Secretion of Thermostable β-Glucosidase by an Intergeneric Bacterial Hybrid Between *Cellulomonas* and *Bacillus subtilis*

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ABSTRACT

The intergeneric protoplast fusion hybrid (Bs/C 005) between *Cellulomonas* sp. and *Bacillus subtilis* produced extracellular aryl β-glucosidase that is otherwise intracellular in parental *Cellulomonas* sp. This extracellular aryl β-glucosidase was active at relatively higher temperature (60°C) and lower pH (pH 5.0) conditions than that of *Cellulomonas* enzyme. It also exhibited increased thermostability and stability over wide range of pH. Cellobiase activity, distinctly different from aryl β-glucosidase detected in both *Cellulomonas* sp. Bs/C 005, was only intracellular. Cellobiase from Bs/C 005, however, was more thermostable than that of *Cellulomonas* sp.

Index Entries: Extracellular aryl β-glucosidase; thermostable enzyme; bacterial protoplast fusion; *Cellulomonas* sp.; intergeneric bacterial hybrid; thermostable glucanase.

INTRODUCTION

The enzyme β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of β-glucosidic bonds between aryl, alkyl, and

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saccharide groups (*1*). The principal role of β-glucosidase (cellobiase) is to catalyze the hydrolysis of cellobiose and cellooligosaccharides. These soluble substrates produced by the action of β-glucanases in the cellulase complex during cellulose hydrolysis are converted to glucose. Since β-glucanases are subject to product inhibition (*2,3*), an adequate amount of β-glucosidase activity is necessary for the efficient functioning of a cellulase system (*4*). Very few bacteria have been reported to produce significant amounts of β-glucosidase. Bacterial β-glucosidases are cellbound (*5*) and therefore can be rate limiting in cellulase preparations obtained from the culture filtrates (*6*). Apart from extracellular production, thermostability is one of the desirable properties of enzymes useful in industrial applications. All enzyme preparations obtained from mesophilic strains, however, are relatively less thermostable than those from thermophiles. Studies on thermophilic bacteria, including *Clostridium thermocellum* (*7,8*) and *Thermomonospora* sp. (*9*), have demonstrated enhanced thermostability of β-glucosidase relative to that from mesophiles.

Cellulomonas is a mesophilic bacterium that produces an active cellulase complex but harbors cellbound (*10*) β-glucosidase. In our earlier report, we demonstrated that the genes coding for the entire cellulase complex of *Cellulomonas* sp. could be transferred and expressed in *Bacillus subtilis* by means of the protoplast fusion technique (*11*). The intergeneric hybrid thus derived produced extracellular aryl β-glucosidase; however, a distinct cellobiase activity originally found in *Cellulomonas* sp. remained cellbound in this hybrid. We report in this paper properties of extracellular aryl β-glucosidase and intracellular cellobiase produced by the stable hybrid in comparison to intracellular aryl β-glucosidase and cellobiase of *Cellulomonas* sp.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from the suppliers indicated: Cellulose powder −123 (Schleicher and Schull, GmbH, FRG; cellobiose and *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma Chemical Co., USA), and Glox reagent (Kabi Diagnostica, Sweden). All other chemicals used were of analytical grade and obtained locally.

Microorganisms

Cellulomonas sp. NCIM 2353, was obtained from the National Collection of Industrial Microorganisms, India. The intergeneric hybrid Bs/C 005 was isolated in our laboratory (*11*). *Cellulomonas* sp. was maintained on LB agar slants containing 1% tryptone, 1% yeast extract, 0.5% NaCl,

and 2% Agar. The hybrid Bs/C 005 was maintained on Dubos minimal medium (*12*) supplemented with 0.5% cellulose powder – 123 and 0.05% yeast extract.

Production of Enzymes

For enzyme production, cultures were pregrown in Dubos liquid medium (10 mL) with 0.1% cellobiose and 0.05% yeast extract at 30°C. Cells after centrifugation were used to inoculate 50 mL Dubos medium supplemented with 0.5% cellulose powder – 123 and 0.05% yeast extract in 250 mL conical flask. Following 7 d of incubation at 30°C on a rotary shaker (250 rpm), cultures were harvested by centrifugation, and supernatants were retained for estimation of extracellular enzyme activity. The pelleted cells were washed twice with 50 m*M* citrate phosphate buffer (pH 6.8), and suspended in a minimal volume (5 mL) of the same buffer. Cell suspension was ultrasonically disrupted at 35 kc for 5 min while cooling on ice. The sonicated suspension was centrifuged and supernatant was used to determine intracellular enzyme activity.

