APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1981, p. 593-596
0099-2240/81/030593-04$02.00/0
Vol. 41, No. 3

Induction, Isolation, and Characterization of *Aspergillus niger* Mutant Strains Producing Elevated Levels of β-Galactosidase

K. M. HELENA NEVALAINEN
Technical Research Centre of Finland, Biotechnical Laboratory, SF-02150 Espoo 15, Finland

An *Aspergillus niger* mutant strain, VTT-D-80144, with an improvement of three- to fourfold in the production of extracellular β-galactosidase was isolated after mutagenesis. The production of β-galactosidase by this mutant was unaffected by fermentor size, and the enzyme was also suitable for immobilization.

The use of β-galactosidase (EC 3.2.1.23) in the hydrolysis of lactose in milk and milk products is one of the most promising applications of enzymes in the food industry (6). β-Galactosidases are produced by many bacteria, yeasts, and molds. However, the properties of the β-galactosidases produced by different microorganisms vary widely, in particular in pH optima. Both intracellular and extracellular production have been observed. In the hydrolysis of lactose to glucose and galactose in acid whey, mold β-galactosidases are the most suitable. These have acidic pH optima and are not sensitive to changes in pH (2.0 to 8.0) or temperature (40 to 65°C) (6). Moreover, the β-galactosidase produced by many *Asperigillus* species (*Aspergillus oryzae* and *Aspergillus niger*) is extracellular, which is an additional advantage in industrial applications.

In this work the production of extracellular β-galactosidase by a strain of *A. niger* was improved by mutagenesis. A rapid and effective screening method for the recognition of mutant colonies with elevated enzyme production is presented.

Indicator plates, on which different mutant genotypes are distinguished by colony appearance, colony color, hydrolysis zones around the growing colonies, etc., have been widely used in bacterial genetics. For the detection of β-galactosidase activity, a variety of indicator plates used for *Escherichia coli* are available (5). Some of these are fermentation indicators, and others are substrate dyes. In both cases, the ability of a colony to hydrolyze or ferment a given substrate is revealed by the color of the growing colony. However, when screening mutants for improved enzyme production, it is often almost impossible to distinguish differences in the color of different colonies as a function of their different enzyme activities. In this work, much attention was given to the development of a simple and effective screening program for testing large numbers of *A. niger* mutant strains for improved β-galactosidase production.

MATERIALS AND METHODS

A. niger VTT-D-79106, a good producer of β-galactosidase (6), was used as initial parent strain for mutagen treatments.

Cultivation media and growth conditions. All strains were stored on potato dextrose agar slants. The following plates were tested for suitability in the screening of β-galactosidase activity: (i) minimum nutrient salts (4) with 2% lactose as the carbon source; (ii) 2% whey permeate with 0.5% yeast extract and 0.5% $(NH_4)_2HPO_4$; (iii) minimum nutrient salts and 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Vega Biochemicals, Tucson, Ariz.), with 1% glucose and with or without 2×10^{-4} M isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.); and (iv) minimum nutrient salts with 6-bromo-2-naphthyl-β-D-galactopyranoside (Koch Light Laboratories Ltd., Colnbrook Bucks, England) and 1% lactose or 1% glycerol as the carbon source.

In all plates, 2% agar was used as the solidifying agent. To keep the size of colonies reasonably small, either 0.1% Triton X-100 (Koch Light) or 0.1 to 0.5% saponin (Weiss rein; Merck, Darmstadt) was added to the media. Screening plates were incubated at 29°C for 2 to 5 days.

Liquid complete medium for shake cultures contained 4% wheat bran, 0.3% distillers spent grain and 1% $(NH_4)_2HPO_4$. The pH was adjusted to 4.0 with 0.1 N HCl (M. Linko and A. Mustranta, FEBS Special Meeting on Enzymes, Dubrovnik-Cavtat, Yugoslavia, abstr. no. s6-27; 1979). Essentially the same medium with slight modifications was also used in a laboratory fermentor (Biotec FL110; working volume, 8 liters), and in a pilot fermentor (Fermac; designed by Rintekno, Espoo, Finland; working volume, 1,200 liters).

