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Characterization of *Aspergillus niger* Mutants Deficient of a Protease

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Aspergillus niger **has been used as a host to express many heterologous proteins. It has been known that the presence of an abundant protease is a limiting factor to express a heterologous protein. The protease deficient mutant of *A. niger* was obtained using UV-irradiation. A total of 1×10^5 spores were irradiated with 10~20% survival dose of UV, 600 J/m^2 at 280 nm, and the resulting spores were screened on the casein-gelatin plates. Ten putative protease deficient mutants showing the reduced halo area around colonies were further analyzed to differentiate the protease deficient mutant from other mutant types. Among ten putative mutants, seven mutants showed significant growth defect on nutrient rich medium and two mutants appeared to be the secretory mutants, which resulted in the impaired secretion of extracellular proteins including proteases. A mutant pro$^-$-20 showed reduced halo zone without any notable changes in growth rate. In addition, the starch-degrading and glucose oxidase activities in the culture filtrate of pro$^-$-20 mutant showed the similar range as that of the parental strain, which suggested that the pro$^-$-20 mutant ought to be the protease deficient mutant rather than a secretory mutant. The reduced proteolytic activity of the pro$^-$-20 was demonstrated using SDS-fibrin zymography gel. The reduced extracellular proteolysis was quantified by casein degradation assay and, comparing with the parental strain, less than 30% residual extracellular protease activity was detected in the culture filtrate of the pro$^-$-20 mutant. The bio-activity of an exogenously supplemented hGM-CSF (human Granulocyte-Macrophage Colony Stimulating Factor) in the culture filtrate of pro$^-$-20 mutant was detected until eight times more diluted preparations than that of the parental strain.**

KEYWORDS: ***Aspergillus niger*****, Protease, UV-mutagenesis**

Filamentous fungi are employed in a variety of industrial processes, including the production of fermented foods, primary metabolites such as organic acids, vitamins, or secondary metabolites (Bodie *et al.*, 1994; Luengo and Penalva, 1994; Sakaguchi *et al.*, 1992). Moreover, filamentous fungi are excellent producers of a broad spectrum of extracellular enzymes and have been used as a host to express many heterologous proteins (Berka *et al.*, 1992; Royer *et al.*, 1995). *Aspergillus niger* has several advantages over other expression systems in that it is a small eukaryotic GRAS (Generally Recognized as Safe) organism with a capacity of secreting a large amount of protein, which can be as high as 20 g/*l* of homologous protein in industrial production strains (Finkelstein, 1987). However, yields of heterologus proteins have generally been low compared to those of homologous proteins, because the presence of an abundant protease is a limiting factor to express a heterologous protein (Carrez *et al.*, 1990; Dunn-Coleman *et al.*, 1991). Recently, we have shown that a fungal expression of mammalian protein with a complex three dimensional structure such as cytokine has an advantage of better specific activity than that from *E. coli* and the expression level can be further improved if using a protease deficient fungal strain (Kim *et al.*, 2000). Accordingly, the improvement of strain has been achieved through various types of strategies including mutagenesis (Mattern *et al.*, 1992), genetic recombination (van den Hombergh *et al.*, 1995) and genetic engineering (Berka *et al.*, 1990; Lenouvel *et al.*, 2001). Thus, the organism of *A. niger* was thought to be a good host for this process.

Although the spectrum of protease in individual *Aspergillus* species can be very diverse and species-specific, nine different proteases, PEPA H and CPY, have been identified in *A. niger* and genes corresponding to those protease have been cloned. Among these, it appeared that acidic extracellular proteases, PEPA, B, F, and G are predominant in *A. niger*. In order to improve the expression of heterologous proteins in *A. niger*, several studies including protease-repressing culture condition (Jarai and Buxton, 1994), classical mutagenesis (Mattern *et al.*, 1992) and construction of null-mutant of either a single (Mattern *et al.*, 1992) or several genes (van den Hombergh *et al.*, 1997) encoding protease have been conducted. Among them, a strain deleting major proteases PEPA, B and E was constructed and very little degradation of susceptible pectinolytic enzyme PELB was observed compared to that found in wild type *A. niger*. However, the engineered strain has a very limited distribution in terms of heterologous expression at the industrial scale. In addition, the facts that it was possible to obtain a strain with 500~1000 times improved protection of the tester protein by a sin-

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gle mutation (van den Hombergh *et al.*, 1997) prompt us to construct a protease deficient mutant by UV-mutagenesis.

