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Repeated Batch Process for Glucoamylase Production by *Aspergillus niger*

Vasanthy Arasaratnam

Department of Biochemistry, Faculty of Medicine, University of Jaffna, Sri Lanka

Abstract: Production of glucoamylase (amyloglucosidase) by *Aspergillus niger* was studied in batch and repeated batch submerged fermentations. In batch culture, growth (biomass 35.8 gL^{-1}) and glucoamylase production (53.2 UmL^{-1}) were highest at 108 h (4.5 days). Addition of spores on the 4th and 8th days to augment the old mycelium extended the glucoamylase production up to 9 days. When the spent medium was supplemented with soluble starch from manioc (50 gL^{-1}) on the 4.5th day, glucoamylase activity increased by 14.6 UmL^{-1} on the 6th day [one and a half day (36 h) after the addition of starch]. Replacement of the spent medium with the fresh fermentation medium on the 4th day, led to 70.3 UmL^{-1} glucoamylase activities on the 7th day (3rd day after the addition of the fresh fermentation medium). The spent medium was replaced with the fresh fermentation medium along with the addition of spores led to an increase in the glucoamylase production by 1.62 and 1.66 folds in the 2nd and the 3rd cycles of that obtained in the first cycle. Thus fed batch process was not possible under the conditions considered for glucoamylase production while repeated batch process seems to be promising.

Keywords: *Aspergillus niger*, Biomass, Glucoamylase, NADPH, Repeated batch process

Introduction

Glucoamylase (1, 4,-α-D- Glucangluco-hydrolase, E.C. 3.2.1.3) is an exo-acting carbohydrase which cleaves glucose units consecutively from the non-reducing end of starch molecule. It is also known as gluc - amylase, amyloglucosidase or λ-amylase. Glucoamylase is produced mainly by fungi such as *Rhizopus* (Inshik and Chunk, 1989) and *Aspergillus* (Lasater and Simith, 1979). Glucoamylase is used for the production of high purity glucose syrups (Mc Mullen, 1977), high fructose syrups (Norman, 1979) high conversion syrups (Norman, 1979), levulose from granular starch (Hebeda and Leach, 1975) and sugar syrups of different dextrose equivalent (DE) values (Normanand Nelson, 1984; Saha *et al.,* 1989; Kumar and Satyanarayan, 2009; Nandy, 2016).

Glucoamylase is produced by solid state fermentation (Ramadas *et al.,* 1996; Arasaratnam *et al.,* 2001; de Souza and Peralta, 2001; Bertolin *et al.,* 2003; Costa *et al.,* 2007; Varzakas *et al.,* 2007; Aithal *et al.,* 2011), continuous cultures (Metwally, 1997; Pedersen *et al.,* 2000), fed batch cultivation (Pedersen *et al.,* 2000; Pederson *et al.,* 2012; Luo *et al.,* 2015; Rani *et al.,*

Corresponding author: Vasanthy Arasaratnam, arva26arva@yahoo.com

1999) cell recycling fermentation (van Verserveld *et al.,* 1991) and with immobilised cells (Konwarth *et al.,* 2010).

The productivity obtained with conventional fermentation processes is limited due to low concentration of microorganism in the system (Arasaratnam *et al.,* 1994). Cell mass and enzyme productivity can be increased by repeated batch process or cell recycling (Ohleyer *et al.,* 1985). Additional advantages of these methods are avoiding product inhibition, broth viscosity (Afschar *et al.,* 1985) and elimination of the time needed for spore germination (Roukas and Alichanidis, 1991). This paper describes the different trails made for the production of glucoamylase continuously from *Aspergillus niger* based on cell recycling, supplementation of nutrients and addition of spores.

Materials and Methods

Chemicals

Manioc flour and soya flour were prepared in the laboratory from local sources. All the other chemicals used were of analytical grade and were purchased from standard sources.

