β-Glucosidase Production and Characterization of Some *Aspergillus* Strains

B. Flachner*, and K. Réczey***
*Department of Agricultural Chemical Technology, Budapest University of Technology and Economics, H-1521 Budapest, Szt. Gellért tér 4, Hungary
**Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapest, P.O. Box 7., Hungary

Original scientific paper
Received: September 18, 2003
Accepted: March 15, 2004

Four *Aspergillus* strains, *A. phoenicus* ATCC1315B, *A. niger* BKM F-1305, *A. foetidus* Biogal 39 and *A. phoenicis* QM 329, were studied for β-glucosidase fermentation and compared with *Trichoderma reesei* RUT C-30. The enzyme productivity of *Aspergilli* was found to be 4.8 times higher than that of *T. reesei.* The fermentation supernatants were examined to find out the kinetical behavior of the non-purified enzyme because under industrial circumstances usually crude supernatant concentrates are used. Experiments were performed with *p*-nitrophenyl-β-D-glucopyranoside (p-NPG) the artificial substrate and cellobiose, the natural substrate of β-glucosidase. In the case of *p*-NPG substrate β-glucosidase follows substrate inhibition kinetics (at 1.5–2.0 mmol L^{-1}) nevertheless in case of cellobiose substrate inhibition was not detected. The unpurified fermentation supernatants from all investigated *Aspergillus* strains are suitable to enzyme supplementation after concentration and its industrial usage may cut the cost of enzymatic hydrolyses of cellulose.

Keywords:
Enzyme production, β-glucosidase, *Aspergillus* sp.

Introduction

Biomass, accumulated by photosynthetic storage of solar energy in green plants, is a significant source of renewable energy. About half of this solar-produced biomass is in the form of cellulose. Great interest has been focused on the hydrolysis of cellulose for the production of glucose, which can be used for fuel production. Ethanol is widely used as a partial gasoline replacement in the USA. Fuel ethanol produced from corn has been used in blends or in oxygenated fuels since the 1980s. Recently, US automobile manufacturers have developed an engine which can use either petrol or petrol-ethanol blend up to 85 % ethanol. The cars with this engine are called FFV (flexible-fueled vehicles). Using ethanol-blended fuel for automobiles can significantly reduce petrol use and exhaust greenhouse gas emission.[1] However, the cost of ethanol compared to fossil fuels is relatively high due to the high raw material costs. One alternative to reduce the cost of ethanol production could be the utilization of cheap lignocellulosic raw materials like agricultural by-products and wastes. Crop residues, grasses, sawdust, wood chips, and waste papers can be used. Pretreated lignocellulosic material can be hydrolyzed to simple sugars and then fermented to ethanol by yeast. Hydrolyses can be performed by enzymatic or by acidic catalysis. Enzymes used in this reaction are called cellulases.

Cellulolytic enzyme systems can be produced by a number of different microorganisms, such as aerobic and anaerobic bacteria,[2,3] white rot fungi,[4] soft rot fungi[5] and anaerobic fungi.[6] The cellulase system consists of three general groups of enzymes: cellobiohydrolyses (CBH I and II), endoglucanases (EG I and II) and β-glucosidase, which act together synergistically to degrade the cellulose to glucose.[7,8] The best characterized and most widely studied system is that of the soft rot fungus *Trichoderma*, particularly *T. reesei*.[9,10] The level of β-glucosidase in the cellulolytic enzyme system of *Trichoderma reesei* is sub optimal for saccharification purposes.[11] β-glucosidase has been recognized to be the limiting enzyme in the complete degradation of cellulosic materials to glucose.[12] A high level of β-glucosidase is needed to avoid the accumulation of cellobiose, which is a strong inhibitor of cellobiohydrolases and endoglucanases.[13,14] Sufficient amount of β-glucosidase is needed also to achieve an optimal product composition.

There are several possibilities to solve these problems. Genetic improvement of good cellulase producers[15,16] or special β-glucosidase inducers in *Trichoderma* fermentation[17,18] enhance the β-glucosidase ratio in the enzyme mixture. There is another possibility to ensure the appropriate β-glucosidase: FPA ratio in the hydrolyzing complex is to supplement the hydrolyzing enzyme mixture with additional soluble or immobilized β-glucosidase produced by another organism.

