Malaysian Journal of Microbiology, Vol 10(1)2014, pp. 48-56

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

Paddy husk support for large scale solid state production and, extraction and stabilization of glucoamylase

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Received 18 June 2013; Received in revised form 1 October 2013; Accepted 9 October 2013

Aims: Determination of the age of the mycelial inoculum is essential for the large-scale enzyme production. For small-scale cultivations of fungi, spore inoculum is generally used. Use of spore inoculum becomes difficult when large-scale cultivations are carried out and mycelia inocula become practically feasible. Similarly enzyme extraction in large-scale processes also makes it difficult to get higher yields of enzyme. Therefore this study was carried out.
Methodology and results: Suitable age of the mycelial inoculum was 24 h and produced the highest glucoamylase activity (1735 U g / DS; Dry Substrate) on the 2nd day. When mouldy medium was used as inoculum, maximum glucoamylase produced in the first, second and third batches were 1205, 2101 and 1360 U / g DS (2nd day) respectively. Glucoamylase obtained by counter current extraction (100%) was the best among one- (33.5%), two- (42.9%) and three- (48.2%) step and counter current extraction procedures. When glucoamylase was diluted six times, the enzyme lost 41% of its activity initially during the first 24h and thereafter no significant loss of activity was observed up to 90 days. Glycerol of 5% (v/v) and above stabilized glucoamylase at 30 °C. Ammonium sulfate inhibited and decreased the stability of glucoamylase while addition of glycerol of different concentrations did not reverse the effect of ammonium sulfate on glucoamylase.
Conclusion, significance and impact study: Suitable age of the mycelial inoculum was 24 h. Highest amount of glucoamylase was extracted by counter current extraction method. Glycerol concentration of 5% (v/v) and above stabilized the glucoamylase at 30 °C.

Keywords: Paddy husk, large scale, solid state fermentation, extraction, stabilization, glucoamylase

INTRODUCTION

Solid-state fermentation offers potential advantages for the filamentous fungi, which are generally capable of penetrating into the hardest of the solid substrates. The inoculum age and size directly affect microbial growth and enzyme production. A larger inoculum size supported the enzyme production while younger cultures entered the growth phase soon (Sharma and Satyanarayana, 2012). Different studies were performed in relation to SSF for glucoamylase production by *Aspergillus niger* (Arasaratnam *et al.*, 1997; Selvakumar *et al.*, 1998; Arasaratnam *et al.*, 2001, Silvinski *et al.*, 2011). Different solid media such as cassava meal (Raimbault and Alazard, 1980), wheat bran (Ramadas *et al.*, 1996; Anto *et al.*, 2006; Balkan and Ertan, 2007; Jacob and Prema, 2008), corncob (Balkan and Ertan, 2007), rye straw (Balkan and Ertan, 2007), wheat straw (Balkan and Ertan, 2007; Iqbal *et al.*, 2011), rice flake (Anto *et al.*, 2006), rice chaff (Gopinath *et al.*, 2011), rice bran (Jacob and Prema, 2008; Rangaswamy, 2012), green gram husk (Sharada *et al.*, 2012), etc. were used in solid state fermentation studies. The use of wheat bran is not feasible in our country due to its unavailability. An alternative source, rice bran was not attractive (Arasaratnam *et al.,* 1997). Hence for glucoamylase production in SSF, paddy husk was used as a support and additional nutrients such as soy meal powder, wheat flour; organic and inorganic nitrogen sources were supplemented (Arasaratnam *et al.,* 1997; Arasaratnam *et al.,* 2001). The medium and culture conditions have been optimized and the glucoamylase activity was increased from 875 to 1853.7 U/g Dry Substance (Arasaratnam, *et al.,* 2001). In this paper we report the large laboratory scale production of glucoamylase under optimized conditions and screening a method for the extraction of glucoamylase from mouldy husk. For small-scale cultivation of the fungi, spore inoculum is generally used. Use of spore inoculum becomes difficult when large-scale cultivations are carried out and mycelial inoculum becomes practically feasible. Thus before going into large-scale glucoamylase production the possibility of using mycelial inoculum was determined.

