). However, *Aspergillus* is by far the most efficient producer of β-glucosidase among the microorganisms investigated (Dan *et al*., 2000). Microbial consortium consisting of two or more different microorganisms is known to be largely responsible for many biotransformations in natural environment. Mixed culture fermentations are widely used in biotechnology for many processes including the production of antibiotics, enzymes, fermented food, composting, dairy fermentation, bioconversion of apple distillery and domestic wastewater sludge (Alam *et al*., 2001). In the present work efforts are made to optimize the cultural conditions for the production of β-glucosidase by using co-culture of *A. niger* and *A. oryzae* and we found the results quite encouraging.

Materials and Methods

Organism and culture maintenance: *Aspergillus niger, A. awamori* and *A. oryzae* were obtained from stock cultures of *Institute of Industrial Biotechnology*, GCU Lahore. Cultures were maintained on potato-dextrose-agar (PDA) slants and stored at 4ºC in a cold cabinet (Model: MPR-1410, SANYO, Japan).

Solid state fermentation: Solid-state fermentation was employed for production of β-glucosidase by fungal consortium. Twenty five milliliter of fermentation medium was transferred to the individual 250 ml cotton plugged Erlenmeyer flasks containing 10 g wheat bran. After autoclaving at 15 lb/inch^2 pressure and 121ºC for 15 min., and cooling the medium at room temperature, 1.0 ml of spore inoculum was transferred to each flask aseptically. The flasks were then incubated at 30±1ºC in an incubator (Model Gallen Kamp, England) for 72 h. All experiments were run parallel in duplicates. Spore count was measured with the help of hemacytometer.

Enzyme extraction: After 72 h incubation period, 100 ml of 0.05 M Sodium citrate buffer (pH 4.8) was added to the fermented substrate in each flask. The flasks were rotated on a rotary shaker at 200 rpm for 1 h at 30±1ºC. The fermented broth was centrifuged at 6000 rpm for 10 min., to get a clear supernatant which was analyzed for β-glucosidase activity.

Beta-glucosidase assay: Beta-glucosidase analysis was performed using pNPG as substrate after Rajoka & Malik (1997).

Enzyme unit: One unit of β-glucosidase is defined as "the amount of enzyme that catalyzes the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (pNPG) to liberate 1.0 µM of *p*-nitrophenol in one min under standard assay conditions"**.** It is expressed as U/g/min (where, g = gram of initial substrate used for growth i.e., wheat bran).

Diluents: Following mineral salt solutions (g/l) were evaluated as diluents for β-glucosidase production. The initial pH (5.0) of diluents was adjusted with 0.1 N NaOH/ HCl.

1. M-1: KH_2PO_4 0.5, KCl 1.0, $(NH_4)_2SO_4$ 0.5, $MgSO_4.7H_2O$ 0.2, L-asparganine 0.5, $CaCl_2$ 0.1 and yeast extract 0.5 (Eggins & Pugh 1962).

2. M-2: $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, urea 0.3, $MgSO_4.7H_2O$ 0.3, $ZnSO_4.7H_2O$ 0.0014, $FeSO_4.7H_2O$ 0.005, $MnSO_4$ 0.0016 , $CoCl_2$ 0.002, $CaCl_2$ 0.002, Tween-80 2.0 ml and polypeptone 1.0 (Mandel & Reese 1960).

3. M-3: $(NH_4)_2SO_4$ 1.4, KH_2PO_4 1.4, urea 0.3, $MgSO_4.7H_2O$ 0.3, $CaCl_2.2H_2O$ 0.3, Tween-80 0.5 ml, proteose peptone 0.75 ml and trace element solution 10 ml (Romanelle *et al*., 1975).

4. M-4: $(NH_4)H_2PO_4$, KH_2PO_4 0.6, K_2HPO_4 0.4, $MgSO_4.7H_2O$ 0.5, yeast extract 1.0, thiamine HCl 100 mg and trace element solution 10 ml (Coutts & Smith 1976).

