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SCREENING FUNGI FOR IMPROVED GLUCOAMYLASE PRODUCTIVITY USING BUFFERED DEXTRAN BROTH CULTURES

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SUMMARY

Improved glucoamylase productivity was screened for using surface cultures in small tubes containing Dextran T-10 broth buffered at a pH supra-optimal for activity of the glucoamylase of the parental strain. Visual identification of improved mutations on microtitre plates is based on their relative abilities to produce residual glucose in culture filtrates. A 141% increase in glucoamylase was achieved.

INTRODUCTION

Traditionally, strain development meant a laborious approach with regard to identification of superior isolates from a mutagen-treated population. Rational selection procedures are more efficient and usually have a biochemical basis (Elander, 1982). In early or primary screening prior to laboratory fermentations, rational selection is achieved by the use of techniques allowing visual identification of superior mutations. The selection of alpha-amylase producers using the size of the zone of hydrolysis of starch is an example. Invariably, strain development of glucoamylase producers employs the same criterion for selection. However, zonation cannot in any way be correlated quantitatively with the amount of glucoamylase produced because of the hydrolytic activity of other amylolytic enzymes such as alpha-amylase. Therefore isolation of improved mutations of glucoamylase using starch plates can only be partially selective.

Dextran can be hydrolysed by glucoamylase to the exclusion of other amylolytic enzymes (Kuek and Kidby, in press). Therefore utilization of dextran can be used as a

rational selection criterion for glucoamylase. The method proposed is a miniaturized screening procedure sucessfully used to select for *Aspergillus phoenicus* mutations with improved glucoamylase productivity.

MATERIALS AND METHODS

Microorganism: *Aspergillus phoenicus* ATCC 15556 from the American Type Culture Collection, Rockville, Maryland, U.S.A, was used as parental strain for mutagen treatment.
Media: Conidia were obtained from seven day old cultures grown on Potato Dextrose Agar (PDA)(Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 10 g $L^{-}1$ agar; Buffered Starch Agar (BSA) contained, per litre; wheat starch, 10 g; K_2HPO_4,70 mg; KH_2PO_4,130 mg; $MgSO_4.7H_2O$, 200 mg; $Na_2SO_4.10H_2O$, 200 mg; $FeSO_4.7H_2O$, 20 mg; $MnSO_4.H_2O$, 10 mg; $ZnSO_4.7H_2O$, 2 mg; $CuSO_4.5H_2O$, 2 mg; monosodium glutamate 550 mg; Yeast extract (Difco Laboratories), 500 mg; Triton X100 (Ajax Chemicals, Sydney, Australia), 1.0 mL (0.1% w/v) and bis(2-Hydroxyethl)imino-tris(Hyroxymethyl)methane (BIS-TRIS) (Sigma Chemicals, 12.55 g (60 mM). The pH of BSA was adjusted to 6.5 using 1.0M HCl before autoclaving for 30 minutes at 108^{o}C; Buffered Dextran Broth (BDB) contained 10 g L^{-1} Dextran T-10 (Pharmacia Fine Chemicals, Uppsala, Sweden). All other ingredients and pH were as for BSA except that no Triton X100 was
added.
Mutagenesis: Conidia from 1 week old cultures incubated at 35^{o}C were suspended in 0.01% Tween 80 solution and this was filtered through a Grade 1 sintered glass funnel (Corning Pyrex) to remove mycelial fragments. This suspension was then stored at 4oC while its conidial concentration was determined by plating appropriate dilutions on PDA+0.1% Triton X100. These plates were incubated for 24 hours at 35oC. Mutagen solution was prepared by dissolving 75 mg of N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG) (Sigma Chemicals) in 23 mL of cold (4^{o}C) 0.05 M tris-maleic buffer at pH 9.0 contained in a flask with a magnetic stirrer. An appropriate volume of spore suspension which would yield 2.5×10^{7} conidia was centrifuged and the conidia were resuspended in 2 mL of the above buffer. This was added to the the NTG solution (spore concentration now 1.0×10^{6} mL^{-1}). Controls contained no NTG. The mixtures were incubated at 30^{o}C for 6 hours with stirring. Under these conditions all the

NTG should be decomposed at the end of the incubation time (Delic *et al*, 1970) and therefore the conidia did not require washing. After appropriate dilution, the mixtures were plated on BSA and incubated for 4 to 5 days at 35°C. With this treatment, less than 2% of the conidia survived.

