0273-2289/96/5758—0399$8.00

Ethanol Production from Cellulose by Two Lignocellulolytic Soil Fungi

LUCIA R. DURRANT

Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP), 13081-970 Campinas-SP, Brazil

ABSTRACT

The present work examines the production of ethanol via direct fermentation of pure celluloses and lignocellulosic wastes by two soil fungi isolated under anaerobic conditions. The strains were cultured on a defined medium containing filter paper slurry as the carbon source under anaerobic, microaerophilic, and aerobic conditions. After complete degradation of the cellulose, lignocellulases and fermentation products were determined. Highest activities for *Trichocladium canadense* (strain Q10) and the basidiomycete strain (strain H2), were obtained when cultures were incubated under microaerophilic conditions and air, respectively. Laccase activity was present in the culture supernatants of both strains, but peroxidase was only produced by strain H2. Ethanol was the major nongaseous fermentation product. Highest conversion of available cellulose to ethanol was obtained with strain Q10 (90–96%), under microaerophilic conditions. Ethanol production decreased when microcrystalline cellulose and lignocellulosic substrates were used.

Index Entries: Cellulolytic fungi; lignocellulose degradation; microaerophilic fungi; fermenting fungi.

INTRODUCTION

The presence of anaerobic cellulose-degrading fungi in the rumen of various animals and in the alimentary tracts of other herbivorous nonruminants is well documented *(1,2)*. They have been isolated from the rumen and stomach of herbivores belonging to a wide range of mammalian families *(3–7)*. These zoosporic fungi have also been isolated from the saliva, and fresh and dry feces from a wide range of animals. All species studied to date utilize cellulose and hemicelluloses for growth, and produce the enzymes necessary for solubilizing both amorphous and highly ordered celluloses present in plant fibers *(8–12)*. Furthermore, they also contribute to lignocellulose digestion in the rumen of herbivorous mammals because they are able to colonize and degrade the lignin-containing tissues of plant cell walls *(11,13–16)*.

Attempts have been made to isolate obligately anaerobic zoosporic fungi from a variety of other anaerobic environments, such as lake and river sediments and sewage treatment plants, but despite an extensive search, these fungi have not been detected in samples from these environments *(17)*. The possibility of the presence of anaerobic fungi in habitats other than the rumen, where they would participate in the degradation and fermentation of lignocellulosic material, is an interesting one.

To determine the presence of free-living anaerobic fungi that participate in the degradation of cellulose, an isolation procedure was developed *(18)*. Two strains of cellulose-fermenting fungi were isolated, under anaerobic conditions, from soil samples collected from poorly drained areas, such as marshes and swamps. The intent of this work was to investigate the presence of lignocellulose-degrading enzymes in culture fluids of these two fungi. The production of ethanol via direct fermentation of pure celluloses and lignocellulosic wastes by these soil fungi isolated under anaerobic conditions was also examined.

MATERIALS AND METHODS

Microorganisms

Trichocladium canadense strain Q10 and strain H2 (a basidiomycete species), isolated under anaerobic conditions, as previously described *(18)*, were used throughout these experiments.

Media and Culture Conditions

A defined liquid medium containing per liter: 1.0 g KH_2PO_4, 0.54 g NH_4Cl, 0.3 g $CaCl_2$, 1.0 g urea, 1.0 g L-cysteine hydrochloride, 0.1 mL resazurin solution (0.1% [w/v]), 1.0 mL/L of a mineral solution (containing 0.22 g/L ammonium tartrate, 0.66 g/L manganese sulfate, 0.15 g/L iron III sulfate, 0.10 g/L cobalt sulfate, 0.10 g/L zinc sulfate, 6.4 mg/L copper sulfate, and 10 mg/L aluminum potassium sulfate), 0.5 mL/L vitamin solution (2.0 mL biotin, 2.0 mL folic acid, 5.0 mL thiamin-HCl, 5.0 mL riboflavin, 10.0 mL pyridoxine-HCl, 0.10 mL cyanocobalamine, 5.0 mL nicotinic acid, 5.0 mL DL-calcium panthothenate, and 5.0 mL thioctic acid), plus cellulose was used throughout these experiments. The cellulose used was filter paper Whatman No. 1, as a slurry prepared by wet ball milling *(19)*. Media for anaerobic cultures were prereduced *(20)* and stored in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI), until the resazurin indicator became colorless.