Materials and Methods

Chemicals and enzymes. Unless otherwise specified, all chemicals, media, and enzymes used in these studies were purchased from Sigma Chemical Co. (MO, U.S.A.), Difco Laboratories (MI, U.S.A.) or Boehringer Mannheim (Mannheim, Germany), respectively.

Isolations. *A. niger* ATCC9642 was used as a parental strain in the UV-mutagenesis experiments. *A. niger* was grown on complete medium (CM) or on minimal medium (MM) with a composition according to Pontecorvo (1953). Protease repressing medium contained 1% yeast extract, 2% peptone, 2% glucose, 0.6% urea and 100 mM NH_4Cl. Casein-gelatin plate consisted of MM plus 50 mM glucose, 0.5% casamino acid, 1% gelatin, 1% casein and 0.01% Triton X-100. Starch plate contained MM supplemented with 0.1% glucose and 1% corn starch. Spores were generated on CM and conidial suspension was made in Tween 20 (0.02%) with vigorous shaking to break conidial chains and to reduce conidial aggregation. This conidial suspension was filtered through a glass-wool plug to remove residual mycerial fragments and used to inoculate 500 m*l* of CM at the concentration of 2×10^8 spores/ml. The inoculated media was incubated for 24 h to 96 h in an orbital shaking incubator at 30°C (150 rpm).

Mutagenesis. A suspension of freshly harvested spores of *A. niger* ATCC9642 was exposed to 600 J/m^2 ultra violet (UV) light from a philips TUV lamp emitting UV-C light at 280 nm. The irradiated spores (survival rate 10~20%) were diluted and plated for single colonies on casein-gelatin plate. After 72 hr of incubation, a halo is formed around the colony due to extracellular proteolytic activity. Colonies with a smaller halo than parental colony were stored on CM and tested again on casein-gelatin plate as well as starch plate in order to distinguish between general secretory mutants and specific protease mutants. Glucose oxidase (GO) activity was measured according to the modified protocol of Sigma. One hundred m*l* of culture filtrate was incubated for 10 min in 0.05 M sodium acetate buffer (pH 5.1) containing 2.4 m*l* of 0.21 mM *o*-dianisidine, 0.5 m*l* of 10% β-D-glucose and 0.1 m*l* of peroxidase solution (60 U/m*l*). The reaction was stopped by addition of 0.3 m*l* of 4 N H_2SO_4 and optical density was measured at the wavelength of 500 nm. The measurements were performed in triplicate and all experiments were carried out at least three times with similar results. GO activity was determined using wild type GO as a standard.

Fibrin zymography. Proteolytic activities in culture filtrates of mutants were observed by fibrin zymogrpahic gel assay (Choi *et al.*, 2001). SDS-Polyacrylamide gel electrophoresis was conducted using 12% acrylamide separating gel co-polymerized with 0.12% fibrinogen and 5% stacking gel cotaining. Culture filtrates of mutant strains were concentrated 20 times using Centricon YM-10 (Millipore, MA, U.S.A.) and subjected to gel eletrophoresis. Electrophoresis was carried out at a constant current of 15 mA for 12 hr at 4°C cold room using a running buffer of tris-glycine containing 0.1% SDS. After electrophoresis, the gel was washed with 50 mM Tris-HCl (pH 8.0) containing 2.5% Triton X-100 for 30 min and washed again with distilled water for 1 hr to remove Triton X-100. After washing, the gel was incubated in a zymogram reaction buffer (30 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3) for 18 hr at 37°C incubator. The gel was stained with 0.025% Coomassie brilliant blue R-250 solution for 30 min and destained with in 100 m*l* of the destaining solution containing 10% methanol and 5% acetic acid.