Enzyme Assays

Aryl β-glucosidase activity was assayed according to the method of Eberhart (*13*), with modification. The assay mixture consisted of 0.1 mL of enzyme solution and 0.9 mL of 1 mg/mL of pNPG in 50 m*M* citrate phosphate buffer. The reaction was carried out for 30 min at 50°C and was stopped by the addition of 2 mL of 2% sodium carbonate. The amount of *p*-nitrophenol produced was measured at 410 nm.

Cellobiase activity was assayed according to the method described by Berghem and Pettersson (*14*), with the following modification. The assay mixture consisted of 50 m*M* cellobiose in 50 m*M* citrate phosphate buffer (pH 6.8) and 0.1 mL of enzyme solution in a final volume of 1 mL. Incubation was carried out at 50°C for 30 min and the reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. The amount of glucose liberated was determined by using Glox reagent (*15*). Protein content in the samples was determined according to Lowry et al. (*16*) with bovine serum albumin as standard.

Unit of Activity

Enzyme activities were expressed in international units (IU). One unit of aryl β-glucosidase or cellobiase was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol or glucose per min, respectively, under standard assay conditions.

Effect of pH and Temperature

Estimation of enzyme activity at different pH values was carried out in reaction mixtures using citrate phosphate buffer (pH 4.0–6.5) or sodium phosphate buffer (pH 7.0–8.0) at 50 m*M* concentration under the assay conditions described. For optimum temperature determination, assays were carried out at different temperatures ranging from 30–70°C.

Stability Experiments

For determination of thermal stability, enzyme solutions were preincubated at specified temperatures in 50 m*M* citrate phosphate buffer. Samples were withdrawn at various time intervals for determination of enzyme activity under the assay conditions described. For determination of pH stability of enzymes, they were preincubated in 50 m*M* buffer of different pH ranging from pH 4.0 to pH 9.0 for 30 min at 40°C and enzyme activity was then determined under the assay conditions described.

RESULTS

From our earlier report (*11*), it was observed that unlike *Cellulomonas*, the hybrid Bs/C 005 secreted a significant amount of aryl β-glucosidase in the growth medium during growth on insoluble substrates such as cellulose powder −123 and Avicel. Both in *Cellulomonas* and hybrid Bs/C 005, cellobiase activity remained cellbound (*17*). This finding led us to characterize these enzymes in culture filtrates of *Cellulomonas* and the hybrid in relation to optimum conditions of temperature and pH for their activities, as well as stability at extreme temperature and pH.

Aryl β-glucosidase and cellobiase from *Cellulomonas* sp. exhibited maximum activity at 45°C, whereas extracellular aryl β-glucosidase of Bs/C 005 was most active at 60°C (Fig. 1A). Intracellular cellobiase (Fig. 1B) of the hybrid exhibited maximum activity at 55°C. Thermostability of these enzymes was determined by preincubating the enzymes at specified temperatures for different time intervals and then assaying the activity under optimal conditions. It was observed (Fig. 2A) that *Cellulomonas* aryl β-glucosidase was less thermostable compared to extracellular enzyme of Bs/C 005, which retained 50% of its initial activity even at 60°C. *Cellulomonas* aryl β-glucosidase lost activity within 5 min when incubated under identical conditions. Cellobiase activity of Bs/C 005 was also more thermostable than that of *Cellulomonas;* however, its 80% activity was lost within 5 min at 60°C (Fig. 2B).