5. M-5: KH_2PO_4 5.0, NH_4NO_3 2.0, $(NH_4)_2SO_4$ 4.0, $CaCl_2$ 0.1, $MgSO_4.7H_2O$ 0.2, yeast extract 2.0, polypeptone 1.0 and trace element solution 10 ml (Saddler 1982).

6. M-6: Na_2HPO_4 1.2, KH_2PO_4 2.0, $(NH_4)_2HPO_4$ 7.0, $CaCl_2$ 0.3, $MgSO_4.7H_2O$ 0.3, yeast extract 0.1, proteose peptone 0.25, tween-80 0.3 ml and trace element solution 10 ml (Macris & Panayatou 1989).

7. M-7: $(NH_4)_3HPO_4$ 0.89, KH_2PO_4 0.6, K_2HPO_4 0.4, $MgSO_4.7H_2O$ 0.5, $FeSO_4.5H_2O$ 0.01, $ZnSO_4.7H_2O$ 0.0044, $MnSO_4$ 0.0025, $CaCl_2.2H_2O$ 0.05, yeast extract 1.0, $CoCl_2.6H_2O$ 0.001 and thiamine HCl 0.0001 (Almin *et al*., 1975).

8. M-8: $NaNO_3$ 2.0, KCl 2.0, NH_4NO_3 1.0, $(NH_4)_2HPO_4$ 1.0, $MgSO_4.7H_2O$ 0.5 and yeast extract 0.5 (Riou *et al.,* 1998).

9. M-9: $NaNO_3$ 0.5, yeast extract 2.0, $CaCl_2$ 0.1, $CoCl_2$ 0.005, K_2HPO_4 2.0, KH_2PO_4 1.0 and peptone 1.0 (Modified Almin *et al.,* 1975).

10. M-10: KH_2PO_4 5.0, $(NH_4)_2SO_4$ 5.0, urea 1.5 and yeast extract 3.0 (Modified Romanelle *et al.,* 1975).

Trace element solution: H_3BO_3 0.06, $(NH_4)_6MO_7O_{24}.4H_2O$ 0.26, $FeCl_3.6H_2O$ 0.10, $CuSO_4$ 0.40, $MnCl_2$ 0.08 and $ZnCl_2$ 2.0.

Statistical analysis: Treatment effects were compared using computer software Costat. Significant differences among the replicates have been presented as Duncan's multiple ranges in form of probability ($p \leq 0.05$) values.

Results and Discussion

Screening of *Aspergillus* sp.: Microbial consortium is ubiquitous in nature and can perform more complicated tasks than monoculture. In the present work, three different strains of *Aspergillus* i.e. *A. niger*, *A. oryzae*, *A. awamori* and their co-cultures were screened for the production of β-glucosidase by solid state fermentation (Table 1). The co-culture of *A. niger* and *A. oryzae* gave the maximum production (1475±5.35 U/g/min) of β-glucosidase.

Table 1. Screening of mono-culture and co-cultures of *Aspergillus* sp., for the production of β-glucosidase using solid state fermentation*.

Organism	Enzyme activity (U/g/min)
A. niger	305 ± 7.07
A. awamori	295 ± 8.12
A. oryzae	875 ± 35.5
A. awamori + *A. niger*	317.5 ± 10.61
A. oryzae + *A. niger*	1475 ± 5.35
A. oryzae + *A. awamori*	462.5 ± 7.67
A. awamori + *A. oryzae* + *A. niger*	82.5 ± 10.61

*Incubation period 72 h, Incubation temperature 30ºC

± indicates standard deviation among the two parallel replicates, which differ significantly at $p \leq 0.05$. LSD=5.225.

Selection of substrate: Selection of agricultural by-products (tea waste, saw dust, wheat bran, wheat straw and rice straw) as substrate for the production of β-glucosidase is shown in Fig. 1. The maximum production of β-glucosidase (1525±6.5 U/g/min) was obtained when wheat bran was used as substrate. It is due to the reason that wheat bran contains adequate amount of carbohydrates, proteins, fats, fiber, ash, Ca, Mg, P, K, S and various amino acids that are essential for the fungal growth and enzyme production. This study is directly substantiated with the findings of other workers (Balkan & Ertan 2006; Rajoka *et al*., 2006).