Protease. The proteolytic activities were measured from 1-, 3-, and 5-day old culture filtrates. The proteolytic activity was determined by monitoring the amount of tyrosine released from casein (Kim *et al.*, 2001). The reaction mixture which consisted of 200 μl of culture medium and 200 μl of 1% casein in 0.25 M Tris-HCl buffer (pH 7.0) was incubated at 37°C. Then 600 μl of 0.4 M trichloroacetic acid (TCA) was added to the mixture to stop reaction and the resulting precipitate was removed by centrifugation. The protease activity was represented as the amount of tyrosine present in the TCA-soluble supernatant, which was determined by measuring at 280 nm. Protein was measured as described by Bradford (1976) using bovine serum albumin as a standard.

hGM-CSF. Degradation of human granulocyte macrophage-colony stimulating factor (hGM-CSF) in culture media was measured by incubation of 100 μl of hGM-CSF solution (containing 2.5 ng hGM-CSF) with 100 μl of culture medium at 30°C for 2 h. The culture filtrates containing exogenous GM-CSF were collected, dialyzed against PBS two times for 2 h at 4°C, and sterilized by 0.2 μm syringe filter. The culture filtrates were serially diluted with sterile PBS and an aliquot of 200 μl of each sterile diluent was prepared for the bioassays. The bioactivity of residual hGM-CSF was measured as described previously (Kitamura *et al.*, 1989). Briefly, the growth factor-starved cellls of hGM-CSF dependent cell line, TF-1, was added to each well of a microtiter plate containing serially diluted sample preparations. The plates were incubated for 48 h, and then 1 μCi of [methyl-^{3}H] thymidine (Amersham Life Science, NJ, U.S.A.) was added to each well.

The plates were incubated for an additional 16 h. The cells were then harvested using an Inotech cell harvester (Switzerland), and the tritium content was measured by liquid scintillation counting. Recombinant *E. coli*-derived hGM-CSF was purchased from Endogene (MA, U.S.A.) and used as a positive control for activity.

Results and Discussion

Isolation of mutants. Various strength of UV from 200 to 1,000 J/m^2 at 280 nm was treated with an interval of 200 J/m^2 in order to determine UV dose to yield 10~20% survival rate. Among the tested, the dose of 600 J/m^2 at 280 nm resulted in 10~20% survival of irradiated spores. Therefore, 400 spores, freshly harvested from CM media, were irradiated and spread on 9-inch diameter petri plate containing MM to obtain the colonies with the size of ≥ 5 mm in diameter. These colonies were then transferred again on MM from which a uniform-size agar plug containing the actively growing hyphal tip was obtained from using a cork-borer and placed on the casein-gelatin plates to see the difference in the extracellular protease activity for each colony.

A total of 1×10^5 spores were irradiated and 1.3×10^4 colonies were transferred to new MM. Among the transferred colonies, more than 400 colonies showed the severely retarded growth on MM indicating there were growth defects in these mutants. Ten putative protease deficient mutants showing the reduced halo zone on casein-gelatin plate were isolated. These ten mutants were further analyzed on the starch plates to differentiate protease mutants from secretory mutants. Out of ten putative mutants, two mutants (pro$^-$-4 and pro$^-$-20) had a reduced or no halo on casein-gelatin plates but a normal halo on starch plates. It was of interest to have more mutants showing reduced haloes on both casein-gelatin and starch plates, which suggested that mutation in a secretory pathway was more common than that in a protease production. The pro$^-$-4, however, showed reduced growth and sporulation rates indicating that the reduced halo zone could be attributed to a mutation other than protease itself (Fig. 1).

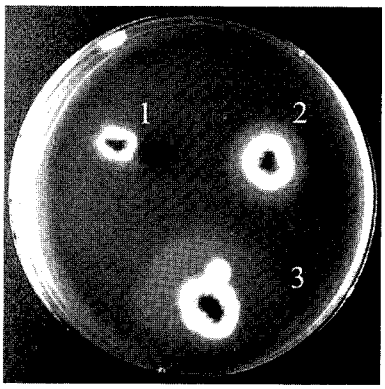

Fig. 1. Proteolytic activity of the parental and mutant strains on the casein-gelatin plate. 1, pro$^-$-4; 2, pro$^-$-20; 3, parental strain.

