J. Microbiol. Biotechnol. (2000), **10**(3), 287–292

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Expression of Murine GM-CSF in Recombinant *Aspergillus niger*

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Received: November 1, 1999

Abstract Recombinant *Aspergillus niger* was constructed to express and secrete a biologically active murine granulocyte macrophage-colony stimulating factor (mGM-CSF). A 500 bp fragment encoding the signal peptide and mature mGM-CSF was cloned between the promoter and terminator of glyceraldehyde-3-phosphate dehydrogenase (*gpd*). The hygromycin phosphotransferase gene (*hph*) was used as a selection marker for the fungal transformants. An expression vector was introduced into *A. niger* ATCC 9642, and a Northern blot analysis indicated the presence of a considerable amount of transcripts from the introduced mGM-CSF. The biological activity of recombinant mGM-CSF (rmGM-CSF) isolated from the culture filtrate was confirmed by measuring the proliferation of the GM-CSF dependent FDC-P1 cell line. It appeared that rmGM-CSF was amenable to the proteolytic activity produced by *A. niger*, since biological activity was only observed when the transformants were grown in a protease-repressing medium, and the activity of rmGM-CSF dramatically decreased with an increase of age of the culture. The yield of rmGM-CSF, as determined by ELISA, was 640 ng/l of culture filtrate. Accordingly, its specific activity is estimated to be approximately two-and-a-half times higher than that of a commercial preparation from *E. coli*.

Key words: *Aspergillus niger*, granulocyte-macrophage colony stimulating factor

Filamentous fungi are used in a variety of industrial processes, including the production of fermented foods, primary metabolites such as organic acids and vitamins, and secondary metabolites [2, 16]. Moreover, filamentous fungi are excellent producers of a broad spectrum of extracellular enzymes [8, 26]. The filamentous fungus *Aspergillus niger* has a number of advantages compared with other expression systems, which include the ability to produce and secrete exceptionally large amounts of proteins, as well as the expression of eukaryotic proteins in a correctly folded and functional form [27]. Furthermore, several production processes involving *A. niger* have GRAS (generally recognized as safe) approval [1] and stable recombinants can be isolated, thus enabling controlled strain breeding [12]. More recently, *A. niger* has been developed as a host organism for the production of heterologous proteins and is an attractive candidate for the large-scale production of such proteins [11].

Cytokines are regulatory peptides that are produced by virtually every nucleated cell type in the body, and they have a pleotrophic regulatory effect on hematopoietic and many other cell types that participate in host defence and repair processes. One such cytokine, a granulocyte macrophage-colony stimulating factor (GM-CSF), can be characterized by its ability to stimulate development of both granulocytes and macrophages [5, 18]. GM-CSFs are one of the first of a large number of cytokines that have been purified and cloned [24]. The structures of GM-CSFs from several organisms, including mice, have been determined by the deduction of the amino acid sequences from the cloned nucleotide sequence, and a mature murine GM-CSF (mGM-CSF), which is preceded by a leader peptide of 25 amino acids, has been found to contain 124 amino acids of 23 kDa with two potential N-linked glycosylation sites [20]. GM-CSFs are involved in the regulation of survival, differentiation, proliferation, and functional activities in granulocyte and macrophage populations. Consequently, there are at least three major areas in which GM-CSFs have been considered for clinical use: the amelioration of acute and chronic states of neutropenia, adjunctive therapy with anti-microbials in nonneutropenic states, and anti-neoplastic effects, by recruitment of leukemic cells to enhance the efficacy of chemotherapy or enhancement of anti-tumor activity [24]. Hence, GM-CSFs are one of the first cytokines to be deployed for clinical use. Recombinant human GM-CSF was approved for use in the U.S.A. since early 1993, as a means of accelerating engraftment following autologous bone marrow transplantation in patients with lymphoid malignancies.

Several studies have shown that it is possible to produce recombinant GM-CSF in different host systems such as *E. coli*, yeast, insect cells, and even plant cells [6, 14, 15,

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23]. However, the expression of GM-CSF in *A. niger* has not been previously reported. As an initial attempt to produce valuable immunological markers with high specific activity, transformants of *A. niger* producing recombinant mGM-CSF were constructed, and the amount and functional activity of mGM-CSF were then determined by ELISA and a bioassay, respectively.

