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Mutation and screening to increase chymosin yield in a genetically-engineered strain of *Aspergillus awamori*

Michael Lamsa and Peggy Bloebaum

Genencor, Inc., South San Francisco, CA, U.S.A.

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SUMMARY

Through the course of five rounds of mutagenesis of a genetically-engineered strain of *Aspergillus awamori*, the yield of a heterologous protein (the acid protease, calf chymosin) increased four-fold. This was accomplished through the use of an agar plate screen incorporating the colony restrictor 2,6-dichloro-4-nitroaniline (dichloran) and the acid protease inhibitor diazoacetyl-norleucine methyl ester (DAN) to reduce high background concentrations of the native acid protease. A miniaturized liquid culture growth method using 24-well culture plates was an intermediate screen between agar plate and shake flask cultures. Analysis of broth samples for active calf chymosin was accomplished with a highly specific, 96-well microtiter plate turbidimetric assay.

INTRODUCTION

The acid protease chymosin, found in the fourth stomach of unweaned calves, is a highly valued commercial enzyme used in the manufacture of cheese. The high degree of specificity of chymosin in cleaving kappa casein helps promote the clotting reactions in milk to form the curds from which cheese is manufactured [1]. Because of this specificity, cheese made with chymosin is less prone to the off-flavors caused by bitter peptides [2,3] released by other clotting proteolytic enzymes. The current method used to obtain chymosin involves extraction of enzyme from the stomachs of unweaned calves [1,4]. This process is expensive and the supply is subject to the fluctuations in the veal market. A commercial process utilizing genetically engineered microorganisims to produce this heterologous pro-

Correspondence: M. Lamsa, Genencor, Inc., 180 Kimball Way, South San Francisco, CA 94080, U.S.A

tein in constant supply and at reasonable cost would therefore be of great value.

In a previous publication [5], we described the expression of the chymosin gene in the non-commercial fungal species, *A. nidulans*, with four different constructions, schematically represented in Figure 1. It was shown that greater than 90% of the chymosin was secreted into the fermentation broth. The same four constructions were used in producing transformants of *A. awamori*, a commercially important organism. After an extensive screening program of these transformants, no strains were identified that could produce sufficient quantities of chymosin. Our objective then was to utilize new and existing techniques in mutagenesis and screening to achieve commercial levels of chymosin. The parent strain for mutants described in this paper was a transformant obtained with pGRG3, in our numbering system, transformant number 107.

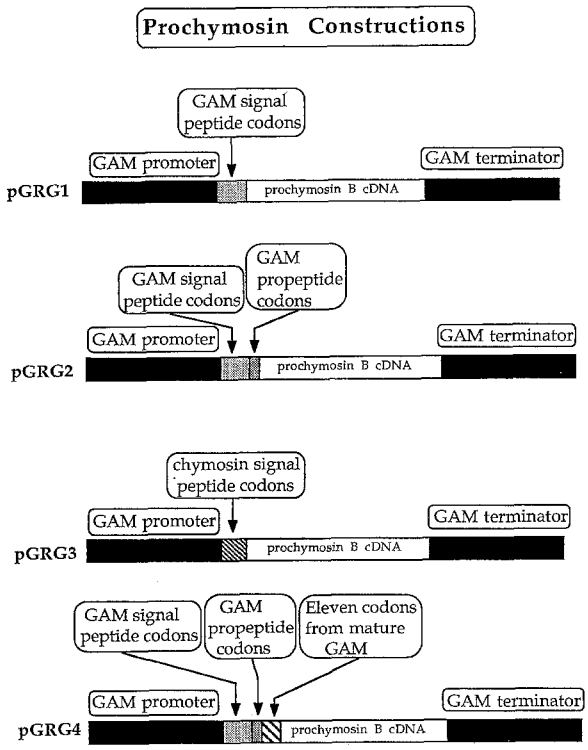

Fig. 1. Schematic representation of constructions pGRG1-4. Chymosin B is the isoenzyme of chymosin we cloned. GAM is an abbreviation for glucoamylase. Our previous publication [5] presents a more detailed map and explanation of these constructions and the plasmid.