Industrially, once the enzyme is produced, its activity has to be preserved and protected until it is used. Storage for long periods and transport over long distances are important for the commercial benefit of most enzymes. Protein engineering by different methods

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

(Liu and Wang, 2003) and O-glycosylation of glucoamylase have improved the stability (Lenders and Crichton, 1988; Williamson *et al.*, 1992). Inclusion of additives to enzyme solution changes its micro-environment and provides a simple but practical means of increasing the stability of the enzyme (Larsson *et al.*, 1989; Bandivadekar and Deshpande, 1994; George *et al.*, 2001). Addition of copper ions has improved the thermo-stability of glucoamylsae from *Aspergillus niger* (Rangab hashiyam *et al.*, 2011). Very few literatures are available on stabilizing enzymes at room temperature and their preservation at ambient temperature. Hence various cheap chemical agents that could stabilize and preserve the enzyme activity were tried for glucoamylase stabilization.

MATERIALS AND METHODS

Microorgansim

Aspergillus niger CFTRI 1105 Central Food Technological Research Institute, Mysore, India was subcultured once in every two weeks on PDA slants and stored at 4 °C. Whenever the spores were required, they were suspended in 1% (v/v) Tween 80 and the number of spores was counted.

Solid-state medium

The *Aspergillus niger* CFTRI 1105 was cultivated in a solid medium consisting of g/kg: paddy husk, 460; soy meal powder, 200; mineral solution, 20 and tap water, 180 at pH 4.0 and 30 °C. The mineral solution contained (g/L): $ZnSO_4 \cdot 7H_2O$, 0.7; $FeSO_4 \cdot 7H_2O$; 0.7; $CuSO_4 \cdot 5H_2O$, 0.7 and 2N HCl, 1000 mL (Arasaratnam *et al.,* 2001).

Extraction of enzyme

Mouldy medium (500 g) was mixed with 2.5 L sterile distilled water for 30 min, at 120 rpm at room temperature and the enzyme was extracted with the help of a screw press. This laboratory scale screw press contains two stainless steel plates attached to the screws, which are operated manually. In between the plates the mouldy medium suspended in distilled water was kept in a muslin cloth and the pressure is applied. The working volume of the screw press is 500 mL to 1 L.

Enzyme assay

The enzyme activity was determined as described previously (Arasaratnam *et al.*, 1997) and the activity of glucoamylase is presented as U/g DS (dry substrate) or Unit per mL.

All the data presented are the mean of three experiment values, where each experiment was carried out in triplicate.

Production of glucoamylase in media inoculated with spores and mycelial inocula of different ages

First the mycelia inocula of *A. niger* from SSF of different ages were developed by inoculating the spores (6 days old, 2 X 10^7 spores g / wet medium) to solid medium and incubating for different periods (0, 24, 48, 72 and 96 h) at 30 °C. Then the solid inocula of different ages were inoculated to solid medium (10%, w/w) and incubated at 30 °C. Glucoamylase produced was monitored.

Continuous batch glucoamylase production with mycelial inoculums

Fresh solid-state medium inoculated (10%, w/w) with mouldy medium of optimized age and incubated for 48 h at 30 °C and the procedure was repeated till the enzyme production reduced.

Extraction of glucoamylase from mouldy medium

One-step extraction

Mouldy medium (1 kg, 60% moisture content) was mixed with sterile distilled water (5.0 L) at 300 rpm for 30 min at 30 °C and the enzyme was extracted with a filter press.

Two-step extraction

Mouldy medium (1 kg, 60% moisture content) was mixed with sterile distilled water (2.5 L) at 300 rpm for 30 min at 30 °C and the enzyme was extracted with a filter press. Then the residue was mixed with sterile distilled water (2.5 L) at 300 rpm for 30 min at 30 °C and the enzyme was extracted with a filter press.

Three-step extraction

Mouldy medium (1 kg, 60% moisture content) was mixed with sterile distilled water (3.0 L) at 300 rpm for 30 min at 30 °C and the enzyme was extracted with a filter press. Then the residue was mixed with sterile distilled water (1.0 L) at 300 rpm for 30 min at 30 °C and the enzyme was extracted. Third extraction was done with 1.0 L distilled water.