Selection of colonies on BSA (Plate Screening): Survivors from the mutagen treated population giving rise to colony: hydrolytic zone diameter ratios of greater than that of ATCC 15556 were selected for secondary screening.

Selection of strains on BDB (Secondary Screening): The isolates from plate screening were inoculated into glass tubes (13 X 100 mm) containing 3 mL of BDB. The tubes were topped with aluminium caps (Cap-O-Test) which do not seal hermatically. Incubation was for 14 days at 35°C at the end of which culture filtrates were obtained by filtration within each tube using filter samplers (Filtrona Plastics, Thomastown, Australia). The filtrates were then assayed for residual glucose.

Assay of residual Glucose: 0.25 ml of double strength glucose oxidase-peroxidase-chromogen solution (Sigma Chemicals) was added to each well of a microtitre plate and warmed to 37°C. Twenty-five microlitre samples of culture filtrates were added to each well using a multi-channel micropipette. The plate was then sealed and incubated at 37°C for 30 minutes. The filtrates were ranked visually using the color produced by *A. phoenicus* ATCC 15556 culture filtrate as standard. Cultures producing filtrates that gave denser color than the standard were selected for assessment of glucoamylase productivity.

Comparison of glucoamylase productivity:

Production of glucoamylase: Production medium consisted of 5% maize flour and 2.5 % corn steep liquor (Sigma Chemicals) which was autoclaved at 121°C for 15 mins. Incubation was in a Gallenkamp gyratory incubator at 35°C and 250 r.p.m. Conidia from 1 week old cultures were suspended in 50 mL of 0.01 % Tween 80 (Sigma Chemicals) solution. 1 mL of this was used to inoculate 50 mL of a production medium in an Erlenmeyer flask. After incubation for 24 hrs. the contents of the Erlenmeyer flasks were transferred to 450 mL of production medium in Fernbach flasks. The cultures were incubated for a further 6 days at the end of which the cultures were filtered to remove biomass and insolubles before assay of the filtrate for glucoamylase.

Assay for glucoamylase: Dextran T-10 (Sigma Chemical) was used as assay substrate (Kuek and Kidby, in press). Reaction tubes consisted of 0.5 mL aliquots of culture filtrate and 2.5 mL of 1 % Dextran T-10 dissolved in McIllvaine (Dawson *et al*, 1969) buffer made to pH 5.0 at 35°C. Blanks consisted of 0.5 mL aliquots of culture filtrate and 2.5 mL of buffer. The tubes were incubated for 1 hour at 35°C after which 3 mL of 6% trichloroacetic acid was added to the reaction tubes whereas 2 mL of 9 % tricloroacetic acid followed by 1 mL of 2.5% Dextran T-10 solution was added to the blanks. Glucose resulting from hydrolysis of Dextran T-10 was assayed using a glucose oxidase-peroxidase-chromogen diagnostic kit (Sigma Chemicals). Optical density was determined at 450 nm after 30 mins. incubation at 37°C. The amount of glucoamylase in 1 mL of culture filtrate that will produce 1 micromole of glucose in the reaction tube in 1 minute is defined as 1 Glucoamylase Unit.