Extracellular aryl β-glucosidase showed pH optimum of 5.0, whereas intracellular aryl β-glucosidase of *Cellulomonas* had pH optimum of 6.0 (Fig. 3A,B). Cellobiase of Bs/C 005 showed a broad range of pH (5.5–7.0) for optimum activity, whereas cellobiase of *Cellulomonas* was most active

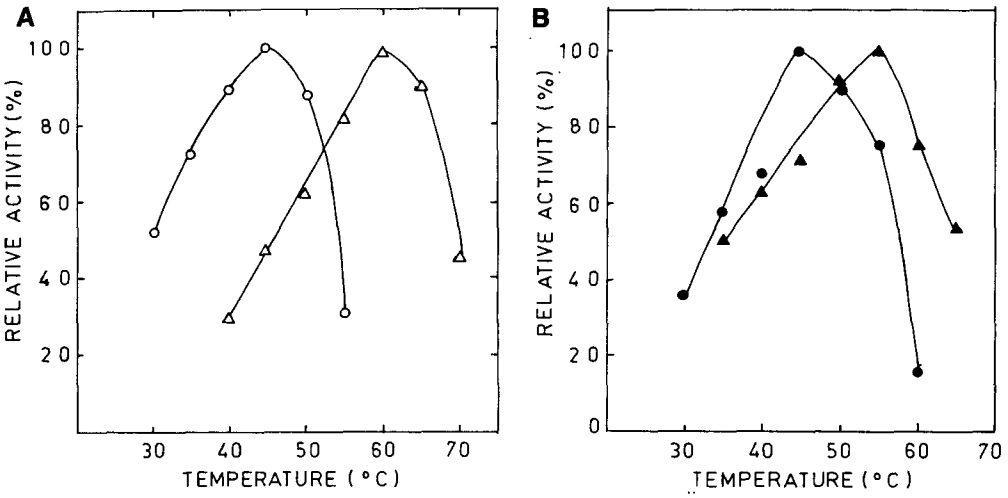

Fig. 1. Effect of temperature on (A) aryl β-glucosidase and (B) cellobiase activities. Incubations were carried out for 30 min at the indicated temperature under standard assay conditions. *Cellulomonas* sp. ○, ●; Bs/C 005 (extracellular) △, (intracellular) ▲.

in a narrow range (around pH 7.0). Both aryl β-glucosidase and cellobiase exhibited pH stability over a broad range (Fig. 4), but the overall range of pH stability in case of aryl β-glucosidase was in acidic region whereas cellobiase enzymes were stable up to pH 8.0.

DISCUSSION

Aryl β-glucosidase is not found extracellularly in *Cellulomonas*, however, this enzyme is externalized in hybrid Bs/C 005 (*11*). Cellobiase activity however, remained cellbound in both *Cellulomonas* sp. and the hybrid as reported earlier (*17*). Extracellular β-glucosidases have been reported in *Clostridium* (*18*), *Bacteriodes succinogen* (*19*), and different *Streptomyces* strains (*20*). In case of *Cl. stercorarium*, Bronnenmeier and Staudenbauer (*18*) have reported that 20% of the total β-glucosidase activity was extracellular. In general, extracellular enzymes are more stable under extreme physical conditions such as higher temperature and they have fairly broad pH tolerance. Their thermostability has been partly attributed to post-translational modifications such as glycosylation (*21,22*). A highly thermostable, intracellular β-glucosidase has also been described by Patchett (*23*) in the case of a gram-negative short, rod-type bacterium from New Zealand. Our finding that the extracellular aryl β-glucosidase of Bs/C 005 was more thermostable than that of *Cellulomonas* suggested possible differences in these two proteins. Thermostable forms of enzymes are also known to be generated by specific amino acid substitutions, mainly of the