Shake cultivations were carried out in 250-ml conical flasks containing 50 ml of medium. The cultivation temperature was 29°C, and the speed of the rotary shaker (New Brunswick MS 2) was 200 rpm. After cultivation for 4 days, the mycelium and medium solids were removed by centrifugation (3,000 rpm, 10 min), and enzyme activities were assayed from the supernatant.

Fermentor cultivations were carried out in the laboratory fermentor as described by Linko and Mustranta (FEBS Special Meeting on Enzymes, Dubrov-

nik-Cavtat, Yugoslavia, abstr. No. s6-27, 1979). Production of β-galactosidase in pilot scale has been described by Mustranta et al. (A. Mustranta, E. Karvonen, H. Ojamo, and M. Linko, Proceedings of the VIth International Fermentation Symposium, London, Ontario, Canada 1980, in press).

Induction and isolation of mutant strains. Mutants were induced from *A. niger* VTT-D-79106 with either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Fluka AG, Buchs SG, Switzerland) or gamma irradiation as described by Nevalainen and Palva (8). The final concentration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was 0.06%, and the amount of irradiation given was 80 to 120 kilorads with a dose rate of 100 kilorads per h. Mutagenized conidia were cloned on potato dextrose agar plates for 5 days. Conidia from the survival level of 0.5 to 5% were then transferred to screening plates by suspending them in 0.9% NaCl, diluting, and plating a dilution to give around 10 to 50 colonies, depending on the type of screening plate used.

The β-galactosidase activity of mutant colonies grown on different indicator plates was detected as follows: on 5-bromo-4-chloro-3-indolyl-β-D-galactoside plates, colonies showing β-galactosidase activity had a deep blue-green appearance caused by the released dye, 5-bromo-4-chloroindigo. When using 6-bromo-2-naphthyl-β-D-galactopyranoside, the detection procedure of Fantes and Roberts (2) was followed. Colonies having β-galactosidase activity developed a deep purple color. On lactose minimal plates rapidly growing colonies formed large, dense, "healthy"-looking clones, and on whey permeate, a transparent hydrolysis zone was formed around the colonies producing β-galactosidase. Colonies showing improved production of this enzyme on the basis of the criteria described above were then picked and cultivated in shake flasks. The best β-galactosidase producers in this scale were then cultivated in a laboratory fermentor, and the β-galactosidase production of the best mutant in the laboratory fermentor was further tested in pilot scale. The best β-galactosidase producer of each mutant generation was used as the parent strain for the next mutagenic treatment.

Assays. β-Galactosidase activity was assayed by using *o*-nitrophenyl-β-D-galactopyranoside (Merck) as the substrate. The reaction mixture contained 0.8 ml of 1.7 mM *o*-nitrophenyl-β-D-galactopyranoside in 0.075 M sodium acetate buffer (pH 4.5) and 0.2 ml of suitably diluted enzyme. The reaction was allowed to proceed for 10 min at 45°C and was stopped by adding 1 ml of 0.1 M Na_2CO_3. The absorbance was measured at 420 nm. β-Glucosidase activity was assayed with an analogous method described by Bailey and Nevalainen (M. J. Bailey and K. M. H. Nevalainen, Enzyme Microb. Technol., in press) by using 1 mM 4-nitrophenyl-β-D-glucopyranoside (Merck) as the substrate. Enzyme activities in both the β-galactosidase and β-glucosidase assays were expressed as nkat per milliliter.

Amyloglucosidase, xylanase, and cellulase activities were assayed as described by Bailey and Nevalainen (in press). The following substrates in solutions with appropriate pH values for each assay were used: 1% Zulkowski starch (Merck; for amyloglucosidase), 1% xylan (Fluka; for xylanase), and soluble 1% alkyl-substituted cellulose (hydroxyethylcellulose, pract. mittelviskos, Fluka; for cellulase). Enzyme activities were measured by determining the reducing sugars liberated from the substrate by using the dinitrosalisylic method (11) for amyloglucosidase and cellulase, and the method of Somogyi (10) and Nelson (7) for xylanase. In the amyloglucosidase and xylanase assays, one unit of enzyme activity was defined as the amount of enzyme releasing 1 mg of soluble protein per ml of reducing sugars in the assay conditions with glucose (amyloglucosidase), or xylose (Fluka; xylanase) as the stardard. Cellulase activities were expressed as nkat per milliliter, with glucose as the standard.

