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# Formulation of Medium and Recycling of Biomass for Glucoamylase Production by *Botryodiplodia theobromae*

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Botryodiplodia theobromae *grown in manioc starch medium supplemented with ammonium phosphate, peptone, tri potassium phosphate, calcium carbonate and soy bean powder, produced 1950 U ml$^{-1}$ glucoamylase in shake flasks at pH 6·0. Fungal biomass could be recycled at least four times without significant loss in enzyme production.*

## INTRODUCTION

Glucoamylase (E.C.3.2.1.3) is an important industrial enzyme, used, for example, in the production of glucose syrups.[1] Various fungal glucoamylases have been employed in the production of sugar syrups with different dextrose equivalents (DE).[1,2] *Aspergillus niger* has been used for the production of glucoamylase in submerged culture[3,4] and in solid state fermentation.[4] *Botryodiplodia theobromae* is a fungus whose potential as a glucoamylase producer has also been investigated.[4] Product formation can be increased by cell recycling[5] in batch process,[3,4] fed batch process[4] and continuous process.[4] Cell recycling has advantages over batch processing in that the biomass is conserved and the time for the fermentation step is generally reduced.[6] This paper reports the formulation of a medium for the production of glucoamylase in submerged culture from *B. theobromae* isolated from a lichen[6] and the recycling of the fungal mycelium for the continuous production of glucoamylase.

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## MATERIALS AND METHODS

### Materials

Glucose and soluble starch (analytical grade) were obtained from BDH Chemical Company Ltd, England. Potato, manioc tubers and soy bean purchased in the local market were milled and pulverised through a domestic sieve. All other chemicals were of analytical grade.

### Analytical methods

Starch[8] and reducing sugar[9] were estimated colorimetrically. Glucoamylase activity was assayed by the method of Bernfeld.[10]

### Glucoamylase activity

One unit of glucoamylase activity is defined as the amount of enzyme that releases 1 nM of reducing sugar per min from starch at pH 5·1 and 52°C.

**Lichen fungus (LF) medium**
Lichen fungus medium contained maltose, 20 g; yeast extract, 20 g and agar, 30 g in a litre of distilled water.

**Organism and isolation method**
*Botryodiplodia theobromae* IMI 334891 was isolated locally.[7] This organism was isolated using lichens from a mango tree from Jaffna, northern part of Sri Lanka. The lichen thallus (crustose type) was surface sterilised with $HgCl_2$ (0·1%, w/v) for 3 min, homogenised with sterile water, streaked on LF plates and incubated at 30°C. Selected colonies were stabilised on potato agar plates at 30°C.

**Manioc and potato starch media**
Manioc powder and peeled potato were boiled, strained through muslin cloth, and the starch in the extracts was diluted to 20 g/litre and 10 g/litre, respectively and used as media. Agar, 30 g/litre was added to prepare potato agar (PA) plates.

**Preparation of inoculum**
Potato starch medium (50 ml) containing starch (10 g/litre) was inoculated with four loops of mycelium from 3 day PA plates and incubated with agitation (160 rpm) at 30°C. The (mycelial) culture at 45 h was centrifuged and the wet volume was noted. The mycelial pellet (3·5 ml) was suspended in 100 ml medium, and a 10% (v/v) of this suspension was used as the inoculum.

**Glucoamylase production**
Glucoamylase production by *B. theobromae* was studied using 125 ml media, in 250 ml flasks plugged with cotton wool, shaken at 160 rpm at 30°C. Glucoamylase secreted into the spent medium was monitored. To improve glucoamylase production, the medium was supplemented as follows:

1. *Effect of $(NH_4)_3PO_4$ and peptone*
Manioc starch medium (control) was supplemented with $(NH_4)_3PO_4$ (2 g/litre) and peptone (3 g/litre).

2. *Effect of $K_3PO_4$*
To manioc starch medium supplemented with $(NH_4)_3PO_4$ (2 g/litre) and peptone (3 g/litre), $K_3PO_4$ (2·5 g/litre) was added. The pH was maintained at 6·0 by the addition of 0·1 N NaOH. The control contained manioc starch medium supplemented with $(NH_4)_3PO_4$ (2 g/litre) and peptone (3 g/litre).

3. *Effect of $CaCO_3$*
The fungus was grown in manioc starch medium supplemented with $(NH_4)_3PO_4$ (2 g/litre), peptone (3 g/litre), $K_3PO_4$ (2·5 g/litre) and $CaCO_3$ (3·6 g/litre). The control contained all of the above constituents except $CaCO_3$, and the pH was maintained at 6·0 by the addition of 0·1 N NaOH.

4. *Effect of soy bean flour*
To manioc starch medium supplemented with $(NH_4)_3PO_4$ (2 g/litre), peptone (3 g/litre), $K_3PO_4$ (2·5 g/litre) and $CaCO_3$ (3·6 g/litre); soy bean flour (20 g/litre) was added. In one flask the pH was maintained at 6·0 and in the second at 5·0. The control contained no soy bean and the pH was maintained at 6·0.