Counter current extraction

This process was carried out at 30 °C. Mouldy medium (1 kg) was divided into five 200 g portions (labeled as A1, to A5). To each 1 L of distilled water was added and the enzyme was extracted as mentioned above. Extract from A1 was preserved (Extract X1) while the extracts of A2, A3, A4 and A5 were added to the residues of A1, A2, A3 and A4 (residues named as B1, B2 , B3 and B4 respectively) and enzyme was extracted as above (Figure 1). The extract of B1 was preserved (Extract X2) and other extracts of B2, B3 and B4 were added to the

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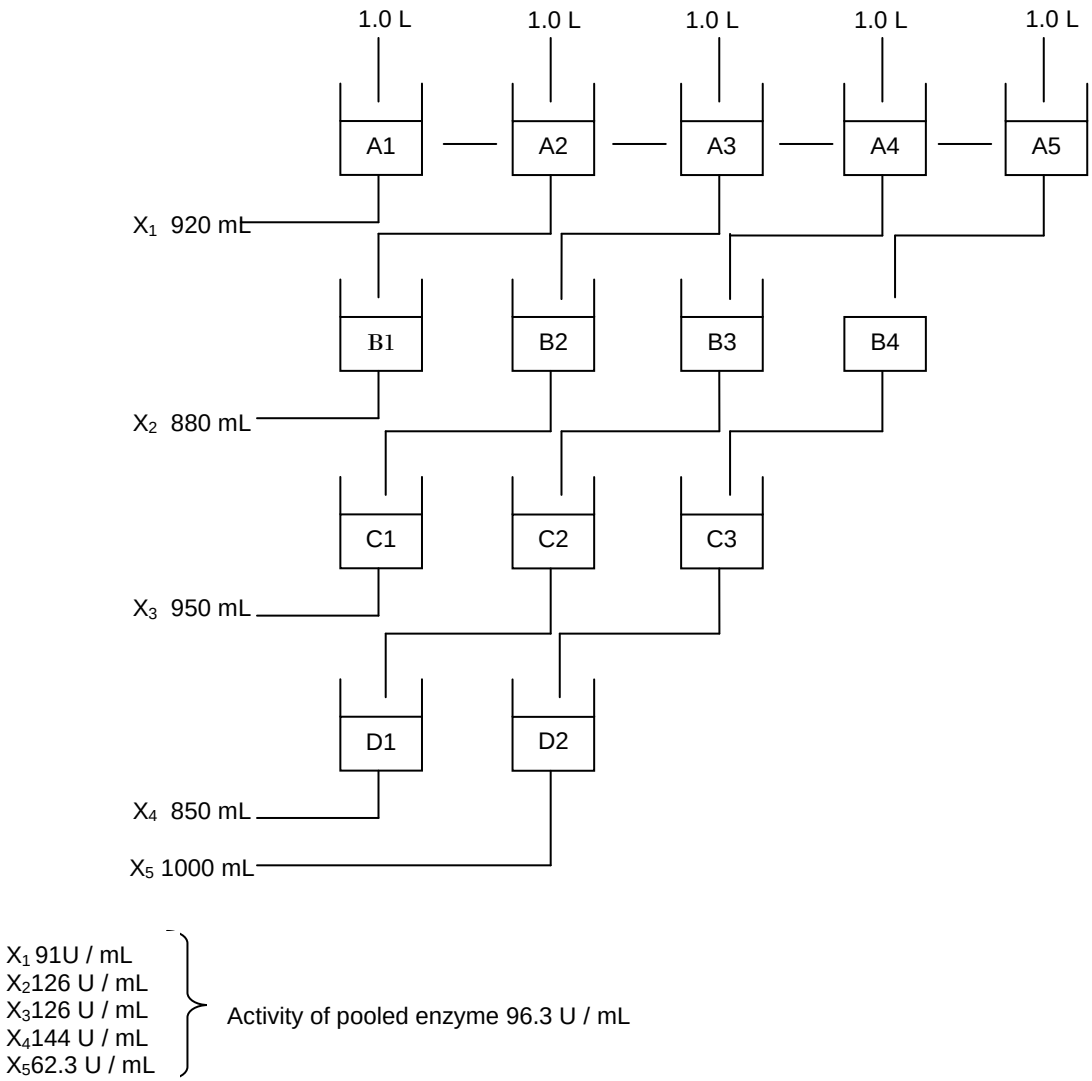

Figure 1: Counter current extraction of glucoamylase from mouldy medium of *Aspergillus niger* CFTRI 1105.

residues of B1, B2 and B3 (C1, C2 and C3) and the enzyme was extracted as mentioned above. The extract of C1 was preserved (Extract X3) and other extracts of C2 and C3 were added to residues of C1 and C2 (D1 and D2) and the enzyme was extracted as mentioned above. Extracts of D1 and D2 were collected (Extract X4 and X5). Glucoamylase activities in extracts X1 to X5 were determined.