Selection of diluent: Fig 2 depicts evaluation of ten different diluents (M-1, M-2, M-3, M-4, M-5, M-6, M-7, M-8, M-9 and M-10) for β-glucosidase production. The maximum enzyme production was obtained with M-2 (containing g/l: $MgSO_4.7H_2O$ 0.03, $CaCl_2.2H_2O$ 0.0002, $(NH_4)_2SO_4$ 0.14, urea 0.03, KH_2PO_4 0.2, $ZnSO_4.7H_2O$ 0.00014, $FeSO_4.7H_2O$ 0.0005, $MnSO_4.7H_2O$ 0.00016, $CoCl_2.2H_2O$ 0.0002, polypeptone 0.1 and Tween 0.2 ml). This might be due to the fact that M2 medium contains all additional nutrients, organic (urea and peptone), inorganic (ammonium sulphate) nitrogen sources and high concentration of Tween 80 that have been reported to increase the production of β-glucosidase (Chellapandi & Jani, 2008).

Effect of different volumes of diluent: The effect of different volumes (5, 10, 15, 20 and 25 ml) of the diluent (M-2) on biosynthesis of β-glucosidase by co-culture of *A. niger* and *A. oryzae* is shown in Fig 3. The maximum production of β-glucosidase was obtained when 10 g of wheat bran was moistened with 10 ml of optimized diluent (M-2) i.e., when substrate to diluent ratio was kept 1:1. It may be because high volume of diluent resulted in insufficient air supply which in turn reduces the microbial growth and enzyme production. Similar findings have been reported by Biesebek *et al*., (2005).

Time course study: In the present study, rate of β-glucosidase production by mix culture of *A. niger* and *A. oryzae* was also investigated (24-120 h). The enzyme production increased with increase in incubation period and reached maximum 72 h after inoculation (Fig. 4). Further increase in the incubation time decreased the production of β-glucosidase. It might be due to the depletion of nutritions, accumulation of harmful by-products in the culture medium and proteolysis of enzyme. This finding is in accordance to the finding of Tsao *et al*., (2000).