MATERIALS AND METHODS

Chemicals and Enzymes

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

Strains and Culture Condition

A. niger strain ATCC9642 was used as the recipient in the transformation experiments. For the preparation of the conidiospores, *A. niger* was grown on an agar-solidified complete medium [22] without nitrate, containing 70 mM NH_4Cl as the nitrogen source, supplemented with 15 nM D(+)biotin and 8 μM pantothenic acid. Standard *A. niger* complete, minimal, and protease repressing media [10] were applied throughout. Fernbach flasks containing 500 ml of the liquid medium were inoculated with 2×10^8 spores/ml and incubated for 24 h to 96 h in an orbital shaking incubator at 30°C (150 rpm).

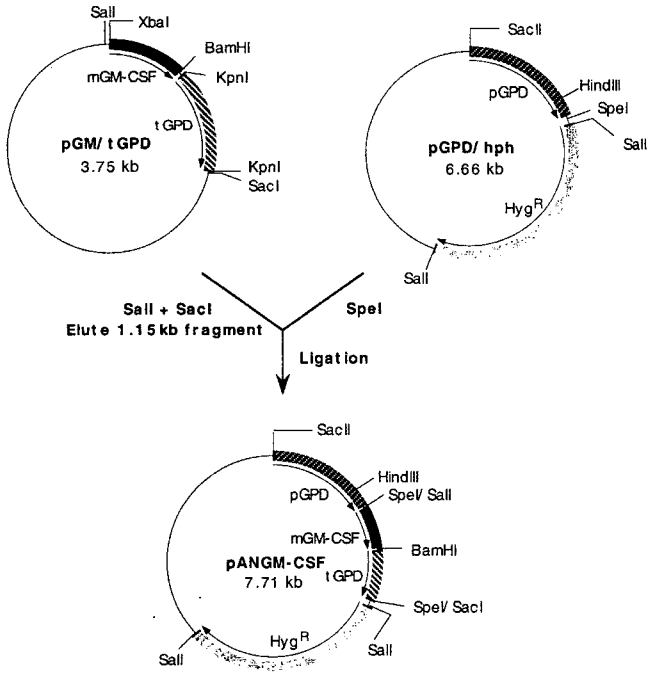

Fig. 1. Cloning strategy to generate the expression vector pANGM-CSF.

The genes of interest are mGM-CSF (murine granulocyte macrophage-colony stimulating factor), pGPD (*A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter), tGPD (*C. parasitica* glyceraldehyde-3-phosphate dehydrogenase terminator), Hyg^R (Hygromycin-B resistance gene). The arrows below the genes indicate the transcriptional direction. Each bar represents a restriction site.

Recombinant Vector Construction

The cloning strategy for the production of the recombinant vector expressing mGM-CSF is depicted in Fig. 1. Briefly, pGM-CSF, a pUC19 vector harboring a cDNA copy of mGM-CSF, was kindly supplied by Dr. D. S. Heo (Seoul National University Medical School, Seoul, Korea). A 600 bp fragment containing the transcriptional terminator of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene from *Cryphonectria parasitica* was inserted into the *Kpn*I site of pGM-CSF to make pGM/tGPD. The *Sal*I and *Sac*I fragment of pGM/tGPD, containing the mGM-CSF gene and *gpd* terminator, was blunt end-ligated into the *Spe*I site of pGPD/hph, which placed the mGM-CSF gene under the control of the *gpd* promoter of the expression vector pANGM-CSF. The hygromycin-B resistance gene (*hph*) was used as a selection marker [9].

Preparation of Protoplasts

A. niger protoplasts were prepared using the procedures of Churchill *et al.* [7] as modified below. A Fernbach flask containing 1 l of a complete medium was inoculated with 10^9 spores and incubated for 16 h at 30°C (65 rpm). The young mycelium was harvested and digested with a suspension of cell wall lysis enzymes containing β-glucuronidase (0.2 ml/g mycelium) and Novozyme (40 mg/g mycelium). The protoplasts, with a concentration of 10^8 protoplasts/ml in a storage buffer containing 4 parts of STC (1 M sorbitol, 100 mM Tris-HCl, 100 mM $CaCl_2$), 1 part of PTC (50% polyethylene glycol 4,000 MW), and 1% DMSO were stored for further experiments at -70°C.