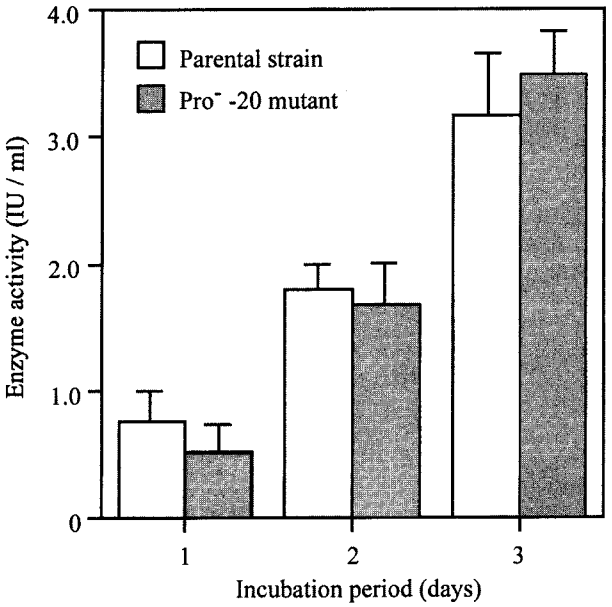

Fig. 2. Glucose oxidase (GO) activity of the parental and pro$^-$-20 strains. One unit of GO activity was defined as the amount of enzyme that will oxidize 1.0 μmole of β-D-gluconic acid and H_2O_2 per minute at pH 5.1 at 35°C. The data are the mean of at least three independent experiments and standard deviations are represented as vertical bars at the top of column.

In order to confirm that the mutant phenotype was due to the protease deficiency rather than the mutation in the secretory pathway, the activity of glucose oxidase, an extracellular enzyme that is well known for *A. niger*, was also examined (Lee *et al.*, 1998; Park *et al.*, 2000). As shown in Fig. 2, no difference in glucose oxidase activities were observed between culture filtrates of parental strain and pro$^-$-20, suggesting that the pro$^-$-20 appeared to

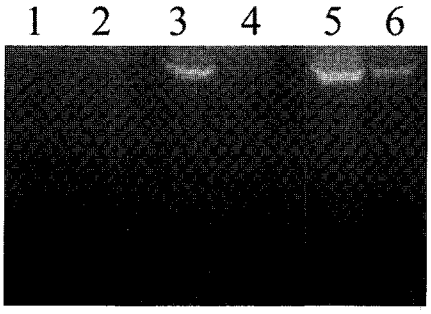

Fig. 3. Fibrin zymogrpahic assay. SDS-fibrin polyacrylamide gel for detection of protease analysis of parental strain and pro$^-$-20. Lanes 1, 3, and 5 contain 1-, 3-, and 5-day old culture filtrates of parental strain, respectively. Lanes 2, 4, and 6 contain 1-, 3-, and 5-day old culture filtrates of pro$^-$-20 mutant, respectively.

be a protease-deficient mutant without any changes in its secretory capability.

Determination of protease activity. Protease activity in the culture filtrate of pro$^-$-20 mutant was observed by measuring the degradation of fibrin on SDS-PAGE gel (Fig. 3). Since this method is using a SDS-polyacrylamide gel that is co-polymerized with a protein substrate, fibrinogen, the protease activity in the culture filtrate is separated by a gel electrophoresis and visualized as a clear zone where the proteolytically active enzyme have degraded the protein substrate in the gel (Choi *et al.*, 2001). As shown in Fig. 3, no proteolytic activities were detected in the 1-day old culture filtrates of parental strain and pro$^-$-20. However, at least two bands were detected from the 3-day old culture filtrate of parental strain in the upper part of fibrinogen gel. Comparing with those proteolytic bands of the parental strain, the culture filtrate of pro$^-$-20 showed the identical, but with a low intensity, band patterns indicating that the proteolytic activities by these proteases were considerably decreased. The 5-day old culture filtrates of parental strain and pro$^-$-20 showed more proteolytic activities comparing to those of 3-day old culture filtrates. However, the protease activities in the culture filtrate of pro$^-$-20 never exceeded those of parental strain. Several studies have shown that wide-domain regulatory mutations resulted in strongly reduced transcriptional levels of all extracellular proteases, which was reflected by a significant reduction of the total extracellular protease activity (van den Hombergh *et al.*, 1996, 1997). Considering that more than two proteolytic bands are simultaneously decreased in the pro$^-$-20 mutant, it appears that the mutation of pro$^-$-20 occurs in a wide-domain regulatory gene rather than a specific structural gene of protease. The expression pattern of proteolytic activities from 1-day to 5-day old cultures of parental strain is in a good agreement with previous studies showing that the heterologous protein product was most at the beginning of culture and decreased thereafter even though there was still a great deal of mRNA transcript (Kim *et al.*, 2000).

