Journal of Industrial Microbiology & Biotechnology (2002) 29, 70–74
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www.nature.com/jim

Amylase hyperproduction by deregulated mutants of the thermophilic fungus *Thermomyces lanuginosus*

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Thermomyces lanuginosus **was subjected to three cycles of mutagenesis (UV/NTG) and a selection procedure to develop amylase-hyperproducing, catabolite-repression-resistant and partially constitutive strains. One of the selected derepressed mutant strain III_{51}, produced ~7- and 3-fold higher specific activity of α-amylase (190 U/mg protein) and glucoamylase (105 U/mg protein), respectively, compared to a wild-type parental strain. Further, the effect of production parameters on mutant strain III_{51} was studied using a Box–Behnken design. The regression models computed showed significantly high R^2 values of 96 and 97% for α-amylase and glucoamylase activities, respectively, indicating that they are appropriate for predicting relationships between corn flour, soybean meal and pH with α-amylase and glucoamylase production.**
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 70–74 doi:10.1038/sj.jim.7000270

Keywords: *Thermomyces lanuginosus*; mutagenesis; amylase hyperproducing; catabolite repression

Introduction

Thermomyces lanuginosus, a thermophilic deuteromycete fungus, is an excellent source of amylases [12] that have been purified and characterized [14]. The glucoamylase from *T. lanuginosus* can quantitatively convert starch into glucose [15,25], whereas the α-amylase yields maltose as the principal final product of raw potato starch hydrolysis [19]. The enzymes are reportedly thermostable, with the half-life of α-amylase and glucoamylase being 0.6 and 4 h, respectively at 70°C. Due to their increased thermostability, these enzymes are potentially useful in the starch industry for production of maltose and glucose [16,19]. However, *T. lanuginosus* is a poor producer of amylase when compared to the commercial strains of *Aspergillus* sp. and, therefore, requires improvement by strain manipulation methods like mutagenesis, protoplast fusion, cloning and transformation, as well as media optimization [17,20] to achieve higher enzyme titers. Addition of a low molecular weight dextran and Tween-80 to the culture medium results in increased production of α-amylase by *T. lanuginosus* [2,14]. We have reported interspecific protoplast fusion as a method of developing amylase-hyperproducing, deregulated strains of *T. lanuginosus* [26]. In addition, some of the hygromycin-resistant transformants with improved amylase titers have also been reported [8]. In the present study, a strain-development programme involving repeated mutagenesis and selection was followed to generate amylase-hyperproducing, catabolite-repression-resistant and partially constitutive strains of *T. lanuginosus.* The selected strain was further studied for the effect of production parameters upon titer using the statistical approach of response surface methodology.

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Received 5 July 2001; accepted 16 April 2002

Materials and methods

Microorganism

T. lanuginosus, a thermophilic fungus, was isolated from composting soil and cultured on yeast starch YpSs agar [6] at 50°C for 7 days. The culture was purified on the same medium by serial subculturing, and was stored at room temperature.

Mutagenesis

In the first step of mutagenesis a spore suspension (1×10^7/ml) of *T. lanuginosus* wild strain W_o was exposed to UV radiation for 45 min, using a Philips germicidal lamp (30 W) emitting primarily at 254 nm, from a distance of 20 cm [7]. Treated spores were suitably diluted and plated on starch complete medium [12] containing sodium deoxycholate (0.1% w/v) as a colony growth restrictor. Plates were incubated in the dark at 50°C for 7 days and were then flooded with iodine solution (0.3% KI and 0.15% I_2). The colonies showing higher amylase titers (ratio of colony diameter to that surrounding the halo zone) were selected as amylase-hyperproducing mutants and further screened by the plate assay method. To select for deregulated mutants the amylase hyperproducers were subjected to secondary screening by plating them on starch complete medium containing 0.1% (w/v) 2-deoxy-D-glucose (2 DG). The 2-DG-resistant mutants were further screened for amylase production on a medium containing 1.5% starch and 3% glycerol.