Enzyme Preparation and Assays

The production of enzymes capable of degrading cellulose, xylan, and lignin was investigated in cultures incubated anaerobically, microaerophilically, and in air. The defined liquid medium, as just described, containing 0.25% (dry w/v) ball-milled filter paper slurry was used to culture the two fungal isolates. Medium for anaerobic cultures was prereduced, placed in an anaerobic chamber, inoculated, and incubated for 4–5 wk. Medium for microaerophilic cultures was prereduced, prior to inoculation, which was carried out in air with cells cultured on plates of cellulose agar medium under microaerophilic conditions (achieved by using BBL CampyPak Plus gas generator envelopes for the production of a reduced oxygen atmosphere [BBL Microbiology Systems, Becton Dickinson,Cockeysville, MD]). Flasks containing these cultures (800 mL medium in 1-L flasks were used for all three conditions) were incubated for 3 wk in tightly closed jars containing the BBL gas generator envelopes. For aerobic cultures, aerated medium was inoculated in air, and flasks were stoppered with loosely fitting aluminum lids and shaken every other day of the incubation period (up to 3 wk). After growth and complete disappearance of the cellulose present in the medium, the supernatant fluids were collected, filtered, and concentrated by ultrafiltration using a Novacell stirred cell

(Filtron Technology, Clinton, MA) equipped with a 10-kDa mol-wt cutoff membrane. Avicelase, carboxymethylcellulase (CMCase), and xylanase activities were investigated by measuring the production of reducing sugar from either avicel, carboxymethylcellulose (CMC; sodium salt, low viscosity; Sigma), or oat spelt xylan (Sigma), by means of the dinitrosalicylic acid-reducing sugar method *(21)*. Activities were determined using 1% of each substrate in 0.05*M* acetate buffer, pH 5.0, and a final volume and reaction time of 0.3 mL and 1 h for avicelase and 0.6 mL and 30 min for CMCase and xylanase, carried out at 50°C. β-glucosidase activity was assayed using 0.9 mL of 5 m*M* *p*-nitrophenyl-β-D-glucopyranoside in 0.05*M* acetate buffer, pH 5.0, and a final volume and reaction time of 1.0 mL and 20 min at 50°C. The *p*-nitrophenol produced by the addition of 2.0 mL 1*M* $NaCO_3$ was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the number of micromoles of reducing sugar or micromoles of *p*-nitrophenol produced/mL of enzyme. Protein was determined by the method of Bradford *(22)*, with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin as the protein standard.

The detection of enzymes involved in lignin degradation was carried out using syringaldazine for the detection of laccase *(23)*, and syringaldazine plus hydrogen peroxide for the detection of peroxidases *(24)*. The presence of peroxidases was also determined using *o*-dianisidine to replace syringaldazine.

Growth on Lignin-Derived Compounds

Lignin sulfonic acid sodium salt, at final concentrations of 0.5% (w/v) and lignin organosolv 2-hydroxyethylether (0.2% [w/v], final concentration), were used to test for the possible utilization of lignin-derived compounds by the isolates. Test tubes containing the lignin source prepared in 10 mL of the defined liquid media were inoculated and incubated under anaerobic, microaerophilic, and aerobic conditions, as described. After growth, the supernatants were collected and subjected to high-performance liquid chromatography (HPLC) analysis, using a fast acid column (Bio-Rad) for the determination of aromatic compounds. The elution buffer used was 15% acetonitrile plus 85% 5 m*M* H_2SO_4.

Fermentation Products

The two strains were inoculated on 20 mL of the defined liquid media containing the following carbon sources: filter paper slurry (0.25% [w/v]), microcrystalline cellulose (0.25% [w/v]), coarse pine sawdust (1% [w/v]), coarse maple sawdust (1% [w/v]), and fine pine sawdust (1% [w/v]). Incubation was carried out under microaerophilic conditions, except for filter paper slurry, which was under the three oxygenation conditions described. For ethanol yield calculations, the amount of cellulose in the lignocellulosic substrates used here was considered as 50%. The conversions of cellulose to ethanol were calculated based on the assumption that 1 g of cellulose on hydrolysis would yield 1.1 g of reducing sugars *(25,26)*.