Soluble protein was assayed by using a slightly modified method of Lowry et al. (3) after precipitation with trichloroacetic acid. Bovine serum albumin (Sigma) was used as the standard.

RESULTS AND DISCUSSION

Choice of screening method. After mutagenic treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and cloning on potato dextrose agar, conidial suspensions were plated on different screening media as described above. After a suitable incubation time of 2 to 5 days, 100 colonies with possible enhanced enzyme production based on the information given by the screening plate in question (see above) were picked from each type of screening plate. The β-galactosidase production of these clones was tested in shake flask cultivations in a medium optimized for β-galactosidase production by *A. niger* (Linko and Mustranta, FEBS Special Meeting on Enzymes, Dubrovnic-Cavtat, Yugoslavia, abstr. no. s6-27, 1979).

Results from these experiments (not shown) indicated that the best correlation between behavior on the screening plate and β-galactosidase production in shake cultivations was obtained from the colonies picked from plates containing minimum nutrient salts with 2% lactose as the only carbon source. However, the indicator plates tested also showed that the presence of an inducer (lactose or isopropyl-β-D-thiogalactopyranoside) was not necessary for the synthesis of β-galactosidase by *A. niger*.

In all plates the colony diameter could be restricted to 0.2 to 0.5 cm with 0.1% Triton X-100 or 0.1 to 0.5% saponin. No difference in colony size was observed when different concentrations of saponin were used.

Isolation and characterization of mutant strains. After mutagenic treatment, conidia from the survival level of 0.5 to 5.0% were cloned and screened on minimal-lactose plates as described above. Vigorously growing clones were then cultivated in shake flasks, and, in addition to β-galactosidase activity, several other enzyme activities (see Materials and Methods) were assayed to obtain some idea of the specificity of the mutations. About 1,000 colonies were

screened at each mutation step, and 50 to 100 of the most promising colonies were cultivated in shake flask scale.

Shake flask cultivations. The first isolated mutant strain with clearly elevated β-galactosidase production was AH-54 (Fig. 1).

When strain AH-54 was isolated, its β-galactosidase production was about 185% compared with that of the parent strain VTT-D-79106 (100%). However, this mutant was found to have a wide variation (135 to 185%) in its β-galactosidase production, and 6 months after the original isolation the production level stabilized at 135% of that of the parent strain (Table 1). Soon after its isolation strain AH-54 was used as the parent strain for further mutagenic treatments, and the second mutant generation, with the best mutant strains BH-57 and BH-26, was induced (Fig. 1). The β-galactosidase activity of this series of mutants was 220% when compared with the initial parent strain VTT-D-79106. An interesting feature of strains BH-57 and BH-26 was their clearly elevated cellulase and xylanase activities, indicating an upward mutation in the system coding for the production of these enzymes (Table 1). Strains BH-26 and BH-57 were then used as parent strains for further mutagenic treatments, now changing the mutagen from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to gamma irradiation in BH-26. However, no further improvement in β-galactosidase production in the descendants of these two strains (in about 4,000 colonies) was obtained in two successive mutagen cycles with either mutagenic treatment.

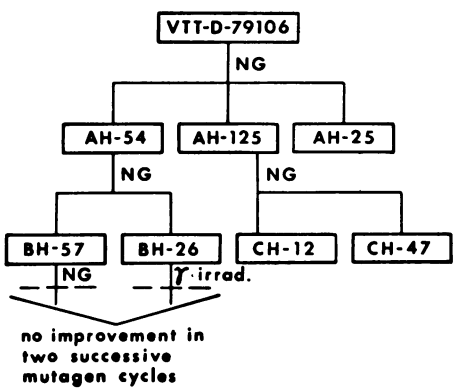

FIG. 1. *Stepwise selection of A. niger mutant strains with improved β-galactosidase production.*

Since the mutant line derived from strain AH-54 (Fig. 1) appeared to give no more enhancement of β-galactosidase production after a reasonable number of colonies (about 5,000) were tested, and as the mutant strain AH-54 had too high a variation in production properties, strain AH-125 was chosen as the parent strain for further strain improvement work. This strain appeared to be a rather specific mutant with respect to β-galactosidase production (Table 1).