The second step of mutagenesis involved exposure of a selected amylase-hyperproducing, partially deregulated mutant strain, designated I_{615}, to UV radiation for 1 h. The survivor colonies were selected on starch complete medium containing 2-DG (0.2% w/v). The 2DG-resistant mutant strains were screened for amylase hyperproduction and resistance to catabolite repression using the plate assay method on media containing (a) 1.5% starch and (b) 1.5% starch and 5% glucose. The selected amylase-hyperproducing, catabolite-repression-resistant mutant strain designated II_{19} was further subjected to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)

treatment at a concentration of 350 μg/ml prepared in 0.5 M Tris–maleate buffer (pH 7.0) [27]. The spore suspension was washed twice with sodium phosphate buffer (0.05 M; pH 5.5), serially diluted and plated on starch complete medium. The survivors were selected on starch complete medium supplemented with 2-DG (0.2% w/v). The 2-DG-resistant colonies were individually plated on three different media types containing (a) 1.5% starch, (b) 1.5% starch plus 5% glucose and (c) 1.5% glucose to select for amylase-hyperproducing, catabolite-repression-resistant and constitutive mutants by the plate assay method.

Plate assay method

For the plate assay, each potential mutant strain was individually plated on starch complete medium (20 ml in 90-mm-diameter petri plates) without a colony growth restrictor and incubated at 50°C for 7 days. A 1-cm^2 agar plug was subsequently cut from the growing edges of the colony and suspended in 4 ml of acetate buffer (0.05 M; pH 5.5) for 1 h at 50°C. The contents were centrifuged at 8000 rpm for 5 min and the extract was measured for α-amylase and glucoamylase activities [26].

Shake flask culture for amylase production by wild and mutant strains

Three media were used to screen mutant strains for amylase production in shake flask cultures. The media contained either 1.5% starch, 1.5% glucose or 1.5% starch and 5% glucose as carbon sources and were prepared in basal medium with the composition (%): yeast extract, 0.4; K_2HPO_4, 0.23; KH_2PO_4. 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; citric acid, 0.057. The pH of each medium was adjusted to 5.5.

Culture conditions

Shake flask cultures were grown in 250-ml conical flasks with 50 ml of medium. Each flask was inoculated with 1 ml of freshly grown mycelium obtained by cultivating aleurospore suspension (1×10^7/ml) at 50°C for 48 h in a shake flask, in basal medium containing glucose. In a few experiments, starch/maltose was used in place of glucose for developing the inocula. The production was carried out in an environmental shaker (220 rpm) at 50°C for 96 h. The contents of flasks were harvested and assayed for enzyme activities and extracellular protein. All experiments were performed in triplicate, and the standard error (SE 5%) was calculated.

Determination of K_m and V_{max}

K_m and V_{max} for α-amylase and glucoamylase in crude enzyme extracts was determined using Line Weaver Burk plots.

Enzyme activities

α-Amylase activity was estimated spectrophotometrically by reading the reduction in the blue colour of the starch–iodine complex [19]. Glucoamylase activity was analysed using DNS [24]. One unit of α-amylase is defined as the amount of enzyme required to hydrolyze 1 mg of starch per minute at 50°C under the assay conditions. One unit of glucoamylase is defined as the amount of enzyme required to release 1 μmol of glucose equivalent per minute when incubated at 50°C. The enzyme activities were expressed as units per milligram of protein. The concentration of extracellular protein in the culture filtrate was assayed by the method of Lowry *et al* [18].

Studies on morphology

The morphology of wild and selected mutants was studied using slide cultures. For slide cultures, each strain was grown at 50°C on a slide containing a thin YpSs agar layer and photomicrographs were taken of 24- to 48-h-old mycelium using a 40× phase-contrast objective.

Statistical analysis

Response surface methodology (RSM) using the Box–Behnken design [21] was used on the amylase-hyperproducing mutant strain III_{51} to determine effects of three independent variables on production of α-amylase, glucoamylase and protein. The three independent variables were corn flour (X_1), soybean meal (X_2) and pH (X_3). Low, middle and high concentration levels of each variable were designated as -1, 0 and $+1$, respectively, and shown in Table 3 [4].

Results and discussion

Stepwise mutagenesis

The survivors of UV mutagenesis of wild-type *T. lanuginosus,* were selected for amylase hyperproduction by observing the clear zone diameter on starch complete medium that was flooded with iodine solution. Of the 85 mutants selected, 33 exhibited resistance to 0.1% 2-DG, an analogue of D-glucose, added to starch medium (Table 1). A similar approach has been used for isolating mutants resistant to catabolite repression of cellulase-producing fungi, *Penicillium purpurogenum* [1], *Penicillium pinophilum* [5], *Trichoderma reesei* [9], alkaline protease-producing *Thermoactinomyces* sp. E 79 mutant [28], citric acid-producing *Aspergillus niger* [29], glucoamylase producing *Rhizopus* sp. [30] and inulinase producing *Kluveromyces* sp. [31]. In *A. nidulans,* the gene loci, *cre*A, *B* and *C*, involved in carbon catabolite repression, have been characterized. The *cre*A gene is likely to be a negative-acting regulatory gene directly involved in regulation of the synthesis of many carbon catabolite-repressible enzymes and permeases [3]. The mutations in *cre*B and *cre*C confer resistance to 2-DG, leading to reduced uptake of carbon catabolite-repressing sugars and derepression of enzyme activities [13]. To ascertain the selected phenotype, 33 2-DG-resistant mutants were screened for