Stabilization studies

Effect of pH on the stability of glucoamylase

The crude enzyme extract containing 2 g/L azide was incubated at different pH values and 30 °C. The activity was monitored.

Effect of dilution of enzyme extract

Stability of glucoamylase on the crude enzyme extract (diluted) containing different concentration of protein (0.1, 0.3 and 0.6 mg/mL) with sodium azide (2.0 g/L) was determined at 30 °C.

Effect of exogenous protein addition on enzyme stability

Stability of glucoamylase was studied after the addition of different amounts of egg albumin (0.75 and 1.0 mg/mL) to crude enzyme extract in presence of 2 g/L azide. The crude enzyme extract was considered as the control.

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Effect of glycerol on the stability of glucoamylase

Stability of glucoamylase in the crude enzyme extract with different amount of glycerol (0, 5, 15, 25, 40 and 50% v/v) and 2.0 g/L and sodium azide was studied.

Effect of $(NH_4)_2SO_4$ on the stability of glucoamylase

Stability of glucoamylase in the presence of different concentration of $(NH_4)_2SO_4$ (0.5, 1.0, 2.5, 5.0%, w/v) and 2.0 g/L sodium azide at 30 °C was determined.

Stability of glucoamylase in presence of 0.5% (w/v) $(NH_4)_2SO_4$ and different concentrations of glycerol

To the crude enzyme extract containing 0.5% (w/v) $(NH_4)_2SO_4$, 2.0 g/L sodium azide and different concentration of glycerol (0, 5, 10 and 15%, v/v) were added and the glucoamylase stability was determined at 30 °C.

RESULTS AND DISCUSSIONS

Production of glucoamylase in media inoculated with mycelial inocula of different ages

In fungal cultivation studies, spores are usually used as inoculum. Several studies have been published in optimizing the spore inoculum size on enzyme / protein production (Ramachandran *et al.*, 2004). Usually the inoculum size used in these studies is in the range from 10^6 to 10^9 / mL. In large-scale processes, it is difficult to have such a big number of spores. Thus it is important to find the possibility of using mycelial inoculum for large-scale operations. In solid-state fermentation the spore number of 4.5 x 10^8 mL was reported as the best inoculum size for glucoamylase production (Ramachandran *et al.,* 2004). To scale up the SSF process, a study was made to use mycelial inoculum as an alternative, for which the age of the mycelium is also very critical. Here to the solid medium (100 g) spores of the *A. niger* (6 days old, 2 x 10^7 spores/g wet medium) was mixed and incubated for different periods. These were used as inocula and added to solid medium (1000 g). Glucoamylase production increased up to 2nd day in the media, which were inoculated with 0 and 24 h old inoculum. While the enzyme production was highest on the 1st day in the media, which were inoculated with the mycelia inocula of 48, 72 and 96 h of ages (Figure 2).

Delay in the highest enzyme production was delayed with 0 (i.e. spore mixed medium) or 24 h old inocula could be due to the time taken by the spores to grow / mycelial development and to produce the enzyme, while the highest enzyme production at 24 h was obtained in the media inoculated with 48, 72 and 96 h old mycelial inocula. At 48, 72 and 96 h, the organism might have been in the late log phase or early stationary phase and hence they were able to utilize the fresh nutrients provided in the medium and quickly produced the enzyme. Among the mycelial inocula, 48 h old inoculum gave the highest glucoamylase activity at 24 h (1205 U/g DS). However the activity obtained with 0 h old mycelial inoculum (spores) was highest on the 2nd day (1735 U/g DS).