Microorganism

Aspergillus niger CISIR N_4 was used. The organism was maintained on Potato Dextrose Agar (PDA) slants at 4°C and subcultured monthly.

Analytical methods

Reducing sugar in the medium was estimated by dinitrosalicylic acid method (Miller, 1959).

Measurement of glucoamylase activity

The activity of glucoamylase was determined with p-nitrophenyl α-D-glucopyranosideas substrate (Miranda *et al.,* 1987). One unit of glucoamylase is the amount of enzyme which release one m mole of p-nitrophenol from p-nitrophenyl α-D-glucopyranoside in one minute at 60°C and pH 4.0.

Analysis of growth - Biomass

Microbial growth was estimated by measuring biomass dry weight. Samples were centrifuged at 6000 rpm for 10 min, washed with distilled water and dried at 105°C to obtain constant weight (Stone *et al.,* 1992).

NADPH level

Aliquots (5 mL) removed were centrifuged (MSE model 2) at speed 7 for 10 min. Residues were washed three times with 8 mL of 0.02M acetate buffer (pH 4.0). The residue (mycelium) was homogenized with Triton-100 (5 mL, one gL^{-1}) and centrifuged as above. The NADPH content of the supernatant was estimated in a fluorescence spectrophotometer at excitation and emission wave lengths of 360 and 450 nm respectively against Triton X-100 (1 gL^{-1}) 0.02M acetate buffer (pH 4.0) as blank (Taya *et al.,* 1986).

Batch Cultivation

The fermentation medium contained (gL^{-1}) soluble starch from manioc flour, 20;$(NH_4)_2SO_4$, 5;KH_2PO_4, 0.5;$MgSO_4.7H_2O$, 0.5;KCl, 0.5;$FeSO_4.7H_2O$, 0.01;soya flour,20 and peptone, 10.0 at pH 6.5. Fermentation medium (200 mL) was inoculated with spores (1.25 mL, 108 spores mL^{-1} in 0.01%, v/v, Tween). The flasks were incubated at

37°C in an orbital shaker (150 rpm) and biomass, NADPH level, reducing sugar and glucoamylase activity were monitored.

Addition of spores

Experiment was carried out as said in batch cultivation and spores (108 spores mL^{-1}) were added to the medium when the NADPH level reached about 82 mL^{-1}. Samples were analysed.

Supplementation of spent medium with soluble starch

The spores were cultivated as said above in 200 mL fermentation medium. Soluble starch solution (50 gL^{-1}) was added to bring back the starch concentration to 10 gL^{-1} when the reducing sugar concentration was decreased to about 0.0 gL^{-1}. Samples were taken and analysed for reducing sugar, glucoamylase activity and NADPH level.

Replacement of the spent medium with fresh fermentation medium

Batch cultivation was carried out and when the reducing sugar concentration was decreased to about 0.0 gL^{-1}. The spent medium was strained through a sterile muslin cloth and the enzyme trapped in between the mycelia was removed by pressing the mycelia in between the muslin cloth. Fresh sterile fermentation medium was added to the mycelium. All these steps were carried out under aseptic conditions. The cultivation was continued and samples were analysed.

Addition of spores with replacement of spent medium with fresh fermentation medium

Batch cultivation was carried out by replacing the spent medium with fresh fermentation medium and the spores were added as said in the above experiment. The replacement was carried out, when the reducing sugar level was decreased to about 0.0 gL^{-1}.

Results and Discussions

The aim of this work is to produce higher titer of glucoamylase by *Aspergillus niger*. The experiments were carried out to improve the glucoamylase production by supplementing the spent medium with the carbon source or by repeated batch cultivation and by introducing the spores into the medium during the replacement of the spent medium with fresh fermentation medium.

Batch Cultivation

The fungus was cultivated in batch process at the laboratory level in shake flasks containing 200 mL of the fermentation medium. Samples were withdrawn time to time under aseptic conditions for enzyme activity, residual sugar content, NADPH level and the dry weight measurements.