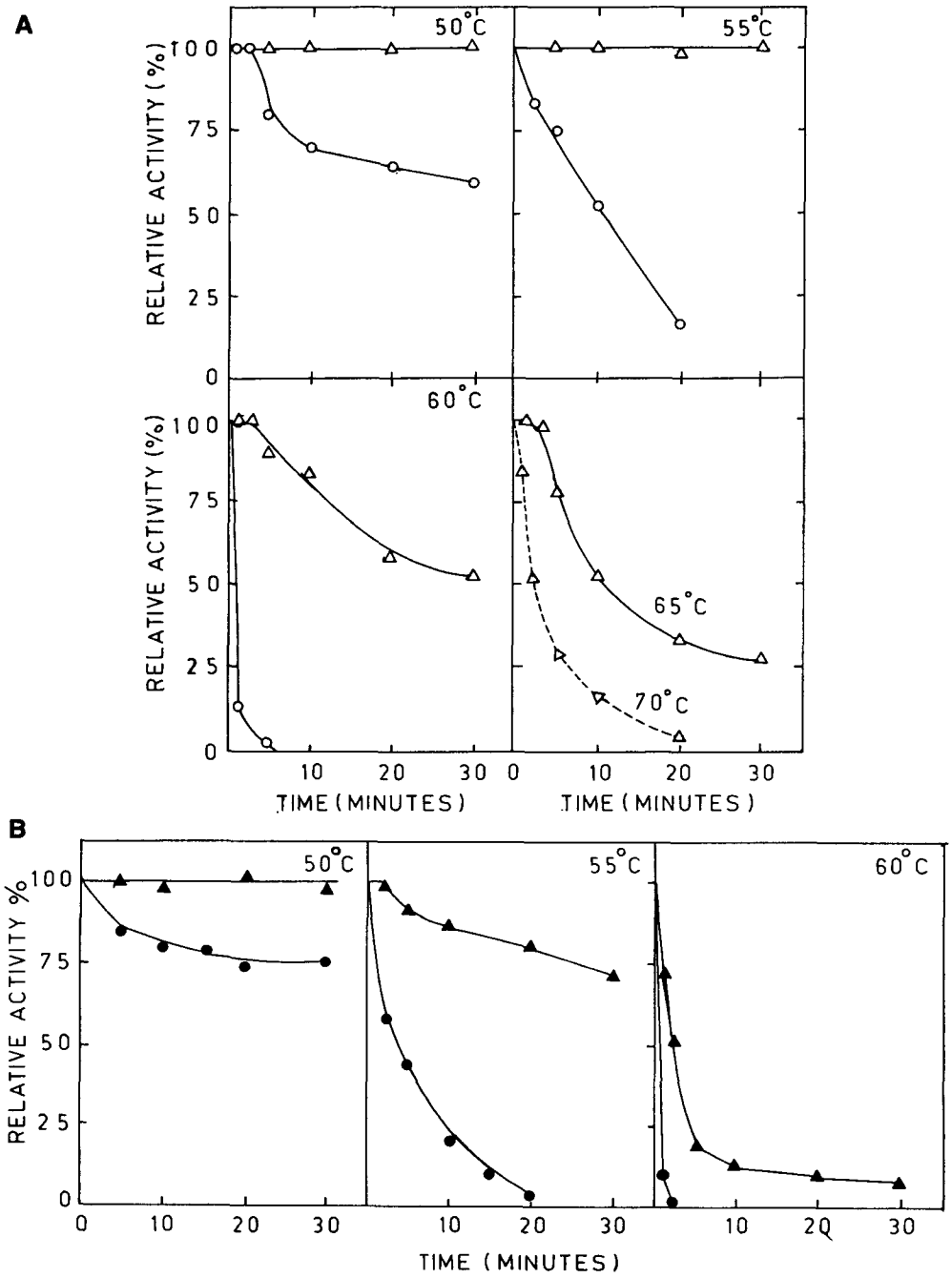

Fig. 2. Thermal stability of β-glucosidase activity. Enzyme solutions were preincubated at indicated temperatures in 50 m*M* citrate phosphate buffer. Samples were withdrawn at different time intervals for determination of activity under standard conditions. Residual activity was expressed as a percentage of untreated control. (A) Aryl β-glucosidase activity: *Cellulomonas* sp. ○, Bs/C 005 (extracellular) △; (B) Cellobiase activity: *Cellulomonas* sp. ●, Bs/C 005 (intracellular) ▲.