Chemicals and Cellulosic Substrates

All chemicals used were reagent grade. Avicel (microcrystalline cellulose type PG-105) was a gift of FMC Corp. (Philadelphia, PA). CMC (sodium salt, low viscosity) and oat spelt xylan were obtained from Sigma. Syringaldazine, lignin sulfonic acid-sodium salt, and lignin organosolv 2-hydroxyethylether, were purchased from Aldrich (Milwaukee, WI).

Table 1
Cellulase and Xylanase Activities in Cultures of Strains H2 and Q10

Growth conditions[b]	Enzyme activities, μmol/mL/min[a]			
	CMCase	Avicelase	β-glucosidase	Xylanase
Strain H2				
Anaerobic	1.20	0.038	0.069	1.65
Microaerophilic	1.50	0.047	0.078	2.74
Aerobic[b]	2.11	0.067	0.050	3.13
Strain Q10				
Anaerobic	0.91	0.017	0.23	1.72
Microaerophilic	1.61	0.013	0.38	2.46
Aerobic[c]	0.80	np	0.10	0.89

[a]The SD of each of the values is <2%. np, not present.
[b]Growth substrate: 10 mL of a 0.2% (dry w/v) ball-milled filter paper medium.
[c]Nonagitated cultures.

Table 2
Detection of Enzymes Involved in Lignin Degradation
After Growth of Strains H2 and Q10 Under Microaerophilic Conditions

Substrates[b]	Enzyme activities, μmol/mL/min[a]		
	Laccase	Peroxidases	
	Syringaldazine	Syringadazine plus H_2O_2	*o*-Dianisidine plus H_2O_2
Strain H2	2.77 (0.07)	4.15 (0.09)	10.20 (0.51)
Strain Q10	6.46 (0.12)	np	np

[a]Numbers in parentheses are standard deviations. np, not present.
[b]Growth substrate: 10 mL of a 0.25% (dry w/v) ball-milled filter paper medium.

RESULTS

Enzyme Activities

CMCase, β-glucosidase, avicelase, and xylanase activities present in the culture supernatants of the two strains grown under the three oxygenation conditions described, are shown in Table 1. Strain Q10 exhibited highest activities after growth under microaerophilic conditions. Strain H2 showed highest levels of enzyme activities after growth under aerobic conditions. As shown in Table 2, laccase was detected in culture supernatants of strains H2 and Q10 by the reaction using syringaldazine as the substrate. Strain Q10, however, produced negative results with syringaldazine plus hydrogen peroxide. Peroxidase was detected in strain H2 after reaction with either syringaldazine plus hydrogen peroxide or *o*-dianisidine plus hydrogen peroxide.