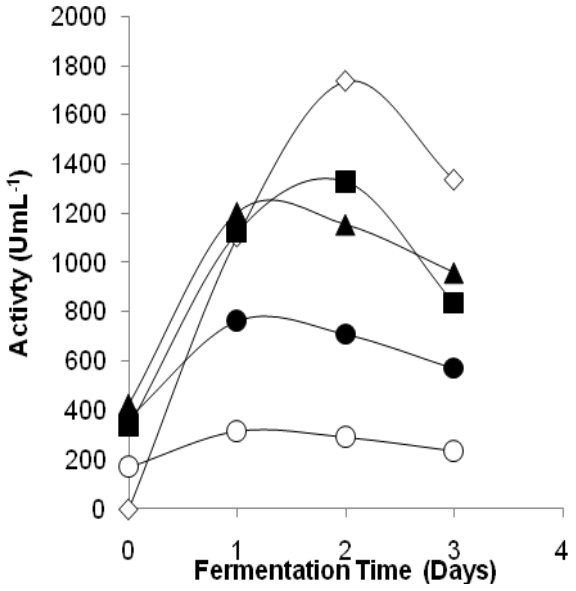

Figure 2: Effect of the age of mycelia inocula on glucoamylase production by *Aspergillus niger* CFTRI 1105. (◊) 0h; (■) 24h, (▲) 48h, (●) 72h and (○) 96 h.

Among the activities obtained, highest activity was obtained with the inoculum of 0 h of age and the total time required was 2 days (0 h for inoculum preparation and 2 days for enzyme production). With the increase in the age of the mycelial inocula (24 to 96 h), highest enzyme activity was obtained on the first day but all these activities were less than that obtained with the spore inoculum. It was observed that a solid mycelial inoculum of 10% (w/v) and 60 h old was the best for α-amylase production by *Aspergillus oryzae* (Shanmuganathan *et al.*, 1994). From the results can also be concluded that, to continue the process, from the mouldy medium, sample can be collected on the 2nd day and used as inoculum for the following batches. Therefore an experiment was carried out to find the feasibility of such continuous batch procedure.

α-Amylase production by *Aspergillus oryzae* was 1372 U/ g DS after 24 h and increased to 1752 U/g DS after 72 h, beyond 72 h the enzyme level has decreased (Ramachandran *et al.*, 2004). The reason could be due to the denaturation of the enzyme caused by the interaction with other components in medium (Ramesh and Lonsane, 1987). In this study too, the organism was in its exponential phase up to the second day of fermentation and produced maximum enzyme activity. After third day the organism would have reached its stationary phase and could have started to produce secondary metabolites, resulting in a lower yield of enzyme.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Continuous batch glucoamylase production with mycelial inoculums

The solid medium inoculated with 2-day-old mouldy medium produced highest glucoamylase activity (1205 U/g DS). At the end of the 1st batch, mouldy medium sample was collected and inoculated to the fresh solid medium and the highest glucoamylase activity was produced on the 1st day (Table 1).

Table 1: Glucoamylase produced from *Aspergillus niger* CFTRI 1105 in a continuous batch process.

Batches	Glucoamylase activity		Total time taken for a batch (Day)
	Activity (U / mL)	Relative activity (%)	
1	1205	57.35	1
2	*2101	100.00	1
3	1362	64.83	1
4	1010	48.07	1
5	652	31.03	1

**Activity obtained in the 2nd batch was considered as 100% and relative activity is presented in percentage.*

When the procedure was continued, in the 3rd and 4th batches highest glucoamylase was produced on the 1st day but it has started to decline from the 3rd batch. The results indicated that such continuous operation with the mouldy medium (containing mycelial) as inoculum is feasible for three batches and the enzyme production was reduced to 31.03% in the 5th cycle. The results indicated the feasibility of using mycelial inoculum continuously for three batches of operation.