Transformation of *A. niger*

The transformation of *A. niger* was carried out by modification of the procedures of Churchill *et al.* [7]. pANGM-CSF DNA (10 μg) was used for each transformation, and the treated protoplasts were regenerated in a potato dextrose agar (PDA) supplemented with 0.5 M of sucrose as an osmotic stabilizer. After 12-18 h, a minimal top agar containing 800 μg/ml of hygromycin B was overlayed, and the plates were incubated at 30°C for an additional 2-3 days until outgrowing colonies were visible. These colonies were transferred and single-spored on a minimal medium containing 200 μg/ml of hygromycin B.

Southern and Northern Blot Analyses

Total DNA and RNA were prepared from lyophilized and ground mycelia according to the procedures of Kim *et al.*

[13]. Five micrograms of DNA and 30 μg of RNA were used for the Southern and Northern blot analyses, respectively. The DNA or RNA was transferred to Hybond-N membranes (Amersham, U.K.) and UV cross-linked. The blot was then hybridized to the radiolabeled mGM-CSF cDNA.

Measurement of Biological Activity of rmGM-CSF and Quantitative Analyses by ELISA

Culture filtrates of *A. niger* were collected, dialyzed twice against PBS for 2 h at 4°C, and sterilized by a 0.2 μm syringe filter. The total protein in the filter-sterilized culture filtrates was measured by a Bradford assay using a Bio-Rad Protein Assay Kit [4] and normalized to 15.5 μg/ml for further analyses. An aliquot of 100 μl of each sterile culture filtrate was used for the bioassays. In order to measure the biological activity, the GM-CSF-dependent cell line FDC-P1 was used, which was kindly provided by Dr. D. Metcalf [19] (The University of Melbourne, Australia) and the tritium uptake by the treated FDC-P1 was measured using a liquid scintillation counter (Packard, U.S.A.) [14]. The concentration of recombinant murine GM-CSF (rmGM-CSF) produced from *A. niger* was measured by mGM-CSF specific ELISA kit (Endogene, Woburn, MA) according to the procedure provided by manufacturer. In this experiment, a recombinant *E. coli*-derived mouse GM-CSF purchased from Pharmingen Inc. (San Diego, CA, U.S.A.) was used as a standard.

RESULTS AND DISCUSSION

Transformation of *A. niger*

The pANGM-CSF expression vector, which contains the promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *A. niger* and the terminator of the *gpd* gene of *Cryphonectria parasitica,* was constructed, as indicated in Fig. 1. A recombinant vector identical to pANGM-CSF, except lacking a functional GM-CSF gene, was used for a mock transformation. Ten to thirty putative transformants were obtained per transformation, and each transformant was single-spored prior to further analyses. The mitotic stability of each transformant was confirmed by successive transfers to alternating selective and nonselective media.

RNA Analyses of Transformants

To determine the expression of the mGM-CSF gene at the transcriptional level, seven transformants growing actively on selective media were chosen. Total RNA from all seven transformants was extracted from the mycelia harvested 3 days after the liquid culture, and Northern blot analyses were performed. mGM-CSF transcripts were observed in pANGM-CSF transformants, whereas no transcripts were evident in either the wild-type strain or the mock transformant (Fig. 2). In order to determine the temporal expression of mGM-CSF mRNA, the mycelium of G10-1 was harvested 1, 3, and 5 days after the initiation of the liquid cultures, followed by a Northern blot analysis with equal amounts of RNA loaded in each lane (Fig. 3). As shown in Fig. 3, the RNA accumulation of GM-CSF that was barely detectable after 1 day, reached a peak at day 3, and gradually decreased thereafter. No transcript, however, was observed from the mock transformant as expected.

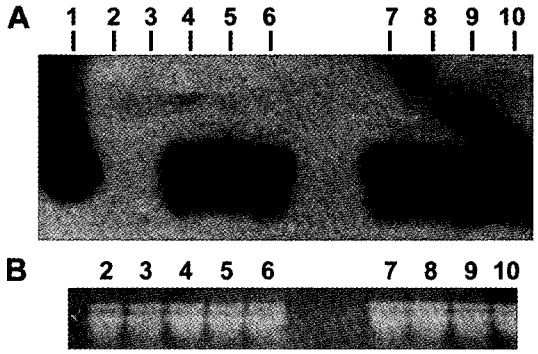

Fig. 2. Northern blot analysis of rmGM-CSF.
(A) Lane 1 contains the 0.5 kb cDNA fragment of mGM-CSF as a positive control. Lanes 2 to 10 contain the total RNA from the wild-type strain, mock transformant, and transformants G4-1, G6-1, G10-1, G7-1, G8-1, G9-1, and G11-1, respectively. Panel (B) shows the gel stained with ethidium bromide, indicating that an equal amount of RNA has been loaded for each sample.