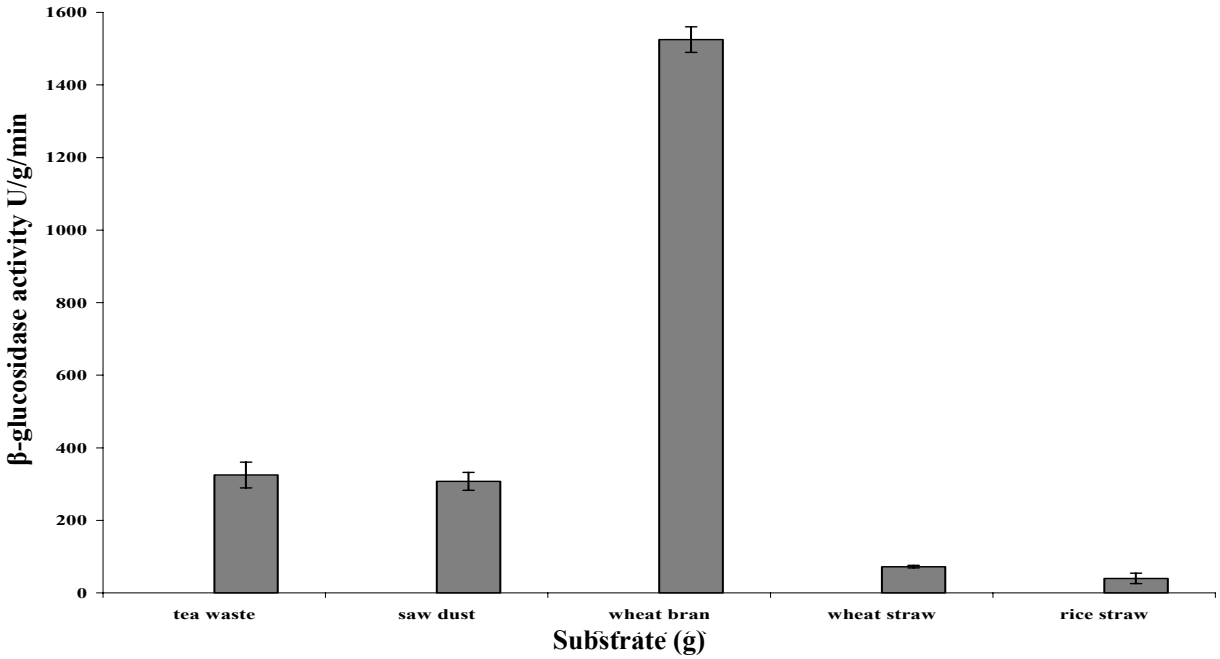

Fig. 1. Selection of substrate for the production of β-glucosidase by co-culture of *A. niger* and *A. oryzae**.
*Incubation period 72 h, Incubation temperature 30˚C
Y-error bars indicate the standard deviation (±SD) among the two parallel replicates, which differ significantly at p0.05.LSD=6.149.

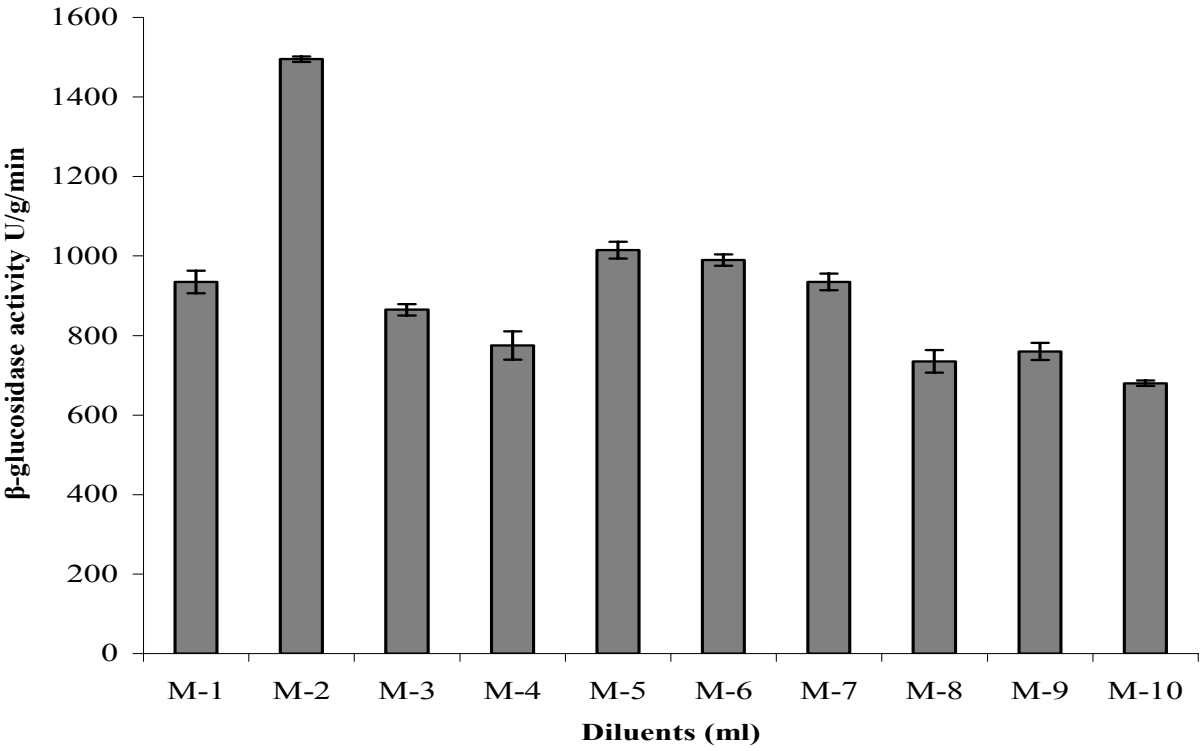

Fig. 2. Selection of diluent for the production of β-glucosidase by co-culture of *A. niger* and *A. oryzae**.
*Incubation period 72 h, Incubation temperature 30°C
Y-error bars indicate the standard deviation (±SD) among the two parallel replicates, which differs significantly at p≤0.05.LSD=4.409.