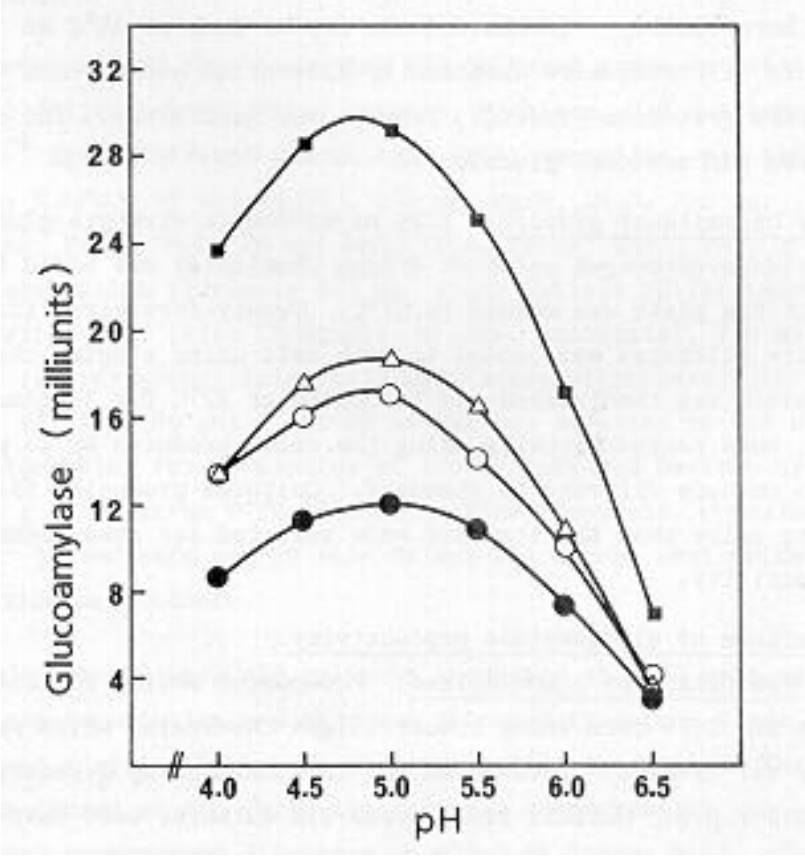

Fig. 1. The relative glucoamylase productivities after six day culture of three selected isolates compared with*Aspergillus phoenicus* ATCC 15556.
■ = R4M5.10 ∇ = R4M5.19 ○ = R1M1.1 ● = ATCC 15556

RESULTS AND DISCUSSION

Six hundred cultures were isolated from BSA plates for secondary screening. Growth on BDB in the small tubes took the form of mycelial mats. Three of the isolates that gave the highest color densities in microtitre plate assay of glucose were selected for assessment of glucoamylase productivity. Fig. 1 shows the relative productivities of the three tested isolates compared with ATCC 15556. Glucoamylase assay was performed over a range of pH to check for mutations with a pH shift. All four cultures had peak activities around pH 5.0. The improvement in glucoamylase productivity was approximately 14 % in R1M1.1, 15 % in R4M5.9 and 141 % in R4M5.10. It is thus demonstrated that this method is capable of selecting superior isolates such as R4M5.10.

With Buffered Starch Agar, more isolates than are necessary are selected for secondary screening. Plate screening can be made more efficient if dextran can be substituted for the starch. However, the hydrolysis of dextran in agar cannot be visualized. Theoretically, this problem could be overcome by attaching a chromogen to the polymer or by making it insoluble. But both methods are relatively difficult to realize because the modifications have to avoid interference with glucoamylase activity. There are currently no suitable specific substrates for glucoamylase that could be used in a plate screening medium.

Selection in Buffered Dextran Broth is effected in two ways. First is the incorporation in the medium of a specific substrate which is also the sole carbon source. Cultures unable to hydrolyse Dextran T-10 in a major way are unlikely to possess much glucoamylase and therefore will either not grow well or produce little residual glucose in their culture filtrate. Secondly, the hydrolytic activity of ATCC 15556 glucoamylase in the growing culture is lowered to the extent that all the glucose produced will be utilized for growth and little residual glucose will result in the culture filtrate. This is achieved by shifting and then buffering the pH of the medium at a higher point than is optimal for ATCC 15556 glucoamylase. In this case, pH 6.5 buffered by 60 mM BIS-TRIS was sufficient to ensure that ATCC culture filtrate produced no color on microtitre plate assay of glucose. Mutations improved in glucoamylase productivity will be able to overcome the pH disadvantage and produce glucose in excess of their growth requirements and thus be identified on that basis on the microtitre plate. In this manner, quick and easy identification of improved mutations is possible with nothing more than visual assessment. This system will of cause select for mutations with pH optima for glucoamylase

shifted towards 6.5. The method proposed was in fact designed for that purpose but such mutations were not found. The specificity of selection can also be affected if mutations with dextranase activity arise.

The advantage gained in secondary screening using dextran broth is that the number of cultures isolated from plates that would require testing of productivity via shake flask culture is reduced in a very specific manner. This is a significant contribution to making screening of glucoamylase producing fungi more efficient. Besides enzyme productivity, other characteristics can be screened for using this method. Conditions of incubation such as temperature and medium composition in the tube cultures can be easily changed to apply different selection pressure. When the selection criterion can only be applied after growth such as in the screening of thermostable glucoamylase, enzyme assay can also be performed on a miniaturized scale on microtitre plates which can be incubated at the temperature desired.

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