**Biomass recycling**
The fungus was grown in 125 ml optimised medium at pH 5·0 with agitation. For biomass recycling, the spent medium was replaced with fresh medium at 70 h intervals and glucoamylase production monitored.

## RESULTS AND DISCUSSION

**Glucoamylase production**
When *B. theobromae* was grown in manioc starch medium, maximal glucoamylase activity (280 U/ml) was produced at 46 h (Fig. 1(a)). This low enzyme activity could be due to the lack of nutrients in the medium, as manioc tuber contains mainly starch (797 g/kg), little protein (11 g/kg) and ash (24 g/kg).[11] Although manioc is poor in nutrients, it was selected as a carbon source that it is readily available and cheap in Sri Lanka.[12] Since the nitrogen content in the manioc powder was very low (0·16 g/litre),[11] it was supplemented with $(NH_4)_3PO_4$ (2 g/litre) and peptone (3 g/litre). Enzyme production decreased to 160 U/ml, presumably due to the decrease in pH of the medium from 6·0 to 1·8 (Fig. 1(b)). Stanbury and Whitaker[6] reported that the decrease in the pH of medium supplemented with $(NH_4)_3PO_4$ and peptone could be due to the depletion of ammonium ions. $K_3PO_4$ (2·5 g/litre) alone did not maintain the pH (Fig. 1(b)). When the pH was maintained by addition of 0·1 N NaOH the gluco-

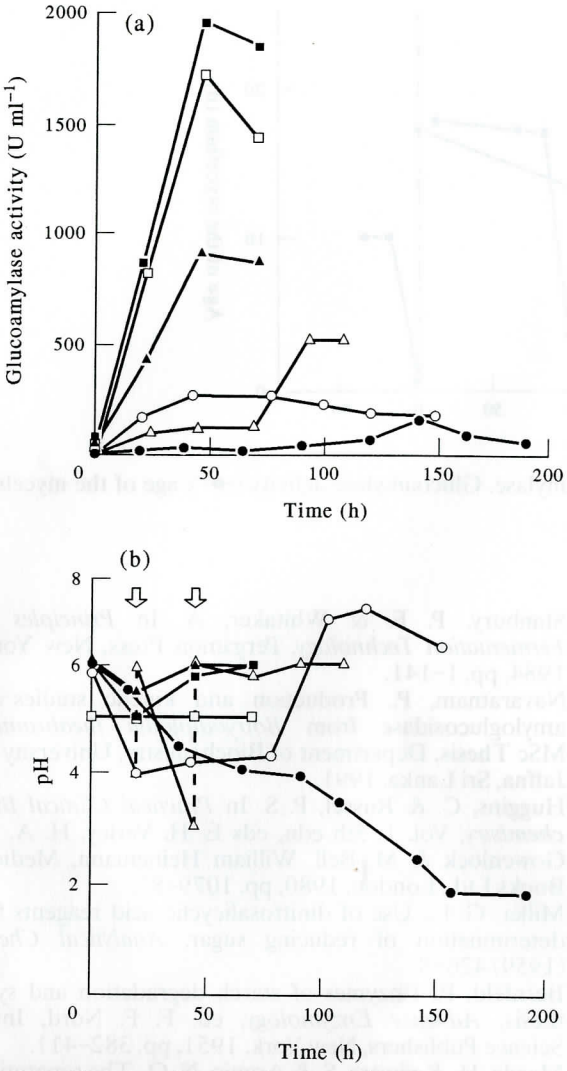


**Fig. 1.** Glucoamylase production (a) and change in pH (b) with time in submerged culture by *Botryodiplodia theobromae*. Basic manioc starch medium (-○-); supplemented with $(NH_4)_3PO_4$, 2 g/litre and peptone, 3 g/litre (-●-); $(NH_4)_3PO_4$, 2 g/litre, peptone, 3 g/litre and $K_3PO_4$, 2·5 g/litre and pH adjusted to 6·0 by intermittent addition of 0·1 N NaOH (-△-); $(NH_4)_3PO_4$, 2 g/litre, peptone, 3 g/litre, $K_3PO_4$, 2·5 g/litre and $CaCO_3$, 3·6 g/litre at pH 6·0, (-▲-); $(NH_4)_3PO_4$, 3 g/litre, peptone, 3 g/litre, $K_3PO_4$, 2·5 g/litre, $CaCO_3$, 3·6 g/litre and soy bean powder, 20 g/litre at pH 6·0, (-■-); $(NH_4)_3PO_4$, 2 g/litre, peptone, 3 g/litre, $K_3PO_4$, 2·5 g/litre, $CaCO_3$, 3·6 g/litre and soy bean powder, 20 g/litre at pH 5·0, (-□-); addition of NaOH (0·1 N) to adjust the pH to 6·0 (↓).