In order to measure the protease activity, casein degradation assay was performed (Fig. 4). The proteolytic activities of culture filtrates from parental strain increased as the culture proceeded. However, the proteolytic activity of pro$^-$-20 mutant reached the peak at 3 days of cultivation and then gradually decreased. Comparing with the parental strain, less than 30% of the proteolytic activity was observed in the culture filtrate of pro$^-$-20 mutant without any notable changes in cell growth and secretory mechanism. In the previous study, it was possible to obtain a UV-induced mutant of *A. niger*, which had only 1~2% of the extracellular protease activity in the parent strain (Mattern *et al.*, 1992). However, the discrepancy

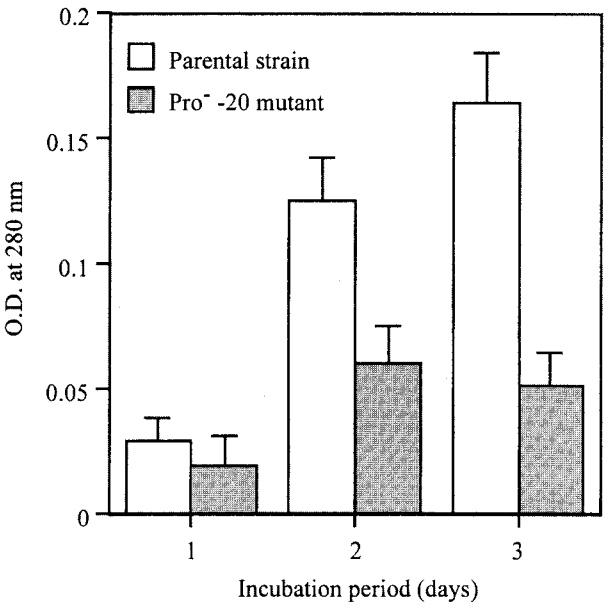

Fig. 4. Casein-degradation assay for the parental and pro$^-$-20 strains. Proteolytic activity was measured by monitoring the amount of tyrosine released from casein. The data are the mean of at least three independent experiments and standard deviations are represented as vertical bars at the top of column.

between the Mattern and our studies seems to be due to the use of different strains and/or reaction conditions using different substrates. In case of the parental strain, the zymographic assay showing the largest clear zone at 5-day old culture filtrate coincided well with the results of casein-degradation assay. However, the zymographic assay of the culture filtrate of pro$^-$-20 mutant did not: the pro$^-$-20 mutant showed more proteolytic activity in 3-day old culture while the clear zone at 5-day old culture was most obvious in zymogram. Considering that six out of nine different proteases are extracellular proteases in *A. niger*, the zymogram made only a view of activities which were strong enough to be seen as a clear zone in the substrate gel. Thus, two or three major protease activities of pro$^-$-20 mutant were represented in zymogram and they were increased as the culture period. However, the casein-degradation assay, measuring the degraded amino acid monomer out of the polymeric casein, resulted from a sum of proteolytic activities of all possible extracellular enzymes, which explained the discrepancies between the zymogram and casein-degradation assay.