Two high β-galactosidase producers, strains CH-12 and CH-47, were isolated from strain AH-125 by using nitrosoguanidine (Fig. 1). CH-47 produced about 4 times the amount of β-galactosidase compared with the original parent strain, VTT-D-79106 (Table 1). All other enzyme activities measured from the culture supernatant of strain CH-47 were clearly under the corresponding activities of strain VTT-D-79106, with the exception of cellulase activity, which was about at the level of strain VTT-D-79106. Strain CH-12 resembled the strain CH-47.

Fermenter cultivations. The best β-galactosidase-producing mutant in shake flask scale, strain CH-47, coded in the strain collection of

TABLE 1. *Measured enzymatic activities of the initial parent strain A. niger VTT-D-79106 and some of the best β-galactosidase-producing mutant strains*[a]

Strain	β-Galactosidase activity		β-Glucosidase activity		Amyloglucosidase activity		Cellulase activity		Xylanase activity	
	nkat/ml	%	nkat/ml	%	U/ml	%	nkat/ml	%	U/ml	%
VTT-D-79106	40	100	62	100	26	100	45	100	130	100
AH-54	54	135	72	116	20	77	40	89	140	108
AH-25	55	138	89	144	32	119	37	82	130	100
AH-125	72	180	73	118	21	81	30	67	130	100
BH-26	88	220	97	156	25	96	74	164	228	175
BH-57	88	220	90	145	28	108	80	178	260	200
CH-12	97	243	67	108	22	85	59	131	148	114
CH-47	166	415	58	94	14	54	48	105	111	85

[a] Strains were grown in shake flasks on a complete medium containing 4% wheat bran. Enzyme activities presented in the table are mean values of 3 to 10 cultures.

this laboratory as VTT-D-80144, and the initial parent strain, VTT-D-79106, were then cultivated in a laboratory fermentor and in a pilot fermentor to investigate possible scale-up problems. The results are shown in Table 2. Detailed information concerning the fermentor cultivations has been given by Mustranta et al. (in press).

The results indicated that scaling up to 1.2 m^3 had no major positive or negative effect on the production of β-galactosidase by *A. niger* (Table 2). The amount of soluble protein found in the culture medium of the mutant strain VTT-D-80144 was higher than in that of strain VTT-D-79106, being 0.66 and 0.2 mg/ml, respectively, after a cultivation time of 3 days in a laboratory fermentor. The β-galactosidase enzyme produced by both strains was suitable for immobilization (Mustranta et al., in press). Thus, the β-galactosidase enzyme of the high-producing *A. niger* mutant VTT-D-80144 appeared to be fully suited to industrial-scale production.

Compared with that of bacteria, the production of industrially important extracellular enzymes by molds is a relatively slow process partly because of their slower growth rates. In recent years, several different yeast and fungal enzyme structural genes have been cloned in bacteria (e.g., 1, 9, 12). In the future we plan to use recombinant deoxyribonucleic acid techniques to allow more efficient production of fungal enzymes, either in bacteria or in fungi containing multiple copies of cloned fungal genes.

TABLE 2. *β-Galactosidase production by strains VTT-D-80144 and VTT-D-79106*[a]

Strain	β-Galactosidase activity (nkat/ml) in:		
	Shake flask (0.05 liter)	Laboratory fermentor (8 liters)	Pilot fermentor (1,200 liters)
VTT-D-79106	40	50	45
VTT-D-80144	166	165	150

[a] Strains were grown in shake flasks (4 days), a laboratory fermentor (4 days), and a pilot fermentor (3 days) in wheat bran medium.

ACKNOWLEDGMENTS

I thank Annikka Mustranta for helpful discussions and Kirsti Leskinen for excellent technical assistance.

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