We developed a screen for chymosin production by *Aspergillus* colonies growing on solid medium which utilizes the zone of clotting produced in overlays containing milk as the detection system. *Aspergillus awamori* produces a native acid protease which clots milk and can interfere with this screening procedure. This problem was overcome by the use of the acid protease inhibitor diazoacetyl-norleucine methyl ester (DAN). The colony restrictor 2,6-dichloro-4-nitroaniline (dichloran) was used to allow screening of the maximum number of colonies, in general 5 to 50 thousand per screen. An intermediate screen involving the growth of potential mutants in 24-well culture plates in 1 ml of liquid medium was key to the verification of a hyperproducing phenotype prior to evaluation in 50 ml shake flask cultures. In this way 20 to 100 individual isolates were evaluated prior to selecting less than 5 mutants for shake flask evaluation. The best strain from shake flasks was then evaluated in a 10-l fermenter. In this manner, over five generations of mutants were generated in an eight month period, leading to a four-fold increase in chymosin productivity.

MATERIALS AND METHODS

Strains

All strains originated from *Aspergillus awamori* UVK143f, a strain of *A. awamori* used for the production of glucoamylase that was derived by ultra violet light mutagenesis of NRRL3112. Strains 106, 107 and 125 are chymosin producing transformants obtained with pGRG2, pGRG3 and pGRG4 constructions, (Fig. 1), respectively. Strains A96, 1AA84, 2AB26, 2AC109 and 2AD73 are mutants of 107, generated in this study, in order of mutant generation.

Liquid culture medium

The medium consisted of $NaNO_3$ 0.6%, KCl 0.05%, KH_2PO_4 3.4%, $MgSO_4 \cdot 7H_2O$ 0.1%,

Maltose 10%, Malt Extract 2%, Bacto Peptone (Difco) 0.1%, Yeast Extract (Difco) 0.1%, pH 6.0.

Minimal medium agar screening plates

The plate medium consisted of $NaNO_3$ 0.6%, KCl 0.05%, KH_2PO_4 3.4%, $MgSO_4 \cdot 7H_2O$ 0.1%, maltodextrin M-040 (Grain Processing Corp., Muscatine, IA) 10%, agar (Difco) 2%, dichloran 0.002%, pH 5.0.

Complex medium agar screening plates

The plate medium consisted of $(NH_4)_2SO_4$ 0.6%, KCl 0.05%, KH_2PO_4 3.4%, $MgSO_4$ $7H_2O$ 0.1%, maltodextrin M-040 (Grain Processing Corp., Muscatine, IA) 5%, Malt Extract 2%, Bacto Peptone (Difco) 0.1%, Yeast Extract (Difco) 0.1%, agar (Difco) 2%, dichloran 0.002%, pH 5.0.

Skim milk overlays

The overlays consisted of $CaCl_2$ 0.45%, glacial acetic acid 0.6%, agarose 1%, skim milk 1%, pH 5.5 pour 25 ml per 13.8 cm diameter Gel Bond circle (Marine Colloids).

Mutagenesis

Ultraviolet light (UV) and nitrosoguanidine (NTG) mutagenesis experiments were modifications of described procedures [10,13].

Shake flask culture conditions

250 ml baffled flasks containing the appropriate medium were inoculated with equal sized agar plugs in triplicate. Flasks were incubated at 37°C on a rotary shaker at 200 RPM for four days with samples taken at 24-h intervals from day 1 through 3, and the entire flask harvested by filtration through miracloth filters at day 4. Filters were dried and weighed to determine mycelial dry weight.

Microculture conditions

Cultures were inoculated with equal sized agar plugs in 1 ml of modified media in triplicate wells of 24-well culture plates. Plates were incubated at 37°C on an orbital shaker. On the fourth day, the entire plate was harvested and broth supernatants were analyzed for chymosin.

Analytical methods

Enzyme immunoassay (EIA) for chymosin was done as described previously [5]. The concentration of chymosin was calculated from standard curves constructed with authentic calf chymosin (Chris Hansen's Lab, Denmark), where 1 milligram of chymosin B was equal to 76.5 Chris Hansen Units (CHU) [6].

Chymosin activity was measured by a microtiter milk turbidity assay versus authentic calf chymosin as follows: To 25 μl of samples and standards diluted in 10 mM sodium phosphate buffer, pH 6.0, 150 μl of turbidity substrate (1% skim milk, 0.45% calcium chloride, in 0.3% sodium acetate, pH 6.0) is added and the microtiter plate is sealed and incubated in a 37°C water bath for 15 min. At the end of the incubation period, absorbance was measured at 690 nanometers with a microtiter plate reader (Biotek Instruments). Chymosin activity values were calculated from a standard curve that is linear between 100 and 800 ng/ml, generated by plotting absorbance versus the reciprocal of the chymosin concentrations.