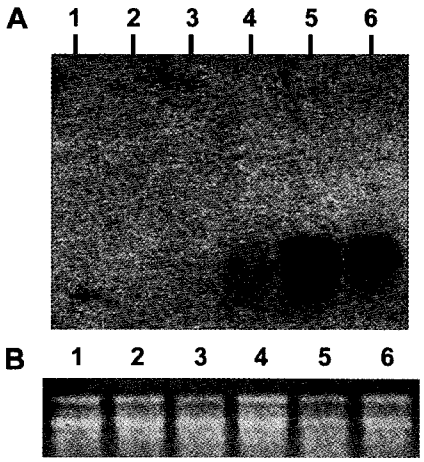

Fig. 3. Northern blot analysis to determine the temporal expression pattern of rmGM-CSF.
(A) Lanes 1, 2, and 3 contain the total RNA extracted from 1, 3, and 5 day cultures, respectively, of the mock transformant. Lanes 4, 5, and 6 contain the total RNA extracted from 1, 3, and 5 day cultures, respectively, of the transformant G10-1. Panel (B) shows the gel stained with ethidium bromide, indicating that an equal amount of RNA has been loaded for each sample.

Southern Blot Analyses

In order to detect the integration of the transforming vector into the chromosome of *A. niger* as well as its copy

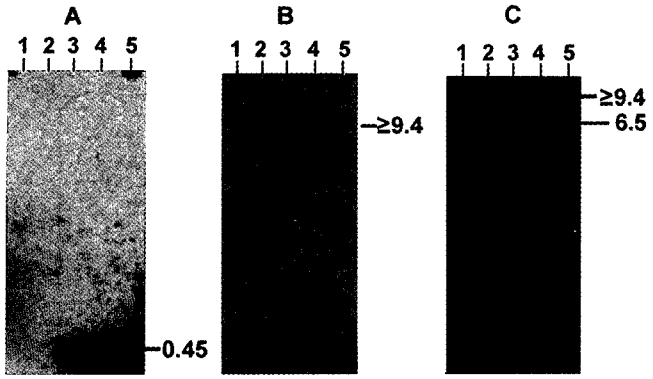

Fig. 4. Southern blot analyses. Panels A, B, and C contain *Xba*I and *Bam*HI-double digested genomic DNA, *Hin*dIII-digested genomic DNA, and *Bam*HI-digested genomic DNA, respectively, of *A. niger* isolates.
Lanes 1 and 2 represent the wild-type strain and mock transformant as negative controls, respectively. Lanes 3, 4, and 5 represent the transformants G4-1, G6-1, and G10-1, respectively. The numbers on the right of each panel refer to the size of the bands in kilobases.

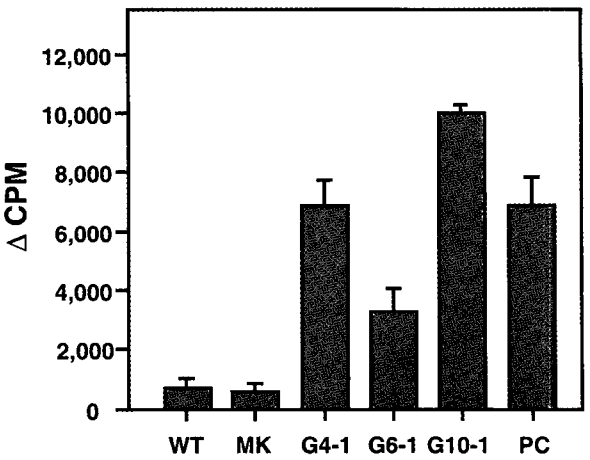

Fig. 5. Measurement of biological activity of mGM-CSF in culture filtrates of *A. niger* isolates.
G4-1, G6-1, and G10-1 represent samples prepared from culture filtrates of transformants grown for 24 h. Samples from 24-h-old culture filtrates of the wild-type (WT) strain and mock transformant (MK) were used as negative controls. *E. coli*-derived rmGM-CSF (Pharmingen, San Diego, CA, U.S.A.) was induced as a positive control (PC). The data represents the mean values of triplicate assays, and the error bars represent the standard errors among the triplicate values. The ΔCPM values represent the difference in the [methyl-^{3}H]thymidine uptake by the sample-treated cells compared with the phosphate buffered saline (PBS) treatment.