Growth of the *Aspergillus niger* was monitored by measuring the NADPH level as well as the dry weight of the mycelium. NADPH level and dry weight of the mycelium showed good correlation (Figure 1). Growth of the mycelium (biomass 35.8 gL^{-1}) and glucoamylase production (53.2 UmL^{-1}) reached maximum at 108 h (Figure 1). Thereafter, no increase in enzyme production was observed. The activity of the enzyme produced in the medium remained constant while growth (both the dry weight and NADPH level) decreased after 108 h. Reducing sugar level of the medium declined rapidly after

48 h (Figure 1). Decrease in the viability of the mycelium could be due to the lack of nutrients or the accumulation of the toxic products would have led to autolysis or death of the mycelium. Glucoamylase production by *Aspergillus niger* requires energy source (van Verseveld *et al.,* 1991). The lack of carbon source was evidenced by a decrease in the reducing sugar level to 0.0 gL^{-1} at 108 h and no further increase in the glucoamylase titre beyond 108 h. As the reducing sugar level was reduced to 0.0 gL^{-1} at 108 h, a study was carried out to supplement the spent medium with carbon source (soluble starch, which also would dilute the toxic substances in the spent medium).

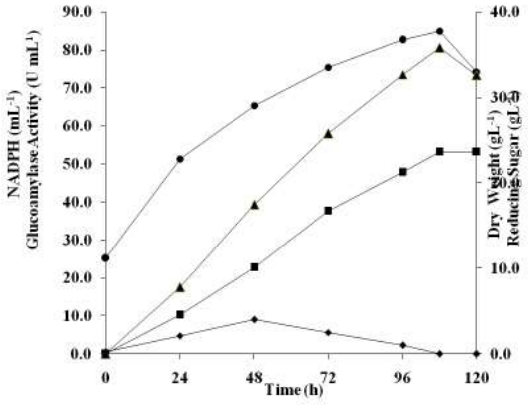

Figure 1: Growth of and glucoamylase production by *Aspergillus niger* in batch cultivation at pH 6.5 and 37°C and changes in reducing sugar and dry weight.
(■) - Glucoamylase activity;
(♦) - Reducing Sugar;
(●) - NADPH and
(▲)- Dry weight

Addition of Spores

Glucoamylase production is also depending on the specific growth rate of the mycelium (Metwally, 1998). The NADPH level increased from 82.6 to 89.2 mL^{-1}, spores were added on the 4th and 8th days (Figure 2). The NADPH level of the mycelium was increased up to 24 hours (5th day of the commencement of the experiment), after the addition of spores on the 4th day. Glucoamylase production (53.8 UmL^{-1}) was increased up to the 6th day, i.e., 48 h after the addition of the spores and stated to decrease on the 7th day to 52.7 UmL^{-1} (Figure 2). Addition of spores on the 8th day showed a slight increase in the NADPH level with slight increase in glucoamylase production (Figure 2). This indicated the inhibition of the germination of the spores as well as the glucoamylase production by the accumulated toxic substance in the spent medium as well as by the deficiency of nutrients.

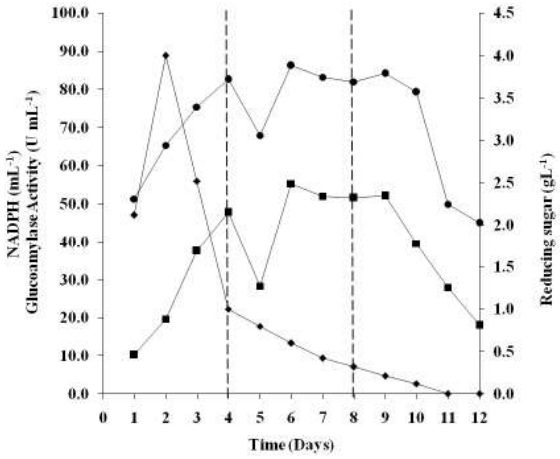

Figure 2: Effect of addition of spores on the changes in glucoamylase production, reducing sugar and growth (NADPH).
The lines indicate the addition of spores (1.25 mL; 108 spores mL^{-1}) to the spent medium on the 4th and 8th days.
(■) - Glucoamylase activity;
(♦) - Reducing Sugar and
(●) -NADPH