Plate screen methods

In the schematic representation of this method (Fig. 2) the fungal colony restrictor dichloran [7], was used to produce hundreds of small (1–2 millimeters in diameter), distinct colonies which were grown on filter paper (Schleicher and Schuell # 404 filter paper circles) overlaid on agar screening plates. After a 3 to 4 day incubation period at 37°C, the filter papers containing the colonies were removed and set aside. Overproducing chymosin mutants were detected using the acid protease inhibitor diazoacetyl-norleucine methyl ester (DAN), and a skim milk overlay poured on Gel-Bond.

Three steps were performed with the 14 cm agar screening plates from which the colonies had been removed. The plates were incubated at room temperature with a copper sulfate solution, followed by incubation with the DAN inhibitor and finally were incubated at 37°C with 1% skim milk overlays. The use of this inhibitor is a two step process requiring copper [8,9], and each step was performed individually to prevent the inactivation of the inhibitor by

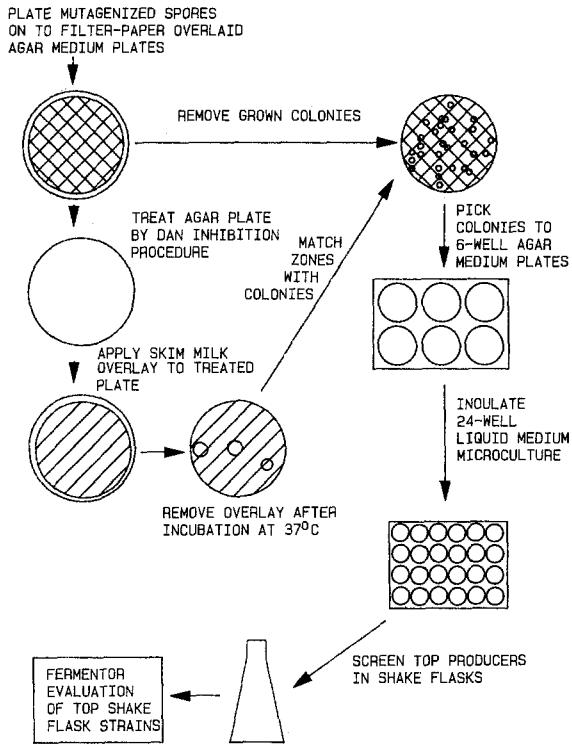

Fig. 2. Schematic representation of the major steps of the screening techniques.

precipitation with the copper. DAN and copper concentrations varied from 2 to 12 mM and were made up in 100 mM sodium acetate buffer, pH 5.5, using 25 ml of each solution per plate. The concentration to use for each screen was determined by titration of control plates. DAN was shown to eliminate the clotting due to the native acid protease in untransformed *A. awamori* UVK143f, while allowing transformants to clot milk in the overlays, presumably due to the presence of uninhibited or partially inhibited chymosin.

Detection of chymosin was seen on 1% skim milk-agarose overlays. After incubation, the overlays were removed and clotting zones were marked on the hydrophobic side of the Gel-Bond. The overlays were aligned with the filter papers on which the colonies had been grown. Individual colonies from these filter papers were picked on to individual wells of 6-well complete medium agar plates and incubated 3–5 days at 37°C. Agar plugs taken from these individual isolates were then analyzed as described under microculture methods.

RESULTS

The overall strategy for screening mutants for chymosin over-production was to culture mutagenized spores on plates of various media and then to detect chymosin activity from the size of zones of clotting in skim milk-agarose overlays. Apparent over-producers detected in this way were grown in 24-well culture plates containing 1 ml liquid medium per well and broth samples were assayed for chymosin activity. The best chymosin producers were subsequently grown in 50-ml shake flask cultures and further analyzed.

Prior to the development of the final plate screen for chymosin production, several transformants were tested to determine the ability to detect differences in chymosin production (Table 1). Strain 107 had previously been shown in shake flask culture to be superior to strains 106 and 125, and this proved to be true when these strains were grown on agar

Table 1

Concentrations of chymosin at the agar plate level[a]

Strain	Construction[b]	Control	Post-incubation	Post-overlay
106	pGRG2	27	23	23
107	pGRG3	62	53	48
125	pGRG4	23	19	21

[a] Values are chymosin concentrations in nanograms per 40 microliter plug taken at the center of unrestricted colonies grown on filter-paper overlaid minimal media agar screening plates, 3 days at 37°C. Control values are for untreated plates, post-incubation values are for plates after the incubations utilizing the DAN inhibitor procedure and post-overlay values are for plates having gone through the inhibitor incubation and activity overlay. These data were generated through the use of an EIA for chymosin. Chymosin was extracted by incubating the agar plug in a 2% bovine serum albumin, 10 mM potassium phosphate, 150 mM sodium chloride buffer, pH 7.2, 0.25 ml per plug, at 37°C for 2 h.