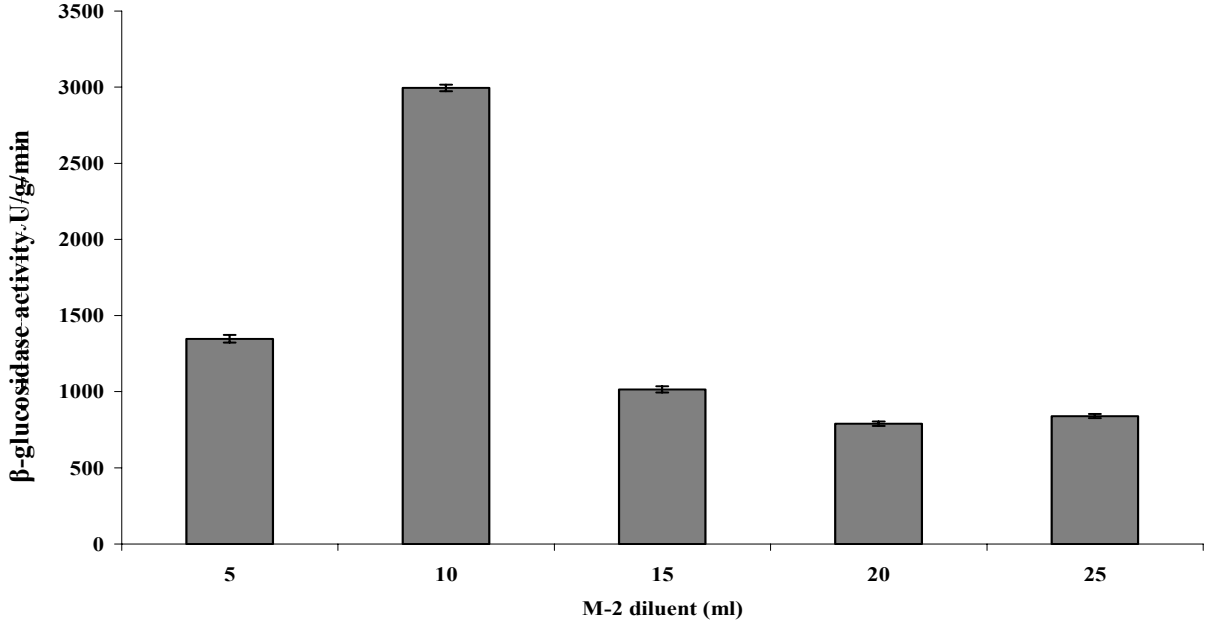

Fig. 3. Effect of different volumes of diluent on the production of β-glucosidase by co-culture of *A. niger* and *A. oryzae**.
*Incubation period 72 h, Incubation temperature 30°C, initial pH 5.0
Y-error bars indicate the standard deviation (±SD) among the two parallel replicates, which differ significantly at p≤0.05.LSD=5.1006.