Aspergilli are widely studied cellulase and β-glucosidase producers. Exo-[19] and endo-β-1,4-glucanase[19–21] activities are weaker compared to *T. reesei*, but it can be genetically improved.[22] Production of β-glucosidase is thoroughly examined, the totally different fermentation methods and different carbon sources resulted in a wide range of productivity data e.g. in case of *A. niger* 2.8–20.1 IU $L^{-1}h^{-1}$,[19,23,24] *A.wentii* 6.6–14.2 IU $L^{-1}h^{-1}$,[25] *A. terreus* 4.9–6.1 IU $L^{-1}h^{-1}$,[26] *A. phoenicis* 16.6–31.9 IU $L^{-1}h^{-1}$, [23,27] *A. foetidus* 14.0 IU $L^{-1}h^{-1}$.[23] These data are significantly larger than the productivity in case of *T. reesei* where 0.7–2.8 IU $L^{-1}h^{-1}$ were determined.[23] Numerous authors published kinetic parameters of the produced β-glucosidase, but because of differences of assay conditions the results are very confusing. In case of *p*-NPG substrate K_m varies between 0.63 and 1.03 mmol L^{-1} for *A. niger* β-glucosidase,[28–31] between 0.075 and 0.88 mmol L^{-1} for *A. fumigatus* β-glucosidase[32,33], and between 0.28 and 3.5 mmol L^{-1} for *T. reesei* β-glucosidase.[36,37] K_m = 1.17 mmol L^{-1} for *A. japanicus*[34] and K_m = 0.55 mmol L^{-1} for *A. oryzae*.[35]

In case of cellobiose K_m varies between 2.3 and 5.6 mmol L^{-1} for *A. niger* β-glucosidase,[28,30] between 0.84 and 1.36 mmol L^{-1} for *A. fumigatus* β-glucosidase[32,33] and between 1.5 and 1.9 mmol L^{-1} for *T. reesei* β-glucosidase.[36,37]

In present work β-glucosidase production of four different *Aspergillus* strains were examined in the same fermentation condition and compared to the β-glucosidase production of *Trichoderma reesei* RUT C30. Kinetical behavior of the fermentation supernatants prepared under same circumstances, was also compared and evaluated.

Materials and methods

Microorganisms

The fungal strains used for β-glucosidase production were obtained from the Department of Agricultural Chemical Technology, Budapest University of Technology and Economics. The strains were maintained on malt agar slants at 30 °C.

Culture and growth conditions

Inoculation was carried out by using 5 % malt inoculum, which constituted 10 % of the medium. Cultures grown in shake-flask (100 ml per 750 ml Erlenmeyer flask) were incubated at 30 °C and shaken at 350–400 rpm. The cultivation medium was Mandels' salt[38] with paper wadding pulp as carbon source (10 g l^{-1}), which was prehydrolysed with Celluclast 1.5 L for 4 h at 50 °C.

Assays

Reducing sugar concentration was determined colorimetrically using dinitrosalicylic acid reagent.[39] Calibration was performed by using glucose.

β-Glucosidase activity was assayed with 5 mmol L^{-1} *p*-nitrophenyl-β-D-glucopyranozide (*p*-NPG) in 50 mmol L^{-1} citrate buffer pH 4.8 as substrate.[7] 1.0 ml substrate solution and 0.1 ml sample were incubated at 50 °C, the reaction was terminated after 10 min with Na_2CO_3 and the absorbance was read at 400 nm. A standard curve was obtained with *p*-nitrophenol.

Cellobiase activity was measured in 2.0 ml reaction mixture, containing 1.0 ml enzyme solution in citrate buffer (50 mmol L^{-1}, pH 4.8), and 1.0 ml 15 mmol L^{-1} cellobiose. After incubation at 50 °C for 30 min, the mixture was immersed for 5 min in boiling water. The glucose concentration was determined colorimetrically using glucoseoxidase (GOD) and peroxidase (POD) enzymes.[40] One international unit (IU) of β-glucosidase and cellobiase activity was defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate per min in the assay conditions.