[b] Refer to Fig. 1 for description.

Table 2

Effect of dichloran on chymosin concentration in agar screen plates, strain 107

[Dichloran][a]	Colony size[b]	Mean value[c]	Standard deviation[d]
Control	8–10	188	37
10	2–3	70	17
19	1	25	5.5
25	<1	7	1.8

[a] Concentration of dichloran in minimal medium screening plates, μg/ml.
[b] Range estimate of size (mm) based on 4 day growth at 37°C.
[c] Expressed as ng chymosin per 45 μl plug by the EIA for chymosin, as described previously.
[d] Four randomly selected plugs per assay.

containing medium. Since dichloran was used to allow the screening of the maximum number of colonies per plate, the use of this colony restrictor made it necessary to determine what its effect would be on chymosin secretion. It was found that the levels secreted were affected uniformly and could be correlated with the decrease in colony size (Table 2).

A series of experiments were performed to determine a protocol for the inhibition of native acid protease. Without inhibitor, untransformed strain UVK143f produced large clotting zones on milk overlays. Intervals of 15, 30, 60 and 120 min, all at room temperature, were used for both the primary incubation with copper sulfate and secondary incubation with DAN. With unrestricted colonies, a minimum treatment of 30 min was required to completely inhibit the *A. awamori* acid protease reaction when the resulting plate was incubated for up to 3 h at 37°C with a milk overlay. When this inhibition procedure was used, it was first necessary to titer a set of control and test plates to determine the correct concentration and time interval to use for optimal results (i.e., to obtain inhibition of *A. awamori* acid protease, but still allow detection of chymosin).

Initial mutagenesis experiments were performed with ultraviolet (UV) light. To achieve a 98% kill the time of exposure with UV increased from 7 min to 250 min between subsequent generations of mutants. Because of this increased difficulty in obtaining high kill rates with UV, nitrosoguanidine (NTG) was substituted for UV. Since NTG is a more potent mutagen [10], it was felt that this mutagen could have a greater impact in yield-improvement.

At least one screen of survivors from each mutagenesis experiment was done with each of the different screen plate media. This method offered the potential to select a wider variety of mutants, possibly selecting mutants from the complex medium screen that would otherwise have been missed in the minimal medium screen. Of the final two mutant strains isolated, 2AC109 was selected by screening on complex medium and 2AD73 was selected by screening on minimal medium plates.

It was difficult to choose potential overproducing mutants at the agar plate level, especially with increasing chymosin yield improvement and the necessity to increase inhibitor concentrations. Adjustments in inhibitor concentration and incubation time for the activity overlay were necessary to minimize the background of endogenous acid protease and weak chymosin producers as yield improvement progressed. Inhibitor concentration started with 2 mM DAN at the beginning of the program and increased to 12 mM DAN when strain 2AD73 was isolated. Although substantial background had been reduced, clotting zones formed after these treatments were often difficult to differentiate based on size and intensity. Incorporation of the microculture technique described proved to be an invaluable tool in reducing the number of mutants screened in shake flasks. Using the microculture method, many more mutants were screened in a short time period and shake flask screens differentiated among true variants. The key strains, mutagens used and number of variants screened with the described methods are summarized in Table 3.

The activity assay for chymosin was highly specific. Nearly 10 micrograms of acid protease from *A. awamori* was required to give the same milk clotting response as 0.2 micrograms of chymosin, a fifty-fold difference (unpublished data). The endogenous acid protease produced by the mutant strains

Table 3

Strains leading to increased chymosin

Strain	Mutagen	Generation number	Number of mutants screened		
			Agar plates	24-well microculture	Shake flasks
107[a]	UV	Parent	350 000	126	4
A96	UV	1	69 000	84	2
1AA84	UV	2	53 000	79	2
2AB26	NTG	3	48 000	115	1
2AC109	NTG	4	26 000	81	2
2AD73[b]	NTG	5	–	–	–

[a] Parent strain, from which all succeeding mutants are derived.
[b] New screening methods were started after this strain.

did not exceed chymosin by more than two-fold on a weight basis (unpublished data). Therefore, samples were always diluted past the threshold of interference.