Extraction of glucoamylase from mouldy medium

Solid-state fermentations require extraction of their products from the solid fermented medium with a suitable extraction method to get maximum yield of the product (Shata, 2005). The common extractant is distilled water or deionized water (Ghidyal *et al*., 1993, Arasaratnam *et al*., 1997; Aikat and Bhattacharyya, 2000; Arasaratnam *et al*., 2001; Silveira *et al*., 2005; Kapilan and Arasaratnam, 2011). To recover glucoamylase from *Aspergillus niger* NRRL 3122, DEAE-cellulose has been reported (Manera *et al.*, 2008). Thus in this studies distilled water was used. With the increase in the repetitive extraction, the enzyme activity extracted also increased (Table 2). Among the extraction procedures, the counter current extraction method extracted highest glucoamylase activity. In all the procedures glucoamylase present in 1 kg of mouldy medium was extracted with 5 L of distilled water. With the increase in the number of extraction steps, glucoamylase activity also increased. Total amount of glucoamylase activity extracted from 1, 2 and 3 step procedures was 33.5, 42.9 and 48.7% respectively while in the counter current extraction procedure the total amount of glucoamylase activity extracted was highest (442,980 U, Table 2). Thus the counter current extraction could be used in large-scale operations to obtain highest recovery of enzymes. Such improved extraction was observed for glucoamylase from wheat bran mouldy medium (Ramakrishna *et al.*, 1982). Even though large volume (5 L) of distilled water was used to extract the glucoamylase in 1 kg of mouldy medium, it is not possible to leach out the entire enzyme from the mouldy medium. In the 2 or 3 step extraction procedures, it is difficult to completely separate the extract from the mouldy medium, because the solvent is absorbed by dry mouldy husk and a portion of the solvent gets adhered to the husk in each step. By counter current extraction method the amount of enzyme lost with the discarded mouldy medium can be reduced.

Stabilization studies

Stability of glucoamylase at different pH values

When the stability of glucoamylase was studied at different pH values (from 3.0 to 7.0) it showed highest stability at pH 4.5 retaining 96.9% of the initial activity on the 10th day (Figure 3). On the 10th day at pH 3.0 and 7.0; 30.7 and 6.1% of the initial glucoamylase activity was respectively retained. Between the pH values of 3.5 and 5.5 above 50% of the initial activity was retained on the 10th day. The best pH range for stability the enzyme was between pH 4.0 and 5.0 (Figure 3). As glucoamylase showed best stability at pH 4.5, it was chosen for storage stability studies at 30 °C. Glucoamylase from *Aspergillus niger* showed greater stability at pH 4.8 than at pH 7.0 and 30 °C (Shenoy *et al.*, 1984).

Effect of dilution of enzyme extract on glucoamylase stability

Stability of glucoamylase decreased with the dilution of the enzyme, i.e. with the decrease in the protein concentration (Table 3). With double dilution, 28% of the enzyme activity was lost on the 1st day while undiluted enzyme extract lost only 9% of the initial activity. The undiluted extract, which contained 0.6 g/L protein, retained 90% of glucoamylase activity on the 90th day (3rd month), while the double diluted enzyme extract retained 71% of the activity. Dilution of enzyme to three times of the original concentration leads to an activity of 59% on the 1st day and to 56% on the 90th day. This clearly indicated that the decrease in protein concentration in the solution decreased the stability of glucoamylase. Thus in the following experiments to the enzyme sample exogenous protein (egg albumin) was added and the enzyme stability was studied.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 2: Activity of glucoamylase extracted from mouldy medium by different methods. The activities of glucoamylase in the extracts obtained at different steps as well as in the pooled samples are presented. Under all conditions glucoamylase in 1 kg mouldy medium was extracted with a total volume of 5 L of distilled water.

Extraction procedure	Extraction (No)	Activity (U/mL)	Volume of extract (mL)	Total Activity (U)	Relative Activity (%)	
					A	B
One -Step	-	30.8	4820.0	148,456.0	100	33.51
Two-Steps	1	70.0	2150.0	150,500.0	79.20	
	2	15.5	2300.0	33,350.0	17.55	
	Pool	**42.7**	**4450.0**	**190,015.0**	-	42.90
Three-Steps	1	59.0	2780.0	164,020.0	76.02	
	2	41.6	980.0	40,768.0	18.90	
	3	13.8	860.0	11,868.0	5.50	
	Pool	**46.7**	**4620.0**	**215,754.0**	-	48.21
Counter current	X_1	91.0	920.0	83,720.0	18.90	
	X_2	126.0	880.0	110,880.0	25.03	
	X_3	126.0	950.0	119,700.0	27.02	
	X_4	144.0	850.0	122,400.0	27.63	
	X_5	62.3	1000	62,300.0	14.06	
	Pool	**96.3**	**4600**	**442,980.0**	-	100