Table 1 Genealogy of the mutant strains selected from the wild strain *T. lanuginosus*

Step no.	Strain	Mutagenesis	Survivors	Colonies selected	Mutant strains selected	Selected phenotype
1	Wild	UV	10,300	33	I_{615}	Increased amylases and partially derepressed
2	I_{615}	UV	5,000	25	II_9, II_{19}	Increased amylases and partially derepressed
3	II_{19}	NTG	5,100	25	III_{51}	Increased amylases, catabolite-repression-resistant and partially constitutive

amylase production in shake culture on media containing 1.5% starch and 1.5% starch plus 3% glycerol. Sixteen mutants showed derepressed amylase production in glycerol-containing medium (data not shown). The selected mutant strain designated I_{615} yielded 2.30- and 1.08-fold higher specific activities and ~6- and 3-fold higher volumetric activities of α-amylase and glucoamylase, respectively, compared to the parent strain in a starch-containing medium (Table 2). In the second step, survivors of the UV mutagenesis of I_{615} were screened on medium containing enhanced levels of 2-DG (0.2%). Twenty-five 2-DG-resistant mutants were screened for amylase hyperproduction and derepression on a medium containing 1.5% starch or 1.5% starch plus 5% glucose, using a semiquantitative plate assay method. We have also used this approach for selecting amylase-hyperproducing recombinant haploid segregants of *T. lanuginosus* [26]. Durand *et al* [9] used a similar approach for selecting hyper β-D-glucosidase-producing mutants of *T. reesei* to eliminate false-positive clones. We found that the flooding of starch plates with iodine results in false-positive clones. Using this approach, mutant strains II_9 and II_{19} were found to be amylase hyperproducing and deregulated. The results of the plate assay were corroborated in shake flask cultures. In shake flask cultures, maximal specific activity of α-amylase (129 U/mg protein) and glucoamylase (69 U/mg protein) in mutant II_{19} was observed, which was approximately five and two times higher, respectively, than wild strain, in media containing starch. Moreover, it was a partially catabolite-repressed mutant as it produced 76 and 39 U/mg protein of α-amylase and glucoamylase, respectively, in starch plus glucose medium. Enzyme activities observed in glucose-containing medium were approximately 5-fold higher as compared to the first-stage mutant, I_{615} (Table 2).

In the third step, mutant strain II_{19} was subjected to NTG mutagenesis. Of the 5100 survivors, 25 2-DG (0.2% w/v) resistant mutants were selected and screened by plate assay for constitutive amylase production in a medium containing 1.5% glucose, in addition to media containing 1.5% starch and 1.5% starch plus 5% glucose. Two mutants, III_6 and III_{51} were amylase hyperproducers, deregulated and partially constitutive. Of the third step mutants, the selected mutant strain III_{51} produced maximal specific activity of α-amylase (190 and 104 U/mg protein) and glucoamylase (105 and 62 U/mg protein) in starch and starch plus glucose media, respectively. Compared to mutant II_{19}, the constitutive levels of α-amylase produced by III_{51} and glucoamylase produced by III_6 were appreciably higher. The overall increase in specific activities of α-amylase and glucoamylase by mutant III_{51} were 7- and 3-fold higher compared to the wild-type W_o, in starch-containing medium. Chadha *et al* [7] reported 2.5-fold higher xylanase production by a *T. lanuginosus* mutant strain, than by the parent strain. *T. lanuginosus* mutant strain III_{51} was stable and did not lose its vigor upon repeated transfers and shake flask culturing during 3 years of experimentation.

Morphology of the mutant strain III_{51}

The mutant strain III_{51}, in addition to being 2 DG-resistant, is morphologically distinct from the wild type and formed pink colonies when observed on starch complete medium after 3–4 days at 50°C. Mutant III_{51} showed vigorous growth and intense sporulation. It had a 1.9-times-thicker hyphal diameter, and a 3.1-times-elongated aleurophore compared to the wild strain (Figure 1).