Kinetic constants were measured using different amount of *p*-NPG (0.1–5 mmol L^{-1}) or cellobiose (1.5–15 mmol L^{-1}), and performing the standard activity assays as described above. Data were analyzed using nonlinear regression method according to Michales-Menten or to substrate inhibition kinetics (Eq. 1). The kinetic quantities were derived from the data-fitting procedure.

$$r = r_{max} \frac{[S]}{K_m + [S] + \left([S]\frac{[S]}{K_1}\right)} \quad (1)$$

where r and r_{max} are the reaction rate, K_m is the reaction equilibrium constant, K_1 is the inhibition constant and [S] is the concentration of substrate.

Result and discussion

Enzyme fermentation

β-glucosidase production of four *Aspergillus* strains and of *Trichoderma reesei RUT-C30* (listed in Table 1) were examined in shake-flask fermentation. The experiments were performed on prehydrolysed paper wadding as carbon source in Mandel's medium. Initial pH was 5.7 in the case of *Aspergillus* strains and 4.5 or 6.0 in the case of *T. reesei*. From each strain three parallel fermentations were performed with daily pH adjustment. Fermentations of *Aspergillus* strains were also carried out without

Table 1 – *Production of β-glucosidase by Aspergillus strains and T. reesei RUT C-30*

Strain	Initial pH	β-glucosidase activity on 9-th day IU mL^{-1}	Productivity P/IU L^{-1}h^{-1}
A. phoenicus ATCC1315B	5.7	3.3	15.3
A. niger BKM F-1305	5.7	3.35	15.3
A foetidus Biogal 39	5.7	3.4	15.7
A. phoenicis QM 329	5.7	3.4	15.7
T. reesei RUT C30	6.0	0.636	2.94
	4.5	0.617	2.86

pH adjustment. In these flasks the pH dropped to 2.3 on the first day and thereafter increased rapidly and reached pH 5.8 on the third day. From the third to the ninth day there was a slow increase in the pH and at the end its value was 6–6.5. Since the different pH adjustment strategies didn't cause any difference in β-glucosidase activity, fermentations were considered as six parallel runs. The uncertainty of parallel fermentations was about 0.028. The different initial pH of the fermentation of *T.* reesei resulted in different pH profile, but at the end the same β-glucosidase activity was achieved in both sets of fermentation. β-glucosidase activities in the function of fermentation time are shown on Figure 1. The productivity of the four *Aspergillus* strains were nearly the same and 4.8 times higher than that of *T. reesei* (Table 1.), which makes them good candidates for supplementation in the enzymatic hydrolysis of cellulose using *Trichoderma reesei* enzyme complex.

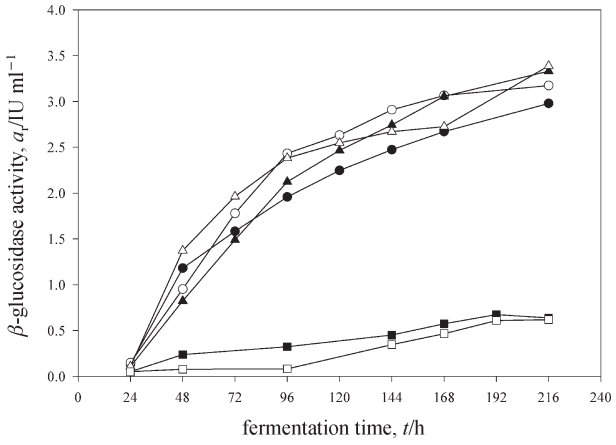

Fig. 1 – *β-glucosidase production of four Aspergillus strains and T. reesei on prehydrolysed paper wadding pulp. (–●– A. phoenicus, –○– A. niger, –▼– A. foetidus, –▽– A. phoenicis, –■– T. reesei RUT initial pH 4.5, –□– T. reesei RUT initial pH 6.0)*

Kinetics studies

For the kinetic studies crude fermentation supernatants were used without any purification. The aim of this study was to get information about the kinetic behavior of the enzymes under industrial circumstances where usually not purified but crude supernatant concentrates are used. Experiments were performed with both p-nitrophenyl-β-D-glucopyranoside (*p*-NPG), the artificial substrate and cellobiose, the natural substrate of β-glucosidase. Both substrates were used in a substrate saturation examination. In the case of *p*-NPG substrate β-glucosidase kinetics do not follow hyperbolic behavior on Michales-Menten diagram and on the Lineweaver-Burk plot do not fit on a single line on Figure 2A, but at high substrate concentrations positively deviate, although enzyme-substrate rate was suitable for Michales-Menten kinetic conditions. Therefore the β-glucosidase follows substrate inhibition kinetics shown in Figure 2, and this kinetic model was significant at 99.9 % confidence level. The fitted kinetic parameters are listed in Table 2. In case of cellobiose substrate inhibition was not detected and kinetics is shown in Figure 3.