Germination of spores is considerably repressed by carbon dioxide (Meyrath and Bayer, 1987) and such toxic substances would have affected the germination of the spores. Spent medium rich in by-products, with increased osmolarity and limited dissolved oxygen would affect the glucoamylase production (Pederson *et al.,* 2012). Hence to have better glucoamylase production it was decided supplement the spent medium with the carbon source, i.e. the soluble starch.

Supplementation of spent medium with soluble starch

Since the growth of the fungus started to decrease at 108 h (Figure 1), and addition of spores at 4th and 8th days did not improve the glucoamylase production (Figures 1 and 2), soluble starch from manioc (50 gL^{-1}) was added at 108 h (4.5 day) when reducing sugar concentration decreased to 0.0 gL^{-1}. When soluble starch (40 mL) was introduced into the spent medium (160 mL) a fall in glucoamylase activity from 53.2 to 42.4 UmL^{-1} was observed (Figure 3). This was due to the dilution of the spent medium. On the 1.5th day after the addition of soluble starch (6th day after the commencement of the experiment) the glucoamylase activity was increased to 54.2 UmL^{-1}. If the dilution factor is considered, addition of soluble starch had increased the enzyme production by 14.6 UmL^{-1}. NADPH level was increased on the 5th day (12 h after the addition of soluble starch) and the levels of NADPH and reducing sugar continued to decrease after the addition of the carbon source (Figure 3). Beyond 7th day, there was decrease in the glucoamylase production. This indicated that in presence of toxic substances in the spent medium, addition of only the carbon source is not useful.

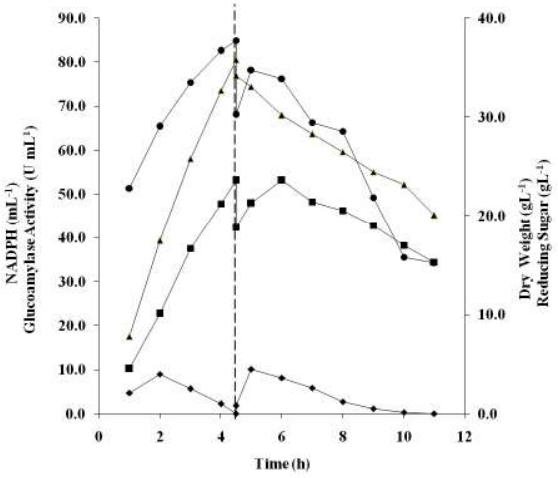

Figure 3: Effect of soluble manioc starch supplementation (50 gL^{-1}) on the changes in glucoamylase production, reducing sugar and growth (NADPH).

The line indicates the supplementation of manioc starch to the spent medium.

(■) - Glucoamylase activity;
(♦) - Reducing Sugar;
(●) - NADPH and
(▲) - Dry weight

Even though there has been a decrease in the glucoamylase production, utilization of the reducing sugar was observed. But the rate of utilization of the carbon source was not the same as that observed in the initial four days (Figure 3). The increase in glucoamylase activity in the spent medium could be either due to the release of intracellular enzyme from the remaining mycelia or the dead or autolysed cells. However other studies had reported an increase in glucoamylase production in fed batch process under controlled conditions (Ramadas *et al.,* 1996, Pedersen *et al.,* 2000) and reduction in cost of enzyme production (Luo *et al.,* 2012). Hence it can

be concluded that the addition starch alone had no significant effect on repeated batch glucoamylase production. Another experiment was carried out by replacing the spent medium with fresh fermentation medium to provide all the required nutrients while removing the toxic substances released into the spent medium.