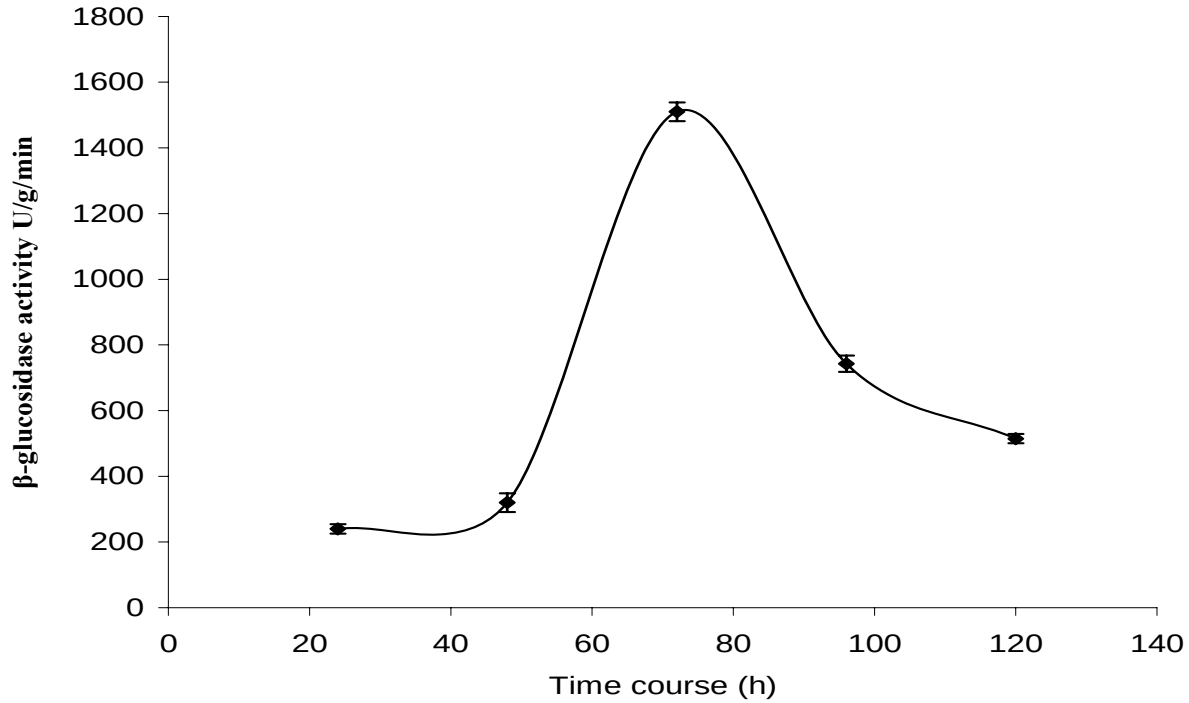

Fig. 4. Time course study for the production of β-glucosidase by co-culture of *A. niger* and *A. oryzae**.
*Incubation temperature 30°C, initial pH 5.0
Y-error bars indicate the standard deviation (±SD) among the two parallel replicates, which differ significantly at p≤0.05.LSD=2.012.