As a consequence of the identical dilution of fermentation supernatants the kinetic parameters on natural and artificial substrates are comparable. On the *p*-NPG substrate the kinetics of β-glucosidase from the fermentation supernatants of *T. reesei* RUT C-30 are similar to that of *Aspergillus* strains. β-glucosidase from *T. reesei* has a lower value of K_m and its affinity is grater than the affinity of en-

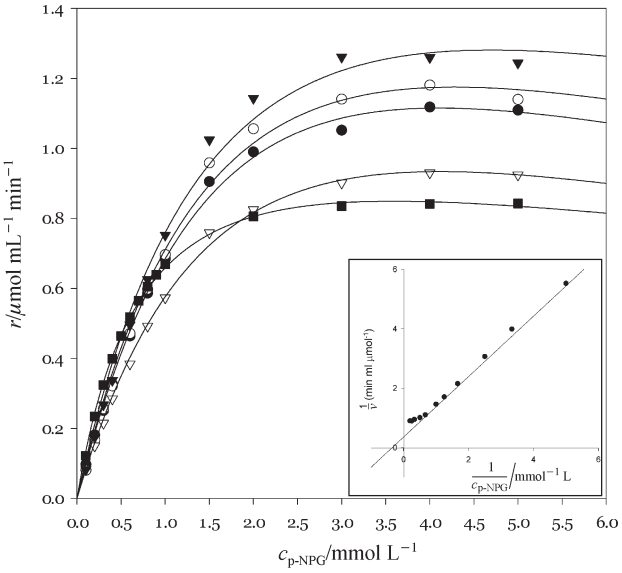

Fig. 2 – *Kinetic behavior of the examined strains on p-NPG substrate. The curves were obtained by fitting the experimental data according to substrate inhibitions kinetics. A one example in the Lineweaver-Burk plot (–●– A. phoenicus, –○– A. niger, –▼– A. foetidus, –▽– A. phoenicis, –■– T. reesei RUT)*

Table 2 – *Kinetic parameters of the fermentation supernatants*

Strain	*p*-NPG			Cellobiose	
	K_m /mmol L^{-1}	r_{max} /mmol L^{-1} min^{-1}	K_1/mmol L^{-1}	K_m/mmol L^{-1}	r_{max}/mmol L^{-1} min^{-1}
A. phoenicus ATCC-1315B	2.18±0.38	2.26±0.28	8.29±2.7	4.64±0.75	1.9±0.11
A. niger BKM F-1305	2.3±0.6	2.44±0.32	7.9±3.8	5.02±0.76	2.05±0.12
A. foetidus Biogal 39	2.54±0.68	2.42±0.30	7.13±3.4	6.0±0.69	2.46±0.12
A. phoenicis QM 329	2.29±0.35	1.97±0.22	7.42±2.0	4.02±0.63	1.51±0.08
T. reesei RUT C-30	0.80±0.04	1.23±0.20	15.9±2.3	1.45±0.23	0.51±0.02

zymes produced by *Aspergilli*. On cellobiose substrate the K_m values are doubled in the case of every strain and the affinity of *T. reesei* enzyme is higher as well. Nevertheless, the β-glucosidase of *T. reesei* is less able to hydrolyze the natural cellobiose substrate than the *p*-NPG used for measuring activity. All four *Aspergillus* strains are able to hydrolyze cellobiose similarly to *p*-NPG. Kinetic behavior of β-glucosidase from fermentation supernatants of different *Aspergilli* is very similar, therefore it is impossible to find the best strain.

We found that *p*-NPG substrate inhibited β-glucosidase activity at 1.5–2.0 mmol L^{-1} contrary to 4 mmol L^{-1} value given in the literature.[28] Figure 4. shows the difference between the curves fitted according to substrate inhibitions kinetics to the theoretical curve calculated on the bases of Michales-Menten kinetics, this difference is similar to all five curves. This fact necessitates revising the accepted method of activity measurement of β-glucosidase because 5 mmol L^{-1} substrate concentration is already strongly inhibitive. Consequently 1 mmol L^{-1} of *p*-NPG substrate is proposed for activity measurement of β-glucosidase.