The EIA for chymosin generally showed good agreement with the activity assay under optimal growth conditions; to avoid false positives, both assays were routinely employed in the evaluation of mutants in the shake flask culture. The focus of the mutagenesis and screening program was to select high producing mutants based on active chymosin secreted into the broth. Under the conditions of growth, the pH was sufficiently low for prochymosin to be processed to the active enzyme. Western blot analysis (unpublished data) showed detectable levels of prochymosin only during the early phases of growth (the first 24 to 36 h) with no detectable levels of prochymosin found in mature cultures. With increasing age of the cultures, the acid protease and possibly other proteolytic enzymes tended to degrade or inactivate the chymosin. EIA values that are substantially higher than the activity values are only seen at these late stages, with excellent agreement in the early time points.

Table 4

Time course of chymosin expression

Strain	Chymosin concentration (mg/l supernatant)							
	Turbidity assay				EIA			
	Day				Day			
	1	2	3	4	1	2	3	4
107	<1	11	11	11	<1	14	21	21
A96	<1	14	17	15	<1	13	20	31
1AA84	<1	15	17	16	<1	17	19	28
2AB26	<1	12	21	20	<1	13	26	42
2AC109	<1	18	33	31	<1	18	37	45
2AD73	<1	8	46	45	<1	10	48	81

Values are average of triplicate shake flasks per strain, rounded to the nearest milligram.

Table 4 shows a time-course analysis of extracellular chymosin found in shake flask culture broths of strain 107 and mutant derivatives in order of generation, using each assay method. It can be seen that the agreement in methods is nearly identical for the first two time points. At day 3, activity values are 75, 85, 89, 81, 89 and 96 percent of those for mass values obtained with the EIA, for the strains in order as listed in Table 4. Most strains show substantial differences by day 4, which is more a function of culture age than any differences due to a change in specific activity of the gene product. In addition, any difference due to a deviation in specific activity of the active enzyme would be observed at all time points. Degradation can in fact be detected by Western blot analysis confirmed by the appearance of lower molecular weight fragments that react with the chymosin antibody. Cultures grown in fermenters under more optimal conditions show much less deviation between the two analytical methods. It is noteworthy that the ranking of strains by order of increasing chymosin production is the same with either analytical method.

For the purposes of comparing chymosin production based on mycelial weight, the flasks were harvested at 95 hours, after no change in active chymosin was detected during a 24-h period (Fig. 3). Early strains A96 and 1AA84 show moderate to no improvement, with more pronounced differences occurring later in the program. This appears to coincide with the change from UV to NTG mutagenesis. In all, the yield improvements achieved were accomplished in the relatively short time frame of eight months. These results are depicted in Fig. 4.

DISCUSSION

Results presented here for this yield improvement program demonstrate the ability of a genetically-engineered filamentous fungus to be further enhanced for the secretion of a heterologous protein

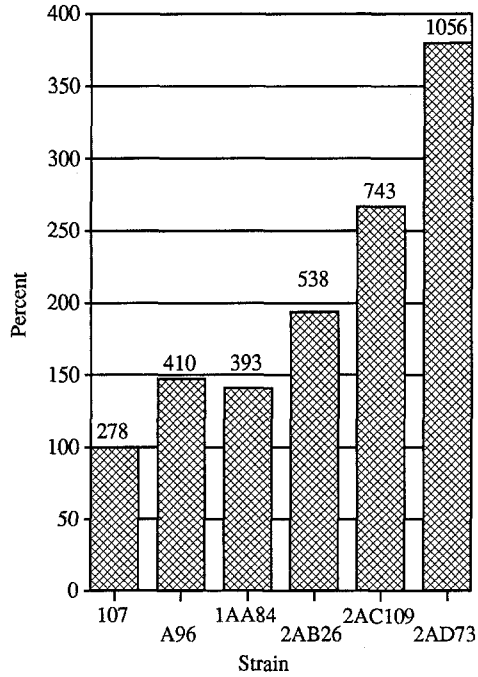

Fig. 3. Chymosin yield improvement as a percent of strain 107. Value above each bar gives micrograms of chymosin produced per gram dry weight mycelia at 95 h.