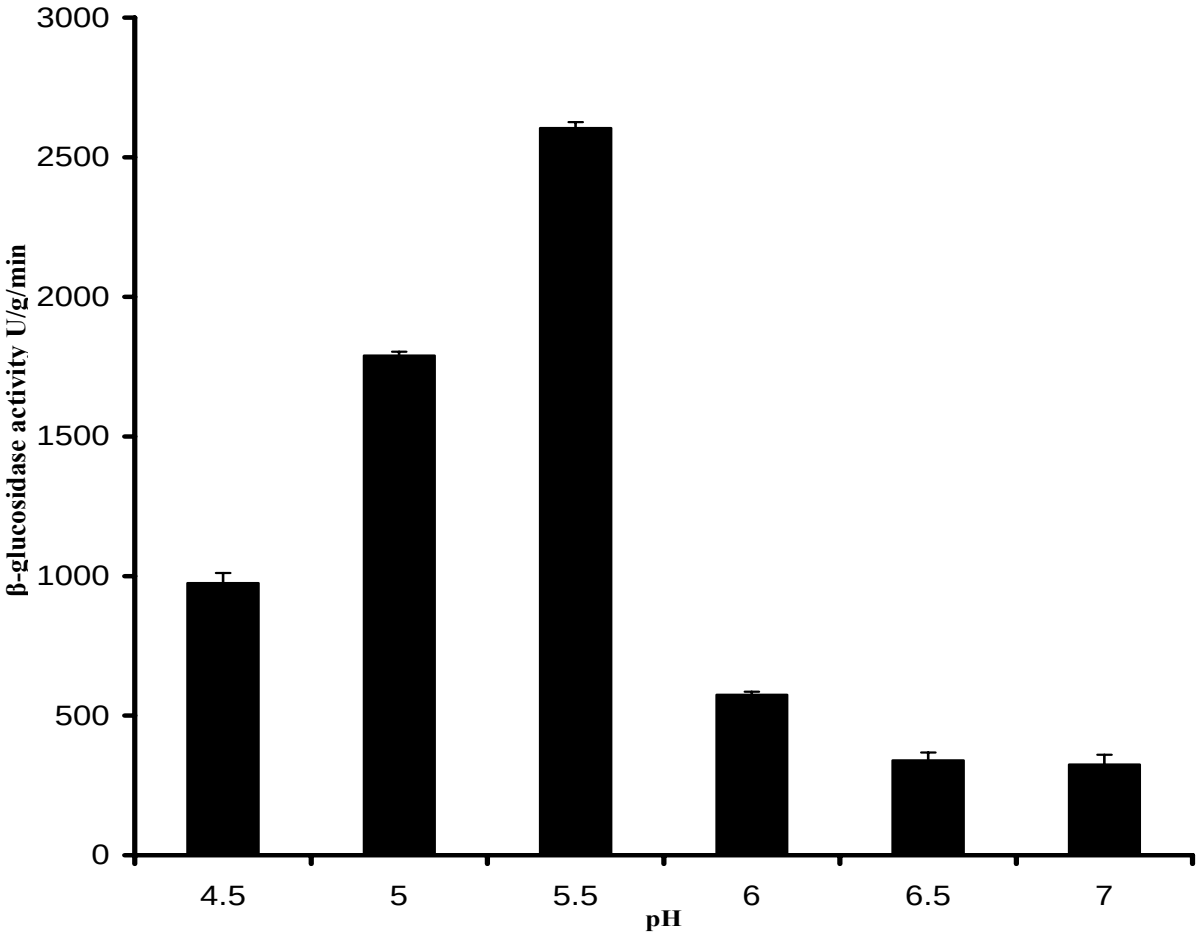

Fig. 5. Effect of initial pH on the production of β-glucosidase by co-culture of *A. niger and A. oryzae**.
*Incubation temperature 30°C, Incubation period 72 hrs
Y-error bars indicate the standard deviation (±SD) among the two parallel replicates, which differ significantly at p≤0.05.LSD=7.173.

Effect of initial pH: In the present investigation, the effect of different initial pH (4.5-7.0) of mineral salt solution was also worked out for β-glucosidase production (Fig 5). The optimal β-glucosidase activity was obtained at initial pH 5.5. Any variation from this optimal pH value resulted in reduced enzyme production. This might be due to the fact that cultivation of fungi at an unfavourable pH value may result in reduced enzyme production by reducing accessibility of the substrate (Bakri *et al*., 2008). In contrast, Abdel-Fattah *et al*., (1997) obtained maximum β-glucosidase production at a pH of 4.0.

Effect of inoculum size: The effect of inoculum size (0.5-2.0 ml/10 g of wheat bran) on the production of β-glucosidase by *A. niger* and *A. oryzae* is shown in Table 2. The maximum production of β-glucosidase (2975±5.35 U/g/min) was obtained in the fermentation flask that was inoculated with 1.5 ml of *A. niger* and 0.5 ml of *A. oryzae* conidia. Further increase or decrease in the size and ratio of inoculum, however, resulted in the decreased β-glucosidase production. Thus, optimum level of conidial inoculum was 2.0 ml (i.e., 3:1 ratio of *A. niger* and *A. oryzae* conidial inoculum) for the production of β-glucosidase. Decreased enzyme production at high inoculum levels might be due to the production of inhibitory metabolites that interfer with the enzyme production (Niladevi & Prema, 2008). Abdel-Naby *et al*., (1999) also reported maximum β-glucosidase activity with 2% spore inoculum.

Table 2. Effect of inoculum size on the production of β-glucosidase by consortia of *A. niger* and *A. oryzae.**

Aspergillus niger (ml)	*Aspergillus oryzae* (ml)	Enzyme activity (U/g/min)
0.5	0.5	1065 ± 14.1
0.25	0.75	1085 ± 2.21
0.75	0.25	1430 ± 9.0
1.0	1.0	875 ± 3.3
0.5	1.5	1740 ± 14.7
0.75	1.25	1235 ± 21.1
1.25	0.75	1190 ± 14.3
1.5	0.5	2975 ± 5.35

*Initial pH 5.5, Incubation period 72 h

The ± indicates standard deviation among the two parallel replicates, which differ significantly at $p \leq 0.05$. LSD=6.176.

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