Bioactivity of exogenously added cytokine hGM-CSF. Degradation in culture medium of exogenously supplemented heterologous protein, human granulocyte macrophage-colony stimulating factor (hGM-CSF), was determined by measuring the bioactivity of hGM-CSF. As shown in Fig. 5, a significant amount of tritium uptake ($\geq$ 5,000 cpm) was observed until 32 times diluents of 1-day old

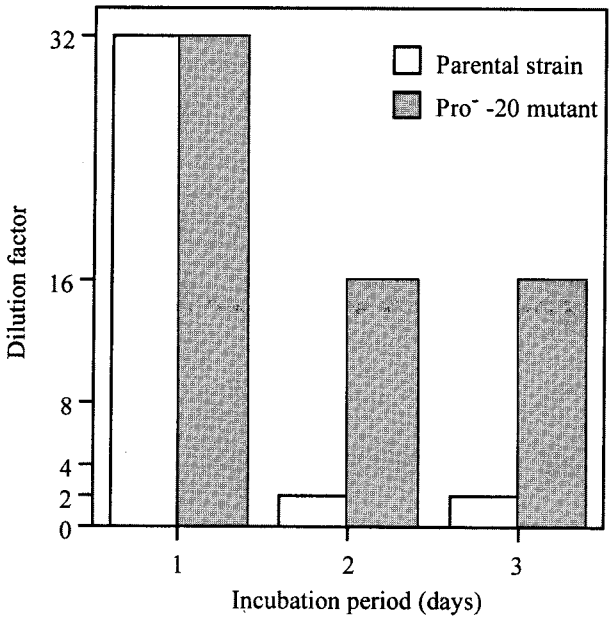

Fig. 5. Degradation of hGM-CSF in culture media of the parental and pro⁻-20 strains. The proteolytic activities in culture media were compared by determining the most diluted ratio of pre-incubated culture filtrates supporting the growth of hGM-CSF dependent cell line. The pre-incubated culture filtrates were prepared as described section in Materials and Methods. The data are the representative of mean of at least three independent experiments.

culture filtrates of parental strain and pro⁻-20 mutant. However, two fold diluents of 3- and 5-day old culture filtrates of parental strain were required to observe significant tritium uptake. A significant amount of tritium uptake (≥ 5,000 cpm) was observed until 16 times diluents of 3- and 5-day old culture filtrates of pro⁻-20 mutant, which suggested the decreased extracellular proteolytic activity of the pro⁻-20 mutant resulted in retarded degradation of the heterologous protein, hGM-CSF, in the culture medium.

In conclusion, a mutant strain pro⁻-20 of *A. niger* have been obtained which have a reduced proteolytic activity in the culture medium. It appears that the mutation occurs in the regulatory part of protease production rather than a specific protease gene. With the pro⁻-20 mutant, less than 30% of the activity in the culture medium of the parental strain remains. The use of pro⁻-20 mutant seems to have advantages for the efficient production of heterologous protein, which are known to be easily degraded in *A. niger*. However, further studies needs to be done to improve the pro⁻-20 mutant since, even in the culture filtrate of the pro⁻-20 mutant, hGM-CSF is still degraded, although at a much lower rate compared to the parent strain.

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References

Berka, R. M., Dunn-Coleman, N. S. and Ward, M. 1992. Industrial enzymes from *Aspergillus* species. *Biotechnology* **23**: 155-202.

_____, Ward, M., Wilson, L. J., Hayenga, K. J., Kodama, K. H., Carlomagno, L. P. and Thompson, S. A. 1990. Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori*. *Gene* **86**: 153-162.

Bodie, E. A., Bower, B., Berka, R. M. and Dunn-Coleman, N. S. 1994. Economically important organic acid and enzyme products. *Prog. Ind. Microbiol.* **29**: 561-602.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Choi, N. S., Yoon, K. S., Lee, J. Y., Han, K. Y. and Kim, S. H. 2001. Comparison of three substrates (casein, fibrin, and gelatin) in zymographic gel. *J. Biochem. Mol. Biol.* **34**: 531-536.

Carrez, D., Janssens, W., Degrave, P., Van den Hondel, C. A. M., Kinghorn, J. R., Fiers, W. and Contreras, R. 1990. Heterologous expression by filamentous fungi: secretion of human interleukin-6 by *Aspergillus nidulans*. *Gene* **94**: 147-154.