Aspergillus strains can produce β-glucosidase not only with better productivity, but also they can much better hydrolyse the natural cellobiose substrate than β-glucosidase by *T. reesei*. Therefore the unpurified fermentation supernatants from all investigated *Aspergillus* strains are suitable to enzyme supplementation after concentration and its industrial usage may cut the cost of enzymatic hydrolyses of cellulose.

ACKNOWLEDGMENTS

The financial support was provided by the grant OTKA (4–157) of the Hungarian National Research Fund. Also tanks to Dr. Mária Vas (Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences) for helping in evaluating enzyme kinetics studies.

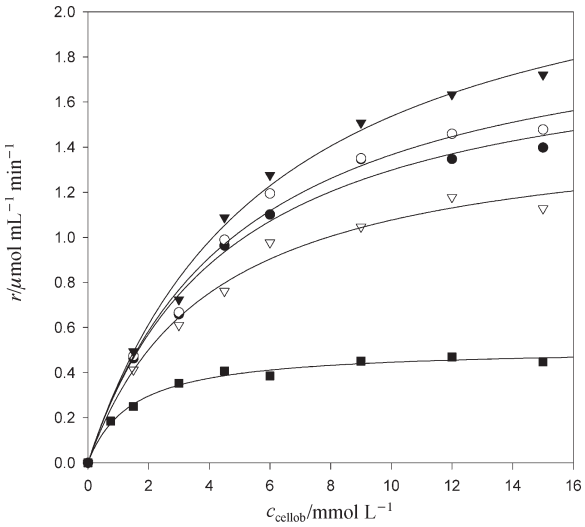

Fig. 3 – *Kinetic behavior of the examined strains on the natural substrate, cellobiose. The curves were obtained by fitting the experimental data according to Michales-Menten kinetics. (–●– A. phoenicus, –○– A. niger, –▼– A. foetidus, –▽– A. phoenicis, –■– T. reesei RUT)*

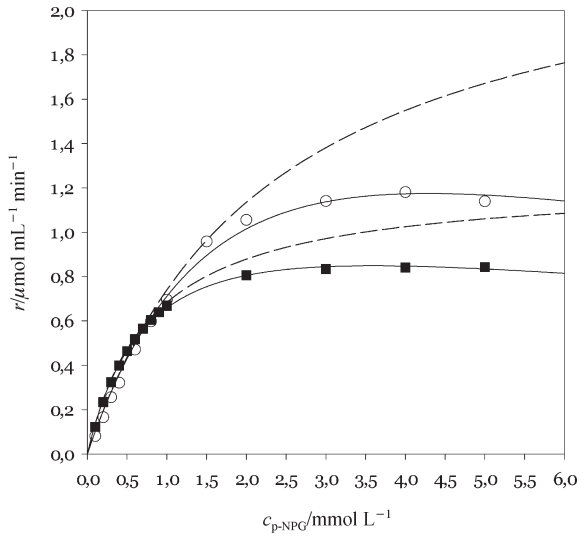

Fig. 4 – *Comparison of the curves fitted according to substrate inhibitions kinetics (solid line) to the theoretical curve calculated on the bases of Michales-Menten kinetics (dashed line) (–○– A. niger, –■– T. reesei RUT)*