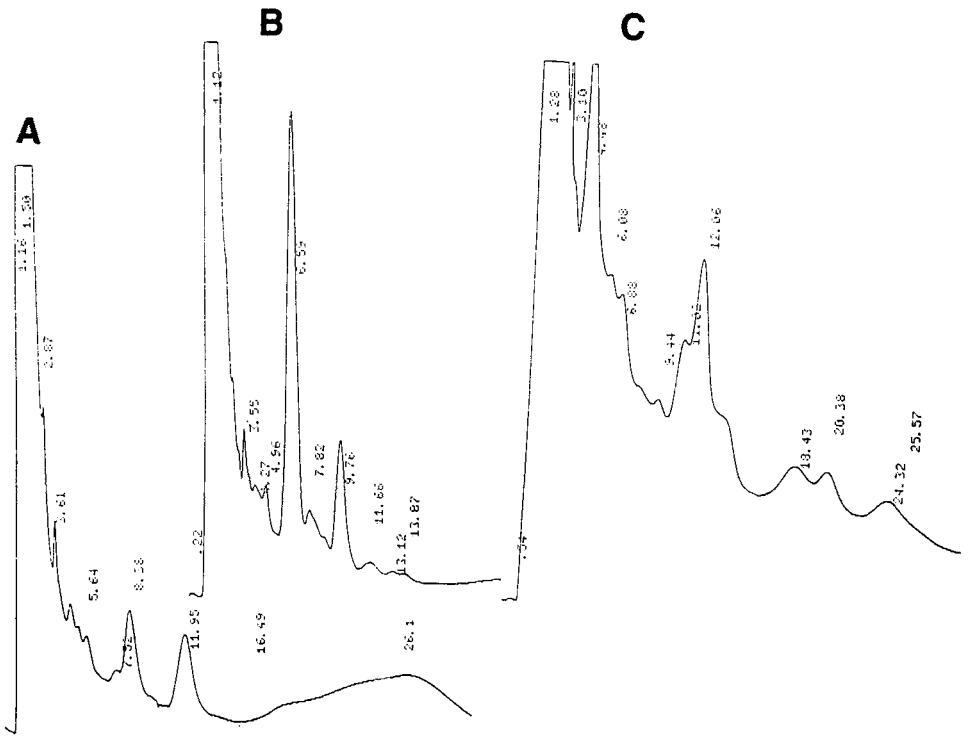

Fig. 1. HPLC chromatograms of supernatant samples of strains H2 and Q10, after growth on lignosulfonate 0.2%, as the sole carbon source, for 3 wk under microaerofilic conditions. Chromatography was carried out using a fast acid column. Aromatic compounds were eluted with a solution containing acetonitrile (15%) and 5 m*M* H_2SO_4 (85%). **(A)** Uninoculated control; **(B)** strain Q10 culture fluid; **(C)** strain H2 culture fluid.

Growth on Lignin-Derived Compounds

In a medium containing lignin sulfonic acid, moderate to good growth of both strains was observed under microaerophilic and aerobic conditions. However, no growth was observed under anaerobic conditions. Only strain H2 was able to use lignin organosolv as growth substrate. The HPLC chromatograms of supernatant samples indicate that the substrates that supported growth were apparently degraded or modified, inasmuch as the composition of the growth medium changed during growth. Moreover, the changes that occurred in the lignin-related substrates were strain specific (Figs. 1 and 2).

Fermentation Products

Both isolates produced ethanol as the major nongaseous fermentation product. Acetate was also produced. Table 3 shows the ethanol produced by strains H2 and Q10 after growth on ball-milled filter paper slurry as the carbon source, and Table 4 shows the ethanol yields obtained after growth of both strains under microaerophilic conditions on lignocellulosic wastes.

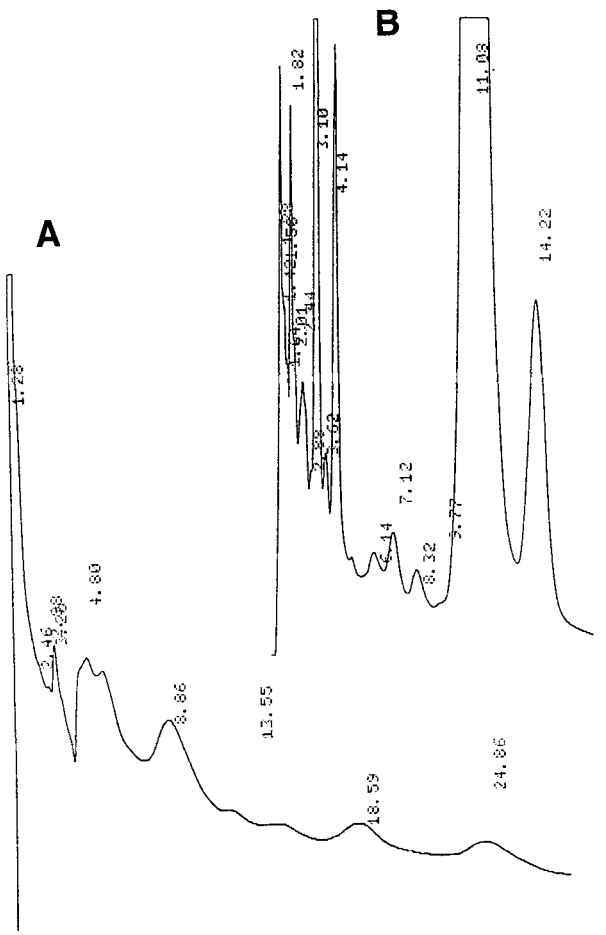

Fig. 2. HPLC chromatograms of supernatant sample of strain H2, after growth on lignin organosolv as the sole carbon source, for 3 wk under microaerofilic conditions. Chromatography was carried out using a fast acid column. Aromatic compounds were eluted with a solution containing acetonitrile (15%) and 5 m*M* H_2SO_4 (85%). **(A)** Uninoculated control; **(B)** strain H2 culture fluid.