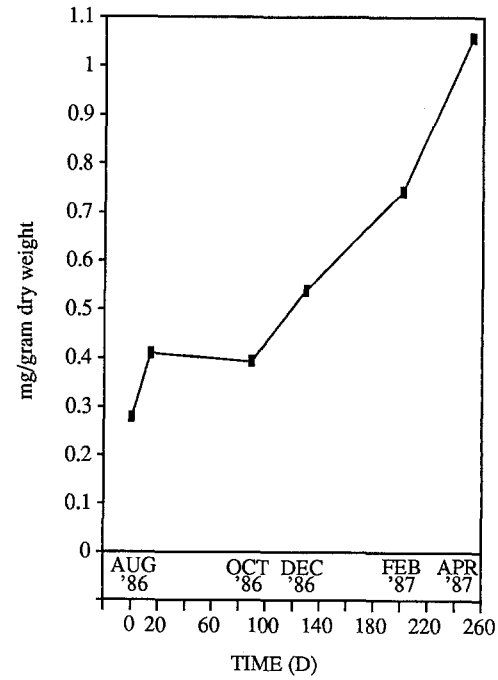

Fig. 4. Increase in chymosin expression with time based on mycelial dry weights. Strains are in order from left to right: 107, A96, 1AA84, 2AB26, 2AC109, 2AD73.

using mutagenesis and screening techniques. Of further importance, the protein secreted is of high commercial value. We have evaluated several of these chymosin producing strains in fermenters, and have achieved yields in excess of 100 mg/l. This is an improvement of nearly five fold over the parent strain 107. Purified and characterized fermentation broths show that the recovered material is identical to native calf chymosin when N-terminal analysis, amino acid composition, Western immunoblotting analysis, specific activity and clotting time characterization in roller-bottle assays are performed [1,17].

One of the major problems we experienced in developing an effective screening method was the presence of the native *A. awamori* acid protease. Although we were able to substantially reduce background with the use of the DAN inhibitor, the use of this inhibitor was labor-intensive and subject to anomalous results. With the use of miniaturized 24-well culture plate techniques, hundreds of mutants were analyzed in liquid culture, increasing our chances of selecting superior strains.

Enzyme immunoassays of the native acid protease show an increase in this enzyme through the first several mutants in this line, then a moderate decrease by the fifth generation, strain 2AD73 (unpublished data). Recently, Berka, et al. [11] have reported deleting the gene for acid protease, solving many of the problems associated with the presence of fungal acid protease on the fermentation broth. There is evidence that acid protease degrades chymosin in vitro (Kirk Hayenga, personal communication). We have observed that chymosin degradation also occurs in fermentation runs that have high acid protease levels. This observation offers one explanation for the differenes in EIA and activity values during late fermentation. These same chymosin constructions in *A. awamori* strains having the acid protease deleted show similar differences in EIA versus turbidity values (unpublished data). Further, these new strains have not undergone any mutagenesis. This suggests that although the acid protease can be demonstrated to cause degradation of chymosin, the action of other proteases, including that of chymosin itself, can not be ruled out as responsible for this degradation. In addition, purified chymosin from fermentation broths has been shown to have identical specific activity to authentic calf chymosin, and exhibits good agreement between activity and EIA values [17].

Other secreted enzymes have been affected by this mutagenesis program. Relative glycoamylase and alpha amylase levels monitored by activity and EIA analysis (unpublished data) changed with each successive generation of mutants. The most dramatic effects were observed with glucoamylase levels, which in strain 2AD73 were only 10% of that produced in parent strain 107. It is not yet clear what has occurred at the genetic level to produce the changes in production of these enzymes. It has been shown that there is abundant chymosin-specific mRNA in strain 107 and its mutants [16]. Mutant 2AB26 has as much chymosin mRNA as glucoamylase mRNA (personal communication, Michael Ward), yet glucoamylase is secreted at ten to twenty times the mass (5 to 10 times on a molar basis) as chymosin. This suggests that the limit to production is not at the level of transcription, but may exist at a post-transcription stage such as mRNA processing, translation or secretion. It is possible that mutations we have induced lead to more efficient translation or secretion.

In summary, the application of mutation and screening techniques for yield improvement in filamentous fungi has a well-founded history [12]. With the advent of cloning and transformation systems for filamentous fungi, further development of these techniques seems natural. Automation and miniaturization has been suggested [13–15] to be key to succes in yield improvement programs.

Appropriately, we are in the process of updating and automating techniques for screening that will allow us to take a more quantitative approach in the primary screen. Many of the techniques we are developing are generic in nature and can be applied to a wide variety of fungi. It is our feeling that yield improvement programs which employ these techniques will play an important role in developing fungal strains for the economical expression of heterologous genes.

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