number, Southern blot analyses were conducted with three transformants (G4-1, G6-1, and G10-1), which showed the highest expression of mGM-CSF with Northern blot analyses. The genomic DNA of the three transformants, a wild-type strain, and the mock transformant was double-digested with *Xba*I and *Bam*HI, transferred to a nylon membrane, and hybridized to a radiolabeled cDNA probe of mGM-CSF. The wild-type strain and mock transformant did not hybridize to the probe, as expected. However, the three transformants (G4-1, G6-1, and G10-1) showed a single band with the expected size of 450 bp (Fig. 4A). Digestion with either *Hin*dIII (Fig. 4B) or *Bam*HI (Fig. 4C) alone, each having a single restriction site within the vector, produced only a single hybridizing band in each transformant, indicating that the transforming vector integrated as a single copy at different sites in the genome. Among the three transformants, G10-1 showed a unique hybridizing band pattern compared with both G4-1 and G6-1. Thus, unlike G10-1, it was hard to discern whether G4-1 and G6-1 were genetically different or clonal since the fragments were too large to separate by size under the conditions presently reported.

Determination of the Biological Activity of rmGM-CSF

The biological activity of rmGM-CSF isolated from the culture filtrates of recombinant *A. niger* was estimated by measuring the proliferation of GM-CSF-dependent FDC-P1 cells. When all strains were grown in either a complete or minimal medium, there was no significant proliferation of FDC-P1 cells (data not shown). However, a significant level of FDC-P1 cell proliferation was observed upon the addition of the culture filtrate of a recombinant *A. niger* strain, only when those recombinant strains were grown in protease-repressing medium. As shown in Fig. 5, both the negative controls, which included a culture filtrate from a wild-type strain and a mock transformant, did not support the proliferation of FDC-P1 cells; additionally, PBS had no effect on cell proliferation (data not shown). In contrast, the positive control rmGM-CSF (commercially-purchased recombinant mGM-CSF from Pharmingen) as well as the culture filtrates of recombinant *A. niger* supported efficient proliferation of FDC-P1 cells. No detectable biological activity was observed in the intracellular preparation of recombinant *A. niger* (data not shown). These results indicate that mGM-CSF is expressed and secreted in a biologically active form from recombinant *A. niger*. The greatest activity was observed in the preparation of transformant G10-1 (Fig. 5). However, the biological activity of mGM-CSF derived from recombinant *A. niger* dramatically decreased (Fig. 6) as the cultivation time proceeded, i.e., the biological activities of the culture filtrates at days 3 and 5 were no better than that of the mock transformant. Interestingly, the transcript levels of mGM-CSF were still high at those times (Fig. 3).

Since it is known that one of the major problems of using filamentous fungi as expression hosts is their abundance of proteolytic enzymes, the proteolytic activity of *A. niger* culture filtrate was analyzed using pepstatin, a specific inhibitor of aspergillopepsin [17]. Thus, pepstatin (0.7 μg/ml) and purified rmGM-CSF (10 ng/ml) were added to the culture filtrate of *A. niger* and incubated for 12 h at 30°C to examine whether the pepstatin prevented rmGM-CSF from being degraded. The pepstatin-treated

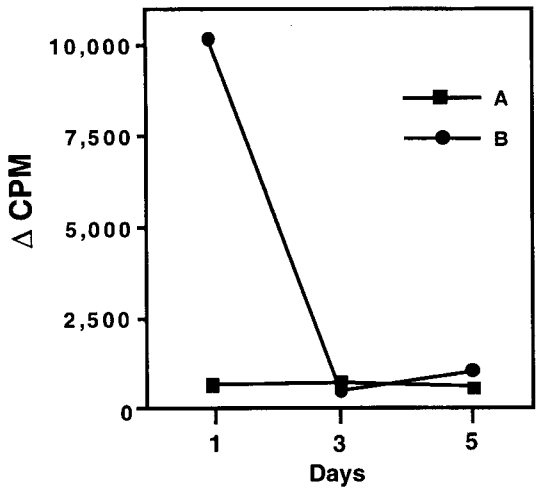

Fig. 6. Time course of biological activity in culture filtrates of *A. niger* isolates.
The ΔCPM values represent the difference in the [methyl-^{3}H]thymidine uptake by the sample-treated cells compared with the phosphate buffered saline (PBS) treatment. A and B represent the samples prepared from the mock transformant and mGM-CSF producing transformant G10-1, respectively.