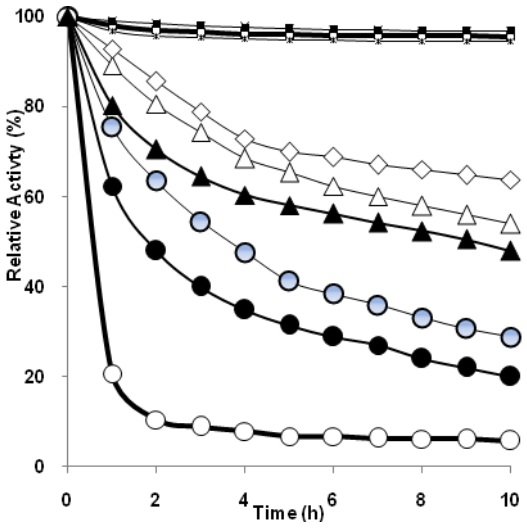

Figure 3: Effect of pH on the stability of glucoamylase produced by *Aspergillus niger* CFTRI 1105. pH (◯) 3.0; (▲) 3.5; (▫) 4.0; (▪) 4.5; (☼) 5.0; (◊) 5.5; (▲) 6.0; (●) 6.5 and (○) 7.0 .

Effect of exogenous protein addition on enzyme stability

When the egg protein was added, stability of glucoamylase was not improved significantly (Table 3). The stabilizing effect of 0.75 and 1.0 mg/mL protein (in addition to 0.6 g/L endogenous protein) was very much similar to 0.6 mg/mL protein (without exogenous protein) in the extract (Table 3). Egg albumin has protective effect on α-amylase (Janecek and Blaz, 1992). However the stability of glucoamylase in undiluted form (presence of 6.0 mg/mL protein) or at higher protein concentration was better than with the diluted enzyme extract. Hence it was decided to use the undiluted enzyme.

Table 3: Effect of different concentrations of proteins [obtained by the dilution of exogenous proteins and by the addition of egg albumin (endogenous protein)] on the stability of glucoamylase at 30 °C. Control did not have any of the additives.

Time (days)	Relative Activity (%)				
	Endogenous protein (g / L)			Exogenous protein + Endogenous protein (g / L)	
	0.1	0.3	0.6	0.75 + 0.6	1.0 + 0.6
0	100	100	100	100	100
1	75.0	90.0	98.0	98.0	98.0
2	65.0	80.0	96.0	97.0	97.5
8	62.0	76.0	95.0	96.0	97.0
16	60.0	74.0	93.0	94.0	94.5
24	59.0	72.0	91.0	92.0	92.0
55	57.0	72.0	90.0	91.0	91.0
90	56.0	71.0	90.0	91.0	91.0

Effect of glycerol on the stability of glucoamylase

The results showed that addition of glycerol to the enzyme extract had helped to improve the glucoamylase stability (Table 4). The effects of 5% (v/v) glycerol and above this concentration showed similar effect. Thus the higher concentration of glycerol is not necessary to maintain the stability of glucoamylase. The glycerol 5%

ISSN (print): 1823-8262, ISSN (online): 2231-7538

(v/v) was considered for the stabilization. Glycerol and other polyalcohols are said to be decreasing the water activity and thereby increasing the enzyme stability. Addition of polyols (1 M) such as ethylene glycol, glycerol and sorbitol improved the enzyme stability (Brumm and Teague, 1989; Pal and Khanum, 2010). Polyols have the capability to form hydrogen bonds that play key role in supporting and stabilizing the native conformation of protein (George *et al.*, 2001).

Table 4: Effect of different concentrations of $(NH_4)_2SO_4$, glycerol and different concentrations of glycerol with 0.5% (w/v) $(NH_4)_2SO_4$, on the stability of glucoamylase at 30 °C. Control did not have any of the additives.