Dunn-Coleman, N. S., Bloebaum, P., Berka, R. M., Bodie, E., Robinson, N., Armstrong, G., Ward, M., Przetak, M., Carter, G. L., LaCost, R., Wilson, L. J., Kodama, K. H., Baliu, E. F., Bower, B., Lamsa, M. and Heinsohn, H. 1991. Commercial levels of chymosin production by *Aspergillus*. *Bio/Technology* **9**: 976-981.

Finkelstein, D. B. 1987. Improvement of enzyme production in *Aspergillus*. *Antoine van Leeuwenhoek J. Microbiol.* **53**: 349-352.

Jarai, G. and Buxton, F. 1994. Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. *Curr. Genet.* **26**: 238-244.

Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y. F., Miyazono, K., Urabe, A. and Takaku, F. 1989. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell Physiol.* **140**: 323-334.

Kim, M. J., Kwon, T. H., Jang, Y. S., Yang, M. S. and Kim, D. H. 2000. Expression of Murine GM-CSF from Recombinant *Aspergillus niger*. *J. Microbiol. Biotechnol.* **10**: 287-292.

Kim, E. H., Lee, J. G., Whang, M. K., Park, H. M., Kim, J. Y., Chae, S. K. and Maeng, P. J. 2001. Controlled expression and secretion of *Aspergillus oryzae* alkaline protease in *Aspergillus nidulans*. *J. Microbiol.* **39**: 95-101.

Lee, S. H., Lee, H. C., Kim, D. H., Yang, M. S. and Chung, B. W. 1998. Overproduction of sodium gluconate using the recombinant *Aspergillus niger*. *Kor. J. Biotechnol. Bioeng.* **13**: 214-219.

Lenouvel, F., Fraissinet-Tachet, L., van de Vondervoort, P. J. and Visser, J. 2001. Isolation of UV-induced mutations in the *are*A nitrogen regulatory gene of *Aspergillus niger*, and construction of a disruption mutant. *Mol. Gen. Genet.* **266**: 42-47.

Luengo, J. M. and Penalva, M. A. 1994. Penicillin biosynthesis. *Prog. Ind. Microbiol.* **29**: 603-638.

Mattern, I. E., Van Noort, J. M., Van den Berg, P., Archer, D. B., Roberts, I. N. and Van den Hondel, C. A. M. 1992. Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Mol. Gen. Genet.* **234**: 332-336.

Park, E. H., Shin, Y. M., Lim, Y. Y., Kwon, T. H., Kim, D. H. and Yang, M. S. 2000. Expression of glucose oxidase by using recombinant yeast. *J. Biotechnol.* **81**: 35-44.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D. and Bufton, A. W. J. 1953. The genetics of *Aspergillus nidulans*. *Adv. Genet.* **5**: 141-238.

Royer, J. C., Moyer, D. L., Reiwitch, S. G., Madden, M. S., Jensen, E. B., Brown, S. H., Yonker, C. C., Johnston, J. A., Golightly, E. J., Yoder, W. T. and Shuster, J. R. 1995. *Fusarium graminearum* A 3/5 as a novel host for heterologous protein production. *Bio/Technology* **13**: 1479-1483.

Sakaguchi, K., Takagi, M., Horiuchi, H. and Gomi, K. 1992. Fungal enzymes used in oriental food and beverage industries. Pp 54-99. *In*: Kinghorn, J. R. and Turner, G. Eds. Applied Molecular Genetics of Filamentous Fungi. Blackie Academic & Professional, N.Y., USA.

van den Hombergh, J. P. T. W., van de Vondervoort, P. J. I., van der Heijden, N. C. B. A. and Visser, J. 1995. New protease mutant in *Aspergillus niger* results in strongly reduced *in vitro* degradation of target proteins; genetical and biochemical characterization of seven complementation groups. *Curr. Genet.* **28**: 299-308.

_____, MacCabe, A. P., van de Vondervoort, P. J. I. and Visser, J. 1996. Regulation of acid phosphatases in an *Aspergillus niger pac*C disruption strain. *Mol. Gen. Genet.* **251**: 542-550.

_____, van de Vondervoort, P. J. I., Fraissinet-Tachet, L. and Visser, J. 1997. *Aspergillus* as a host for heterologous protein production: the problem of proteases. *TIBTECH* **115**: 256-263.