Discussion

Both strains grew and degraded cellulose under anaerobic conditions. Strain H2 exhibited best growth and lignocellulases production under aerobic conditions. The results obtained with strain Q10 suggest that it prefers low oxygen concentrations for growth and degradation of lignocellulosic materials, and that it may be a facultative microaerophilic fungus. In this respect, it should be noted that active aeration did not improve growth and degradation of cellulose by strain Q10, and in cultures incubated in air without agitation, noticeable growth occurred only near the bottom of tubes. Both strains were able to grow in lignin sulfonic acid, but only strain H2 was able to grow on the more complex lignin-related substrates used here (lignin organosolv). The results obtained with the syringaldazine and *o*-dianisidine tests indicate that a different enzyme system is utilized by each strain for growth on lignin-related compounds. Strain H2 produces laccase and peroxidases, enzymes reported to be involved in lignin degradation (*27*). According to the detection tests

Table 3
Ethanol Produced by Strains H2 and Q10
Grown on Ball-Milled Filter Paper Slurry

Growth conditions[a]	Ethanol Yields, %[b]	
	Strain H2	Strain Q10
Anaerobic	36.5 (3.05)	96.0 (4.65)
Microaerophilic	8.0 (3.42)	90.5 (5.83)
Aerobic	81.0 (4.86)	65.6 (2.49)

[a]Growth substrate: 10 ml of a 0.25% (dry w/v) ball-milled filter paper medium.

[b]Values represent % of theoretical yield. Numbers in parentheses are standard deviations.

Table 4
Ethanol Produced by Strains H2 and Q10
Grown on Lignocellulosic Materials

Growth substrate[a]	Ethanol yields, %[b]	
	Strain H2	Strain Q10
Coarse pine sawdust	8.4 (0.79)	20.2 (1.38)
Coarse maple sawdust	8.0 (0.76)	37.6 (1.57)
Fine pine sawdust	67.2 (5.71)	6.2 (0.46)

[a]Growth conditions: Incubation carried out under microaerophilic conditions.

[b]Values represent % of theoretical yield. Numbers in parentheses are standard deviations.

used here, strain Q10 does not produce peroxidases. The lack of peroxidases production by strain Q10 is probably the reason for its incapability to grow on the lignin organosolv substrate. The enzyme systems utilized by these strains to grow on lignocellulosic substrates still need further investigation.

The microbial production of chemicals by lignocellulose fermentation has generated considerable research interest. Since the commercially available celluloses are unlikely to be used as substrates for industrial fermentations, fuel production from biomass in the form of agricultural and forest residues is attractive as an alternative renewable energy resource. Fungi having the capacity to convert carbohydrates to ethanol have been increasingly studied in recent years, and their technological use is being explored *(28,29)*. The production of ethanol and other useful chemical feedstocks from lignocellulosic materials by direct fermentation is, however, more complex than isolated forms of cellulose or hemicellulose, owing to the presence of lignin, the crystallinity of cellulose, and also the presence of a complex mixture of sugars. The two fungal strains used here were able to ferment cellulose and lignocellulosic substrates to ethanol. When lignocellulosic materials were used the ethanol yields were lower than that obtained with filter paper slurry, but strain H2 was able to produce good yields of ethanol when the particle size of the material

was reduced, as in the case of the fine pine sawdust. The two strains are also able to grow on xylan, cellobiose, xylose, glucose, and other monossacharides *(18)* as sole carbon sources. The fact that they are able to grow on lignin-related compounds and also produce enzymes involved in the degradation of lignin when growing on cellulose is an advantage. The main disadvantage is the slow conversion rate when compared to ethanol production by yeast. However, more detailed information on certain growth parameters, such as substrate concentration, aeration conditions, temperature, and pH, may lead to improvement in the conversion rates. For instance, in the case of strain H2, a combination in aeration conditions during growth may offer an easy way to speed up the process and is now under investigation.