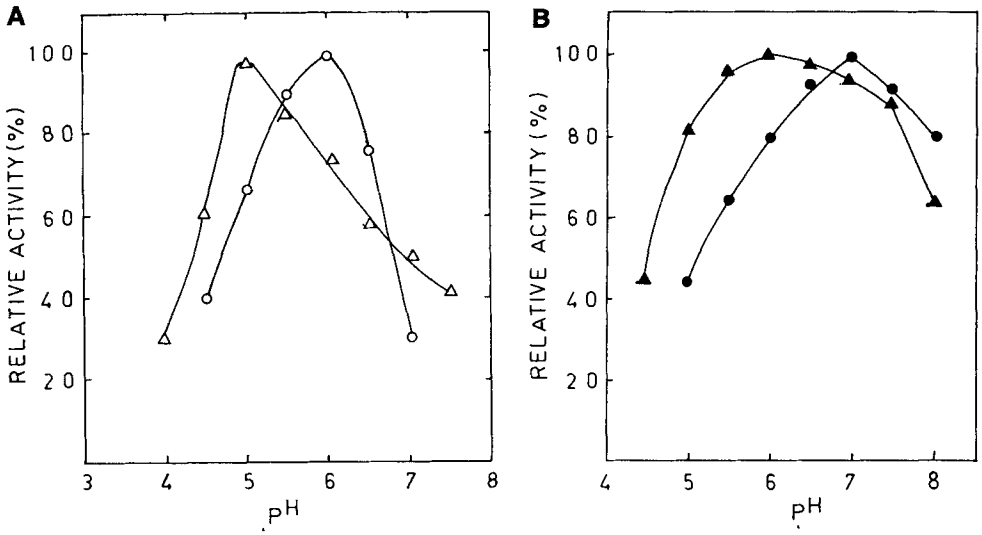

Fig. 3. pH profile of (A) Aryl β-glucosidase and (B) cellobiase activities. Enzyme activity was determined at pH values between 4.0–8.0 (pH 4.0–6.5, citrate phosphate buffer; pH 7.0–8.0 sodium phosphate buffer). *Cellulomonas* sp. ○, ●; (Bs/C 005 (extracellular) △, (intracellular) ▲.

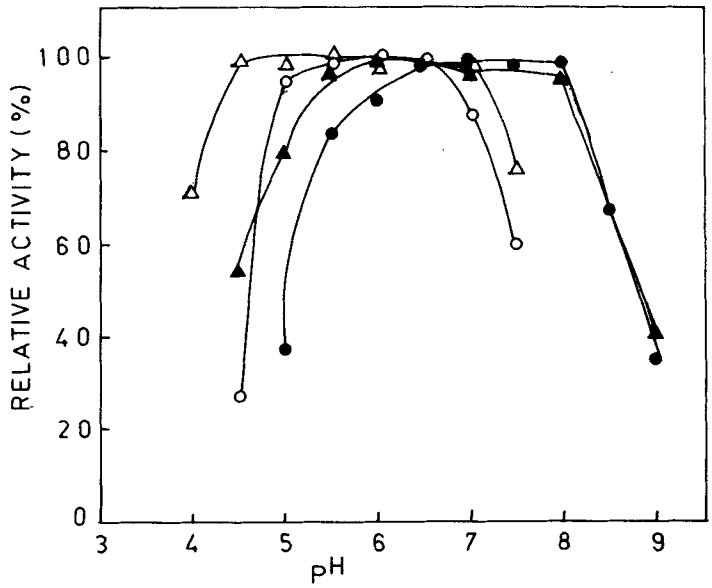

Fig. 4. Effect of pH on stability on Aryl β-glucosidase and cellobiase. Enzyme solutions were preincubated in buffers (pH 4.0–9.0) for 30 min at 40°C and then activity was determined under standard conditions. *Cellulomonas* sp. Aryl β-glucosidase ○, Bs/C 005 Aryl β-glucosidase (extracellular) △; *Cellulomonas* sp. cellobiase ●; Bs/C 005 cellobiase (intracellular) ▲.

hydrophilic to hydrophobic type (*24,25*). Since Bs/C 005 is a protoplast fusion product formed by multiple genomic recombinations (*11*), introduction of new amino acids in the primary sequence of aryl β-glucosidase is a possibility. It is necessary therefore to investigate whether posttranslational modifications such as glycosylation or change(s) in the primary sequence of protein are responsible for increased thermostability of the Bs/C 005 enzyme. The shift in the pH optimum toward acidic side for both aryl β-glucosidase and cellobiase in the hybrid could be owing to many other reasons. Presence of two β-glucosidases (aryl β-glucosidase and cellobiase) in *C. fimi* has been reported by Wakarchuk et al. (*26*). Our results regarding optimum pH and temperature, as well as stability at different pH and temperature conditions of these enzymes, further substantiated the fact that the aryl β-glucosidase and cellobiase are two independent enzymes in *Cellulomonas* sp. studied. Indeed, in culture filtrate Bs/C 005, only aryl β-glucosidase could be detected and cellobiase was present only in trace amounts, further suggesting that expression of these two enzymes is independently regulated, and, that there are at least two independent genes coding for these enzymes. Recent report of detection of two β-glucosidase genes in *C. fimi* (*27*) further confirmed our conclusions of multiple glucosidases in *cellulomonas* sp.