Mutants of *T. lanuginosus* showed altered enzyme kinetic properties both for α-amylase and glucoamylase. The V_{max} values, for α-amylase and glucoamylase was significantly higher in all mutants (data not shown). The glucoamylase of mutant strain III_{51} was also distinct as it showed higher affinity for starch (K_m=0.71 mg) compared to the wild type (K_m=2.5 mg). The V_{max} value of

Table 2 Production of α-amylase and glucoamylase by wild-type and mutant strains of *T. lanuginosus*

Strain	α-Amylase activity (U/mg protein)			Glucoamylase activity (U/mg protein)		
	a	b	c	a	b	c
Wild	28±0.30 (8±0.32)	5±0.37 (2±0.14)	4±0.21 (3±0.29)	32±0.38 (9±0.41)	3±0.32 (2±0.37)	4±0.29 (4±0.21)
First-step mutant						
I_{615}	64±5.92 (48±0.55)	14±0.38 (12±0.43)	13±0.53 24±0.58* (31±0.20) (49±0.60)*	34±0.38 (25±0.10)	7±0.15 (6±0.09)	5±0.36 14±0.52* (12±0.30) (28±0.41)*
Second-step mutant						
II_9	96±2.66 (96±1.30)	55±0.93 (52±0.75)	34±0.65 (98±0.41)	52±0.34 (52±0.32)	55±0.32 (26±0.39)	17±1.37 (48±1.10)
II_{19}	129±4.43 (106±1.25)	103±2.16 (79±0.65)	76±2.49 (113±1.41)	69±3.22 (56±1.19)	38±0.89 (29±0.60)	39±0.97 (58±0.29)
Third-step mutant						
III_6	134±2.16 (84±2.29)	97±1.92 (70±1.80)	92±2.14 (96±2.30)	118±2.15 (74±2.89)	73±1.93 (55±1.89)	72±1.32 (77±1.16)
III_{51}	190±3.42 (142±1.21)	124±2.38 (98±2.35)	104±3.76 (147±2.91)	105±2.61 (80±2.53)	39±3.10 (30±2.42)	62±1.71 (85±0.92)

Enzyme values given in parentheses are in units per milliliter.
a, Medium containing 1.5% starch; b, medium containing 1.5% glucose; c, medium containing 1.5% starch plus 5% glucose.
*Medium containing 1.5% starch plus 3% glucose.

glucoamylase for mutant strain III_{51} was 4.1 times higher than the wild type. In a similar observation, mutants of *P. pinophilum* showed improved V_{max} for β-glucosidase and cellobiase [5]. The V_{max}/K_m ratio calculated for α-amylase and glucoamylase of mutant strain III_{51} was appreciably higher than the wild-type strain. Thus, it is evident that the morphological mutant III_{51} is of considerable interest due to its high amylase production, resistance to catabolite repression, being partially constitutive, and its improved kinetic properties.

Box–Behnken design of experiments for amylase production by mutant III_{51}

In addition to mutagenesis and protoplast fusion [26], formulating a cost-effective medium and optimization of production parameters is an important aspect of a strain-development program. Preliminary studies using a classic one-dimensional approach to the search of variables [32] revealed corn flour and soybean meal as respective suitable carbon and nitrogen sources for amylase production at an initial pH of 5.5 by mutant III_{51}. To predict relationships between the independent variables (corn flour, soybean meal and pH) and the dependent variables (α-amylase and glucoamylase activities), a Box–Behnken design was used to obtain data as shown in Table 3. The coefficient of regression models as a function of corn flour, soybean meal and pH are reported in Table 4.

pH had a pronounced effect on α-amylase and glucoamylase production both in linear and squared terms (Table 4). The initial medium pH ranging between 5.0 and 5.8 was optimal for achieving higher α-amylase and glucoamylase activities. Petrova *et al* [23] found that an initial medium pH of 5.0 was optimal for production of α-amylase by *T. lanuginosus*.

Soybean meal showed a significant effect on α-amylase production and on glucoamylase production ($P<0.05$). Enzyme activities increased with an increase in the soybean meal level up to 0.45%, which may be attributed to the presence of essential amino acids in addition to being a good nitrogen source [11]. Organic nitrogen sources such as yeast extract also support high glucoamylase production by *Aspergillus awamori* [10] and *T. lanuginosus* [12].