Time (Days)	Relative activity (%)												
	Control	Glycerol (%, w/v)					$(NH_4)_2SO_4$ (%, w/v)				0.5% $(NH_4)_2SO_4$ + Glycerol (%, w/v)			
		5	15	25	40	50	0.5	1.0	2.5	5.0	0	5	10	15
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1	98.0	99.5	99.5	99.5.	99.5	99.8	94.0	87.0	85.0	80.0	94.0	94.0	94.0	94.0
2	97.7	99.0	99.0	99	99.0	99.7	82.0	79.0	72.0	70.0	82.0	82.0	82.0	82.0
3	-	-	-	-	-	-	78.0	72.0	67.0	59.0	78.0	78.0	78.0	78.0
7	95.0	98.0	98.0	98.5	98.5	99.6	73.0	65.0	60.0	50.0	73.0	73.0	73.0	73.0
15	92.0	97.0	96.5	97.5	98.0	99.5	69.0	60.0	50.0	44.0	69.0	69.0	69.0	69.0
24	91.0	96.0	96.0	97.0	97.5	99.4	68.0	60.0	46.0	40.0	68.0	68.0	68.0	68.0
55	90.0	96.0	96.0	96.5	97.0	99	65	60.0	46.0	40.0	65.0	65.0	65.0	65.0
90	90.0	96.0	96.0	96.0	96.5	98.8	65	60.0	46.0	40.0	65.0	65.0	65.0	65.0

Effect of $(NH_4)_2SO_4$ on the stability of glucoamylase

$(NH_4)_2SO_4$ was added to the enzyme extract anticipating its effect on reducing the water activity. K^+, Na^+ and $(NH_4)^+$ have protective effect on α-amylase (Janecek and Blaz, 1992; Sivaramakrishnan *et al.*, 2006). However $(NH_4)_2SO_4$ at different concentrations decreased the stability and the activity of glucoamylase (Table 4). When $(NH_4)_{2SO4}$ concentration was increased from 0.5 to 5% (w/v) glucoamylase activity was decreased from 21.7 to 16.3 U/mL. At these concentrations of $(NH_4)_2SO_4$, precipitation of proteins from the crude extract of glucoamylase was not observed. Thus decrease in the activity was due to the inhibitory effect of $(NH_4)_2SO_4$ on glucoamylase. As the stability was not improved with higher $(NH_4)_2SO_4$ concentrations, to the enzyme extract containing 0.5% (w/v) $(NH_4)_2SO_4$ different concentration of glycerol was added to find their combined effect. Here 0.5% (w/v) $(NH_4)_2SO_4$ was selected for further studies as it has helped the glucoamylase to sustain its stability at longer periods of incubation.

Effect of 0.5% (w/v) $(NH_4)_2SO_4$ and different concentrations of glycerol on glucoamylase stability

The results show that (Table 4) addition of glycerol in addition to 0.5% (w/v) $(NH_4)_2SO_4$ to the crude enzyme extract did not improve the enzyme stability. On the 90th day in the absence of 0.5% (w/v) $(NH_4)_2SO_4$ and glycerol, the enzyme retained 90% of the initial activity but after 55th day in presence of 0.5% (w/v) $(NH_4)_2SO_4$ only 65% of the enzyme activity was retained (Table 4). The same activity was retained on the 90th day. Increase in glycerol concentration in presence of 0.5% (w/v) $(NH_4)_2SO_4$ also has not improved the enzyme activity. Thus the results indicated that the $(NH_4)_2SO_4$ would have either blocked the active sites of the enzyme or denatured or inactivated or inhibited the enzyme. Thus enzyme extract should be purified and the purified enzyme should be used for the enzyme stability to studies.

CONCLUSION

Suitable age of the mycelial inoculum was 24 h. Highest amount of glucoamylase was extracted by counter current extraction method. When glucoamylase was diluted six times, the enzyme lost 41% of its activity initially during the first 24 h and thereafter no significant loss of activity was observed up to 90 days. Glycerol concentration of 5% (v/v) and above stabilized glucoamylase at 30 °C. Ammonium sulfate inhibited and decreased the stability of glucoamylase while addition of glycerol of different concentrations did not reverse the effect of ammonium sulfate.

ACKNOWLEDGEMENT

The authors thank Sida/SAREC and International Science Programme in the Chemical Sciences (IPICS), Sweden, for the financial support.

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