Replacement of the spent medium with fresh fermentation medium

As the supplementation of soluble starch to the spent medium did not show significant effect and the toxic substances seems to get accumulated in the spent medium, the spent medium was completely replaced with fresh medium after the reduction of the reducing sugar to 0.0 gL^{-1} (Figure 4). The spent medium after four days of fermentation was strained and replaced with fresh medium as the extracellular glucoamylase production requires energy and requires the supply of sufficient energy sources (van Verseveld *et al.,* 1991). When the spent medium was replaced with the fresh fermentation medium, glucoamylase production started to increase and reached a maximum value (70.3 UmL^{-1}) on the 3rd day (7th day after the commencement of the experiment).

These results emphasized that the reason for the decreased glucoamylase production as the toxic substances present in the medium (Figure 4). Thereafter from the 3rd day (7th day after the commencement of the experiment) the enzyme activity started to decrease. Decrease in the enzyme production beyond the 7th day, i.e. 3rd day after the replacement of the spent medium with the fresh medium could be due to the aging of the mycelium. With increase in the age of the mycelium the viability of the cells might have decreased (Rhem and Reed, 1988). Maximum activity in the 2nd cycle obtained was 70.3 UmL^{-1} and was 1.32 times higher than that of the highest activity (53.2 UmL^{-1}) obtained in the 1st cycle. The time required for maximum glucoamylase production was decreased in the 2nd cycle from 4.5 to 3 days.

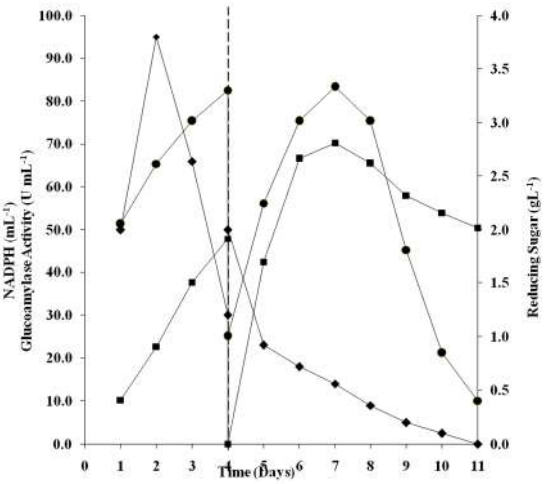

Figure 4: Effect of replacement of spent medium with fresh medium on the changes inglucoamylase production, reducing sugar and growth (NADPH).

The line indicates the replacement of spent medium with fresh fermentation medium.

(■) - Glucoamylase activity;

(♦) - Reducing Sugarand

(●) - NADPH

The results showed that even though the mycelium was relieved from toxic effect which could have been brought about by the removal of the spent medium, the efficiency of glucoamylase production was high for a short while and the enzyme production was reduced. The oxygen limitation, high osmolarity and by-products metabolism can affect the glucoamylase production

(Pedersen *et al.,* 2012). This may be due to the reduced enzyme production and release of the enzyme from the dead mycelium. *Botryodiplodia theobromae* retained the capacity to produce glucoamylase through four cycles with an increase in the age of the mycelium (8 days) (Navaratnam *et al.,* 1996). In continuous batch citric acid production, 70% of the original citric acid production was obtained in the second cycle (Roukas and Alchsndis, 1991). In this studies the rate of reducing sugar utilization as well as the NADPH level were reduced. These can be considered as the indications of the loss of the mycelial viability. Hence a study was carried out to have young mycelium by introducing the spores into the medium while replacing the spent medium with fresh medium to remove the toxic substances and to provide nutrients.