Corn flour had a significant effect on α-amylase and glucoamylase production both in linear and squared terms. Corn flour in interaction with pH also showed a significant effect on

Table 3 Experimental design and test results of mutant strain III_{51}

Trial	Corn flour (%)	Soybean meal (%)	pH	α-amylase activity (U/mg protein)	Glucoamylase activity (U/mg protein)
1	1	0.3	5.5	93	37
2	3	0.3	5.5	94	46
3	1	0.5	5.5	96	51
4	3	0.5	5.5	128	56
5	1	0.4	4.0	72	23
6	3	0.4	4.0	37	8
7	1	0.4	7.0	77	21
8	3	0.4	7.0	77	30
9	2	0.3	4.0	55	7
10	2	0.5	4.0	84	35
11	2	0.3	7.0	73	32
12	2	0.5	7.0	103	56
13	2	0.4	5.5	136	77
14	2	0.4	5.5	136	77
15	2	0.4	5.5	135	74
16	2	0.4	5.5	137	79
17	2	0.4	5.5	142	85

Table 4 Coefficients of regression models for dependent variable of mutant strain III_{51}

Term	α-Amylase	Glucoamylase
Constant	−726	−692
X_1	31*	66**
X_2	801*	900**
X_3	229***	177***
$X_1 \times X_1$	−24***	−21***
$X_2 \times X_2$	−1063*	−949**
$X_3 \times X_3$	−21***	−16***
$X_1 \times X_2$	77*	−7
$X_1 \times X_3$	6*	4*
$X_2 \times X_3$	3	−6
S	9.768	6.677
R^2	96.0%	97.0%

*$P<0.5$.
**$P<0.05$.
***$P<0.005$.
X_1=corn flour; X_2=soybean meal; X_3=pH.

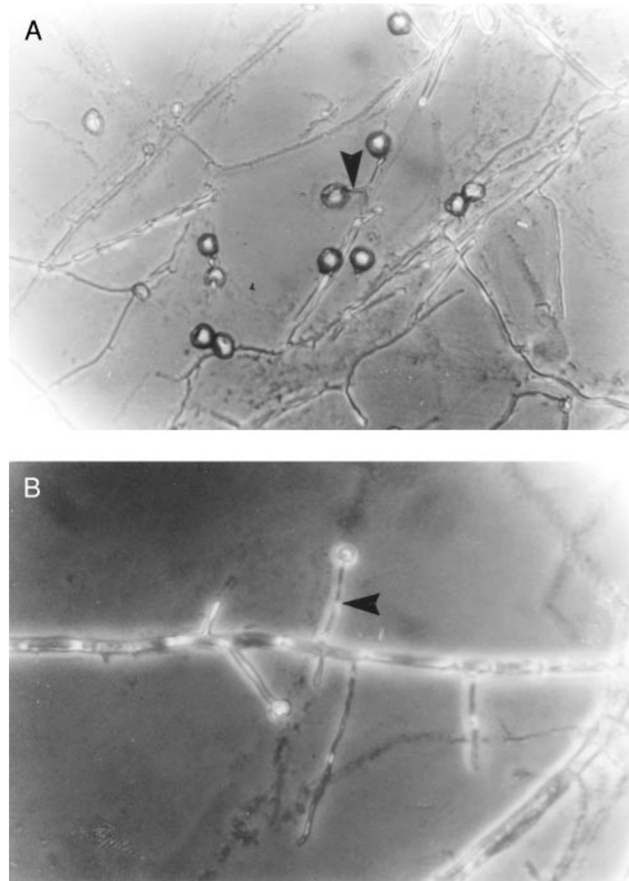

Figure 1 (A) Photomicrographs of mycelium of wild-type strain and (B) mutant strain III_{51} of *T. lanuginosus* (400×).

α-amylase production. Corn flour is a substrate of choice for glucoamylase production by *A. niger* at an industrial level [22]. Our results confirmed that corn flour can be a suitable and cheap alternative to low molecular weight dextran that supports maximal α-amylase production in *T. lanuginosus* [14,23].

The R^2 values of the models showed that they are appropriate and can be used to describe the effect of corn flour, soybean meal and pH on alpha amylase and glucoamylase production by *T. lanuginosus* III_{51}. Using regression models, it can be predicted that 2.1% corn flour, 0.46% soybean meal and an initial pH of 5.8 are optimal for maximal α-amylase activity (192 U/mg protein), whereas maximal glucoamylase activity (99 U/mg protein) could be achieved at 2.0% corn flour, 0.45% soybean meal and pH 5.7.

Acknowledgement

This work was supported by research grant (BT/13/12/94-PID) from the Department of Biotechnology, Government of India.

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