Reference

1. *Sun, Y., Cheng, J.*, Bioresource Techn. **83** (2002) 1.
2. *Gilkes, N. R., Kilburn, D. G., Jr.R.C., M., Warren, R. A. J.*, Biores. Technol. **36** (1991) 21.
3. *Perez-Pons, J. A., Rebordosa, X., Querol, E.*, Biochim Biophys Acta **1251** (1995) 145.
4. *Uzcategui, E., Ruiz, A., Montesino, R., Johansson, G., Pettersson, L. G.*, J. Biotechnol. **19** (1991) 271.
5. *Lymar, E. S., Li, B., Renganathan, V.*, Appl. Enviro. Microb **61** (1995) 2976.
6. *Barichievich, E. M., Calza, R. E.*, Curr. Microbiol. **20** (1990) 265.
7. *Berghem, L. E. R., Petterson, L. G.*, Eur. J. Biochem **46** (1974) 295.
8. *Beguin, P., Aubert, J. P.*, FEMS Microbiol Rev **13** (1994) 25.
9. *Bisaria, V. S., Ghose, T. K.*, Enzym Microb. Technol **3** (1981) 90.
10. *Tomme, P., Claeyssens, M.*, FEBS Letters **239** (1989) 239.
11. *Allen, A., Sternberg, D.*, Biotechnol. Bioeng. Symp. **10** (1980) 189.
12. *Sternberg, D., Vijayakumar, P., Reese, E. T.*, Can J Microbiol **23** (1977) 139.
13. *Philippidis, G. P., Smith, T. K., Wyman, C. E.*, Biotechnol. Bioeng. **41** (1993) 843.
14. *Lee, Y.-H., Fan, L. T.*, Biotechnol. Bioeng **25** (1983) 939.
15. *Barnett, C. C., Berka, R. M., Fowler, T.*, Biotechnology (N Y) **9** (1991) 562.
16. *Brow, J. A., Falconer, D. J., Wood, T. M.*, Enzyme Microb. Technol. **9** (1987) 169.
17. *Sternberg, D., Mandels, G. R.*, J Bacteriol **144** (1980) 1197.
18. *Chen, S., Wayman, M.*, Process Biochem. **26** (1991) 93.
19. *Kim, S. W., Kang, S. W., Lee, J. S.*, Biores. Technol. **59** (1997) 63.
20. *Yamana, Y. I., Fujata, J., Izuwa, S., Fukuchi, K., Shimizu, R. I., Hiyoshi, A., Fukuda, H., Mikami, S., Kimzaki, Y., Wakabayashi, S.*, J. Biosci Bioeng **93** (2002) 479.
21. *Akiba, S., Kimura, Y., Yamamoto, K., Kumagai, H.*, J. Ferment Bioeng **79** (1995) 125.
22. *Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., Kim, S. W.*, Bioresour Technol **91** (2004) 153.
23. *Réczey, K., Brumbauer, A., Bollok, M., Szengyel, Z. S., Zacchi, G.*, Appl Biochem Biotechnol **70-72** (1998) 225.
24. *Martino, A., Pifferi, P. G., Spagna, G.*, J Chem Techn Biotechnol **60** (1994) 247.
25. *Srivastava, S. K., Gopalkrishnan, K. S., Ramachandran, K. B.*, J. Ferment Technol **65** (1987) 95.
26. *Pulshalkar, S., Rao, K. K., Menon, K.*, Curr Microbiol **30** (1995) 255.
27. *Réczey, K., Persson, I., Tjerneld, F., Hang-Hägerdal, B.*, Biotechnol Techniq **3** (1989) 205.
28. *Dekker, R. F. H.*, Biotechnol Bioeng **28** (1986) 1438.
29. *Spagna, G., Romagnoli, D., Angela, M., Bianchi, G., Pifferi, P. G.*, Enzym Microb. Technol **22** (1998) 298.
30. *Watanabe, T., Sato, T., Yoshioka, S., Kuwahara, M.*, Eur J Biochem **209** (1992) 651.
31. *Yan, T. R., Lin, C. L.*, Biosci Biotechnol Biochem **61** (1997) 965.
32. *Ximenes, E. A., Felix, C. R., Ulhoa, C. J.*, Curr. Microbiol **32** (1996) 119.
33. *Rudick, M. J., Elbein, A. D.*, J. Bacteriol **124** (1975) 534.
34. *Sanyal, A., Kundu, R. K., Dube, S., Dube, D. K.*, Enzym Microb. Technol **10** (1988) 91.
35. *Riou, C., Salmon, J. M., Vallier, Z., Gunata, Z., Barre, P.*, Appl Enviroment Microbiol **64** (1998) 3607.
36. *Woodward, J., Arnold, S. L.*, Biotechnol Bioeng **23** (1981) 1553.
37. *Berghem, L. E. R., Petterson, L. G.*, Eur J Biochem **46** (1974) 295.
38. *Mandels, M., Weber, J.*, Adv. Chem. Ser. **95** (1969) 391.
39. *Miller, G.*, Anal. Chem. **31** (1959) 426.
40. *Huggest, A. S. C., Nixon, D. A.*, Biochem J. **6** (1957) 12.