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REFERENCES

1. Woodward, J. and Wiseman, A. (1982), *Enzyme Microb. Technol.* **4,** 73–79.
2. Berghem, L. E. R., Pettersson, L. G., and Axio-Fredriksson, U. B. (1975), *Eur. J. Biochem.* **53,** 55–62.
3. Bisset, F. and Sternberg, D. (1978), *Appl. Environ. Microbiol.* **35,** 750–755.
4. Sternberg, D., Vijaykumar, P., and Reese, E. T. (1977), *Can. J. Microbiol.* **23,** 139–147.
5. Stoppok, W., Rapp, P., and Wagner, F. (1982), *Appl. Environ. Microbiol.* **44,** 44–53.
6. Coughlan, M. P. (1985), *Biotechnol. Genet. Eng. Rev.* **3,** 39–109.
7. Ait, N., Creuzet, N., and Cattaneo, J. (1979), *Biochem. Biophys. Res. Commun.* **90,** 537–546.
8. Ait, N., Creuzet, N., and Cattaneo, J. (1982), *J. Gen. Microbiol.* **128,** 569–577.
9. Hagerdal, B., Ferchak, J. D., and Pye, E. K. (1980), *Biotechnol. Bioeng.* **22,** 1515–1526.
10. Choi, W. Y., Haggett, K. D., and Dunn, N. W. (1978), *Aust. J. Biol. Sci.* **31,** 553–564.

11. Gokhale, D. V., Soo Han, E., Srinivasan, V. R., and Deobagkar, D. N. (1984), *Biotechnol. Let.* **6,** 627–632.
12. Dubos, R. J. (1928), *J. Bacteriol.* **15,** 223–234.
13. Eberhart, B. M. J. (1961), *J. Cell. Comp. Physiol.* **58,** 11–16.
14. Berghem, L. E. R. and Pettersson, L. G. (1974), *Eur. J. Biochem.* **96,** 295–305.
15. Bermeyer, H. U. and Bernt, E. (1974), *in Methods in Enzyme Analysis,* vol. 3, Verlag Chemie Weinheim, FRG. 2nd ed., 205.
16. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
17. Gokhale, D. V. and Deobagkar, D. N. (1989), *Appl. Environ. Microbiol.* **55,** (10), 2675–2680.
18. Bronnenmeier, K. and Staudenbauer, W. L. (1988), *Appl. Environ. Microbiol.* **28,** 380–386.
19. Groleau, D. and Forsberg, C. W. (1981), *Can. J. Microbiol.* **27,** 517–530.
20. Moldoveaulu, N. and Kluepfel, D. (1983), *Appl. Environ. Microbiol.* **46,** 17–21.
21. Langsford, M. L., Gilkes, N. R., Singh, B., Muser, B., Miller, R. C., Warren, R. A. J., and Kilburn, D. G. (1987), *FEBS Lett.* **255,** 163–167.
22. Merivuori, H., Sands, J. A., and Montenecourt, B. S. (1985), *Appl. Microbiol. Biotechnol.* **23,** 60–66.
23. Patchett, N. L., Daniel, R. M., and Morgan, H. W., (1987), *Biochem. J.* **243,** 779–787.
24. Mozhaev, V. V. and Martinek, K. (1984), *Enzyme Microb. Technol.* **6,** 50–59.
25. Suzuki, K. and Imahori, K. (1973), (Tokyo) *J. Biochem.* **74,** 955–970.
26. Wakarchuk, W. W., Kilburn, D. G., Miller, R. C., and Warren, R. A. J. (1984), *J. Gen. Microbiol.* **130,** 1385–1389.
27. Kim, H. K. and Pack, M. Y. (1989), *Enzyme Microb. Technol.* **11,** 313–316.