amylase activity in the medium increased to 520 U/ml, suggesting that the decrease in glucoamylase activity at low pH could be due to decreased enzyme production or denaturation of the enzyme. The incorporation of $CaCO_3$ (3·6 g/litre) not only maintained the pH at 6·0 but also further increased the enzyme activity to 900 U/ml at 46 h (Fig. 1(a)). The amount of $CaCO_3$ added (3·6 g/litre) was based on the theoretical requirement to neutralise the phosphoric acid which could be liberated into the medium. Addition of soy bean powder (20 g/litre) to the manioc starch medium supplemented with $(NH_4)_3PO_4$, peptone, $K_3PO_4$ and $CaCO_3$ further increased the enzyme activity to 1950 U/ml and 1720 U/ml at pH 6·0 and 5·0, respectively. Soy bean contains more than 40% protein, 56–60% carbohydrate and 2% fat,[13] and hence it is a good source of nitrogen and carbon. In addition, its unsaturated fat can enhance microbial enzyme production and secretion.[14,15] Unsaturated fatty acids increase the fluidity of the membrane[4,14] and thereby influence enzyme secretion. In the recycling of biomass, bacterial contamination is a problem.[4,6] As low pH inhibits bacterial growth, pH 5·0 was selected for further experiments as the 'optimised medium'.

The optimised medium is simple, cheap and most of the constituents are available locally. Manioc tubers and soy bean are plentiful throughout the year in the north of Sri Lanka. $CaCO_3$ and peptone could be substituted with natural sources such as powder of cuttle fish endoskeleton and hydrolysate of meat or fish, respectively. Therefore, the optimised medium is economically useful for the production of glucoamylase by *Botryodiplodia theobromae*.

## Biomass recycling

*Botryodiplodia theobromae* was grown in shake flasks in the optimised medium in an orbital shaker (160 rpm) for a period of 70 h at pH 5·0. The recycling of biomass for batch production of glucoamylase was achieved by replacing spent medium with fresh medium (Fig. 2). Enzyme production reached a peak (1750 U/ml) at 48 h in the first cycle and at 32 h in the subsequent three cycles. In the fifth cycle, the enzyme activity decreased to 1000 U/ml and this could be due to a decrease in the viability of the cells. However, *B. theobromae* retained the capacity to produce glucoamylase through four cycles, whereas *A. niger* exhibited a sharp decrease in glucoamylase yield after the second cycle.[4] Another advantage of *B. theobromae* was the reduction in the time required for maximum glucoamylase production (48 h) compared with *A. niger* CISIR $N_4$ (96 h).[16]

In the recycling process, the fermentation time was reduced in successive batches with almost constant production of glucoamylase. Hence, the productivity of glucoamylase was increased. In a recycling process the inoculum needs to be pre-

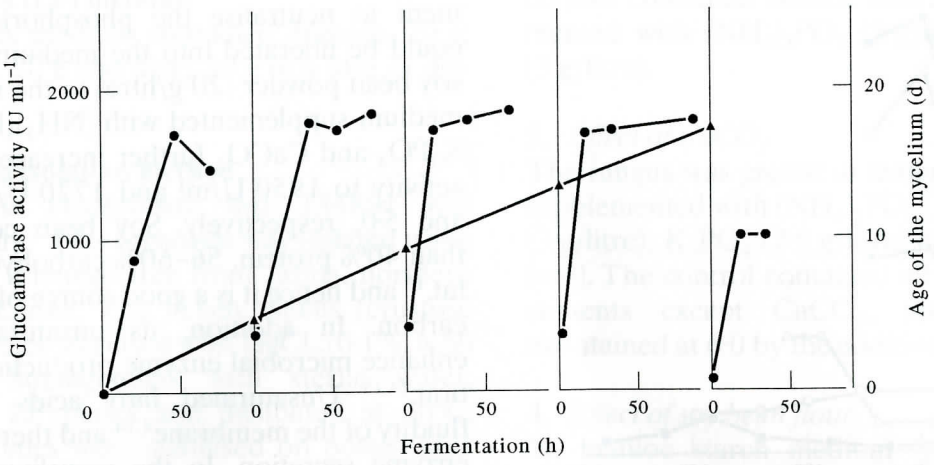


**Fig. 2.** Recycling of biomass for the batchwise production of glucoamylase. Glucoamylase activity (-●-); age of the mycelium (-▲-).

pared for the first batch alone, whereas in batch cultures, each batch needs individual inoculum preparation. Therefore the advantage of recycling (multiple cycle) over single batch submerged culture is that at each cycle the time for log and growth phases is saved, and active fungal cells in the production phase are used. This ensures active enzyme production in a short time period. In addition, when the same mycelium is used repeatedly in large scale operation, the time taken for charging, cleaning and filling the fermenter can be greatly reduced.

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