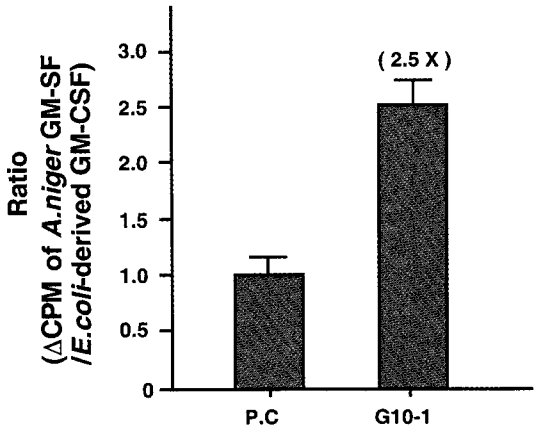

Fig. 7. Quantitative analysis of rmGM-CSF specific activity.
The rmGM-CSF produced from the transformants of *A. niger* was measured by ELISA and the concentration of rmGM-CSF used for biological activity was normalized to 30 pg according to ELISA. The data represent the mean values of triplicate assays. The yield of rmGM-CSF by G10-1 was estimated to be 640 ng/l.

rmGM-CSF supported a considerable amount of proliferation of FDC-P1 cells, whereas there was no detectable proliferation induced by the samples without pepstatin (data not shown). These results, together with the experiments with the growth media, Northern blot analyses, and temporal bioactivity of the culture filtrate from recombinant *A. niger*, suggest that heterologously expressed GM-CSF is labile to proteolytic degradation. Hence, a concerted effort is needed to design methods to prevent heterologous proteins from being degraded by the proteases produced by *A. niger*. A recent study on the molecular breeding of *A. niger* to generate protease deficient mutants by using site-directed recombination demonstrated the feasibility of mass production of many rare proteins by filamentous fungi [27].

Quantitative Analyses of rmGM-CSF

An ELISA was conducted to determine the amount of the mGM-CSF produced by recombinant *A. niger* and *E. coli*-derived rmGM-CSF was used as a standard (described in Experimental protocol). The transformant G10-1 produced 640 ng/l of active mGM-CSF, 1 day after the initiation of the liquid cultures. The yield was less than that from other expression systems, with an exception of plant cell suspension cultures [14]. However, the high transcription level of the rmGM-CSF gene in 3-day culture vs 1-day culture (Fig. 3) suggests that there is a great potential for producing cytokines in filamentous fungi if methods can be devised to prevent recombinant proteins from being degraded by fungal proteases. In addition, the specific activity of rmGM-CSF from a recombinant *A. niger* strain, estimated by the degree of [methyl-^{3}H]thymidine uptake per ng of rmGM-CSF, was two-and-a-half times higher than that of commercial GM-CSF (Fig. 7).

Based on the fact that nonglycosylated *E. coli*-derived and differently glycosylated yeast- or baculovirus-derived recombinant GM-CSF are biologically active, N-linked glycosylation does not appear to be essential for GM-CSF activity. Thus, the rmGM-CSF produced by *A. niger* may have favorable characteristics over that of *E. coli*-derived rmGM-CSF. Cytokines are known to have many pleotrophic effects on immuno responses; however, clinical applications of many recombinant therapeutic human cytokines have been hampered due to the side effects of overdose [3, 21, 25]. Therefore, an increase in specific activity is important for the clinical application of many recombinant cytokines. This study has clearly shown that rmGM-CSF from *A. niger* can be successfully produced and derived for secretion into a culture medium in a biologically active form, and its specific activity is greater than that derived from *E. coli*.

Acknowledgments

We would like to thank Dr. A. C. L. Churchill for her critical review of this manuscript. This work was supported by a grant from the National R&D Program of the Korean Ministry of Science and Technology.

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