ACKNOWLEDGMENTS

The author thanks FAEP (Foundation for the Support of Research and Teaching at UNICAMP), FAPESP (Foundation for the Support of Research in Sao Paulo State), and CNPq (National Coucil for Scientific and Technological Development) for financial support.

REFERENCES

1. Orpin, C. G. (1975), *J. Gen. Microbiol.* **91,** 249–262.
2. Orpin, C. G. (1977), *J. Gen. Microbiol.* **99,** 107–117.
3. Bauchop, T. (1989), *Biosystems* **23,** 53–64.
4. Kostiukovskii, V. A., Okunev, O. N., and Tarakanov, B. V. (1990), *Mikrobiologiia* **59,** 1067–1074.
5. Phillips, M. W. and Gordon, G. L. (1988), *Biosystems* **21,** 377.
6. Teunissen, M .J., Op den Camp, H. J., Orpin, C. G., Huis in't Veld, J. H., and Vogels, G. D. (1991), *J. Gen. Microbiol.* **137,** 1401.
7. Teunissen, M. J., Smith, A. A. M., Op den Camp, H. J., Orpin, C. G., Huis in't Veld, J. H., and Vogels, G. D. (1991), *Arch. Microbiol.* **156,** 290–296.
8. Barichievich, E. M. and Calza, R. E. (1990), *Appl. Environ. Microbiol.* **56,** 43–48.
9. Lowe, S. E., Theodorou, M. K. and Trincy, A. P. (1987), *Appl. Environ. Microbiol.* **53,** 1216–1223.
10. Orpin, C. G. (1988), *Biosystems* **21,** 365–370.
11. Teunissen, M. J. and Op den Camp, H. J. (1993), *Antonie Van Leeuwenhoek* **63,** 63–76.
12. Wilson, C. A. and Wood, T. M. (1992), *Enzyme Microb. Technol.* **14,** 258–264.
13. Akin, D. E. (1987), *Anim. Feed Sci. Technol.* **16,** 273–285.
14. Akin, D. E. and Borneman, W. S. (1990), *J. Dairy Sci.* **73,** 3023–3032.
15. Akin, D. E., Borneman, W. S., and Windham, W. R. (1988), *Biosystems* **21,** 385–391.
16. Akin, D. E. and Rigsby, L. L. (1987), *Appl. Environ. Microbiol.* **53,** 1987–1995.
17. Wubah, D. A., Akin, D. E., and Borneman, W. S. (1993), Critical Rev. Microbiol. **19,** 99–115.
18. Durrant, L. R., Canale-Parola, E., and Leschine, S. B. (1995), in *The Significance and Regulation of Soil Biodiversity,* Collins, H. P., Robertson, G. P., and Klug, M. J., eds., Kluwe Academic, The Netherlands.
19. Leschine, S. B. and Canale-Parola, E. (1983), *Appl. Environ. Microbiol.* **46,** 728–737.
20. Hungate, R. E. (1969), *Methods in Microbiology,* vol. 3B, Norris, J. R. and Ribbons, D. W., eds., Academic, New York.
21. Miller, G. L. R., Blum, R., Glennon, W. E., and Burton, A. L. (1960), *Anal. Biochem.* **2,** 127–132.
22. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
23. Kawai, S., Umezawa, T., and Higushi, T. (1988), *Arch. Biochem. Biophys.* **262(1),** 99–110.
24. Harkin, J. M. and Obst, Jr. (1973), *Experimentia* **29,** 381–508.
25. Christakopoulos, P., Macris, B., and Kekos, D. (1989), *Enzyme Microb. Technol.* **11,** 236–239.
26. Christakopoulos, P., Koullas, D. P., Kekos, D., Koukios, E. G., and Macris, B. (1991), *Enzyme Microb. Technol.* **13,** 272–274.
27. Morohoshi, N. (1991), *Enzymes in Biomass Conversion,* Leatham, G. F. and Himmel, M. E., eds., ACS Symposium Series 460. ACS, Washington, DC.
28. Singh, A., Kumar, P. K., and Schuger, K. (1992), *Biochem. Int.* **27,** 831–839.
29. Singh, A., Kumar, P. K. and Schuger, K. (1992), *Biotechnol. Appl. Biochem.* **16,** 296–302.