Replacement of the spent medium with fresh fermentation medium and addition of spores

On the 4th and 8th days the spent medium was replaced with fresh fermentation medium and spores were added to the medium. When additional spores were introduced on the 4th day the NADPH level and glucoamylase activity started to increase and reached the maximum value of 86.3 UmL^{-1} on the 7th day, i.e., 3 days after the replacement of the medium and addition of spores. Similarly when the spent medium was replaced with the fresh medium and spores were also added on the 8th day, the glucoamylase production has reached the maximum value on the 10th day to 88.5 UmL^{-1} which is 2 days after the replacement of the spent medium and addition of spores (Figure 5).

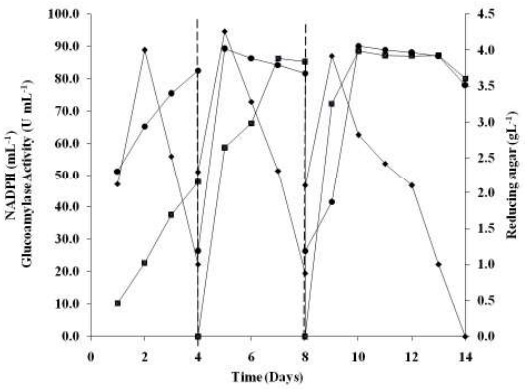

Figure 5: Effect of addition of spores and replacement of spent medium with fresh medium on glucoamylase production, reducing sugar and growth (NADPH).
The lines indicates the replacement of spent medium with fresh medium and the addition of spores (1.25 mL; 108 spores mL^{-1}) on the 4th and the 8th days.
(■) - Glucoamylase activity;
(♦) - Reducing Sugarand
(●) - NADPH

Highest activity was obtained in the 2nd and 3rd cycles were 86.3 and 88.5 UmL^{-1}, respectively. These values were equivalent to 1.62 and 1.66 times of the maximum activity (53.2 UmL^{-1}) obtained in the 1st cycle (Figure 5). Thus the added spores would have grown in the fresh fermentation medium and have given young mycelium to produce glucoamylase in addition to the mycelium which existed. This is somewhat similar to the inoculation of the fresh fermentation medium with the spores, as well as the viable mycelium producing the enzyme.

The glucoamylase production could have been the double the amount of what was produced in the first cycle. Decreased glucoamylase production than that was expected could be because of the

degradation of the old mycelium as well as the inhibitory effect of the derivative products of the mycelium on the growth of the spores.

When the fresh medium was introduced along with the spores, the NADPH level did not reduce substantially by the fourth day (i.e. by the 8th day of the commencement of the experiment) of the introduction of the medium and the spores, indicating the germination and the of the spores and the growth of the mycelium. When the fresh medium was again replaced for the spent medium and the additional spores were introduced, the NADPH level has started to declaim by the 12th day and did not decrease as it was observed in the previous experiments. The decrease in the NADPH level was observed when the reducing sugar level has reached 0.0 gL^{-1} on the 14th day of the commencement of the work and 6th day after the introduction of the fresh medium and the spores (Figure 5). Even though the addition of the spores had not increased the glucoamylase production substantially, it has helped the medium to have viable mycelium and to produce glucoamylase continuously (Metwally, 1998) and has provided sufficient energy for the production of the extracellular glucoamylase (van Verseveld *et al.,* 1991).

Conclusions

This work indicates that glucoamylase production in repeated batch culture was not possible by adding spores to the spent medium or supplementing the spent medium with carbon source or replacing the spent medium with fresh fermentation medium. This study clearly indicated that addition of fresh medium and introducing the spores to the pre-existing mycelium can allow the glucoamylase production to continue at least for three cycles. The study also has indicated that mycelial death after the first cycle and deficiency of nutrients affect the enzyme production substantially. Hence for the production glucoamylase, the organism could be cultured by replacing the spent medium with the fresh medium to remove the inhibitory products and maintaining the viable mycelium by adding spores intermittently.

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