ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2011, том 47, № 1, с. 61–65

UDC 600

STRAIN IMPROVEMENT FOR ENHANCED PRODUCTION OF CELLULASE IN *Trichoderma viride*

© 2011 F. Xu*, J. Wang*, S. Chen*, W. Qin**, Z. Yu***, H. Zhao****, X. Xing****, H. Li*****

*State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100193, China
e-mail: chensf@cau.edu.cn

**Biorefining Research Initiative and Department of Biology, Lakehead University, Thunder Bay, ON, Canada P7B 5E1
e-mail: wqin@lakeheadu.ca

***Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, Hefei, Anhui 230031, China
e-mail: zlyu@ipp.ac.cn

****Department of Chemical Engineering, Tsinghua University, Beijing 100084, China, e-mail: xhxing@tsinghua.edu.cn

*****Department of Engineering Physics, Tsinghua University, Beijing 100084, China, e-mail: liheping@tsinghua.edu.cn

Received January 18, 2010

The filamentous fungi *Trichoderma* species produce extracellular cellulase. The current study was carried out to obtain an industrial strain with hyperproduction of cellulase. The wild-type strain, *Trichoderma viride* TL-124, was subjected to successive mutagenic treatments with UV irradiation, low-energy ion beam implantation, atmospheric pressure non-equilibrium discharge plasma (APNEDP), and N-methyl-N'-nitro-N-nitrosoguanidine to generate about 3000 mutants. Among these mutants, *T. viride* N879 strain exhibited the greatest relevant activity: 2.38-fold filter paper activity and 2.61-fold carboxymethyl cellulase, 2.18-fold β-glucosidase, and 2.27-fold cellobiohydrolase activities, compared with the respective wild-type activities, under solid-state fermentation using the inexpensive raw material wheat straw as a substrate. This work represents the first application of APNEDP in eukaryotic microorganisms.

Cellulose, a β (1 ⟶ 4)–linked glucose polymer, is considered to be the primary product of photosynthesis and the most abundant renewable carbon resource in nature [1, 2]. Economic analyses have indicated that the production cost of cellulase is still the major cost factor in the hydrolysis of cellulosic materials to fermentable sugars. It is therefore imperative to improve the production of cellulase in order to make the process more economically viable [3, 4].

The cellulase complex secreted by filamentous fungi consists of three major enzyme components, an endo-1,4-β-glucanase (EC 3.2.1.4), a 1,4-β-D-cellobiohydrolase (EC 3.2.1.91), and a 1,4-β-glucosidase (EC 3.2.1.21), which act synergistically during the conversion of cellulose to glucose [2, 5, 6]. Among the cellulolytic fungi, *Trichoderma* species have been studied extensively because of their production of efficient cellulases, which are several hundred-fold more active than those produced by bacteria [3, 7]. Although, *T. reesei* produces cellobiohydrolases and endogluconases in high quantities, it is deficient in β-glucosidase, causing the accumulation of the disaccharide cellobiose. The repression and end-product inhibition of cellulase by cellobiose limit the enzyme synthesis and activity [8, 9]. Thus, attempts to use the cellulase system from *T. reesei* mutants have not been successful.

To date, many traditional mutagenesis stratagies have been applied to improve the production of cellulase [10–13]. Nevertheless, despite the efforts of many laboratories, no commercially efficient enzyme complex has been produced. The high cost of enzyme production is still the bottleneck in the production of fermentable sugars from cellulosic materials [3]. Hence, the use of novel mutagens becomes very necessary in enhancing cellulase production.

Mutagenesis via the novel low-energy ion beam implantation and atmospheric pressure non-equilibrium discharge plasma (APNEDP) techniques has attracted great attentation owing to their efficiency, safety, and non-polluting nature [14–16]. Ample evidence has demonstrated that ion-beam mutation has a wide range of biological effects. It also has a very high linear energy transfer and mutation efficiency compared with γ-ray or high-energy particles [14, 15]. APNEDP has been used recently in prokaryotic microbial mutation breeding. For example, Wang et al. [8] used APNEDP to induce mutations in *Streptomyces avermitilis* and obtained mutants with higher avermenctin B1a productivity than that of the wild-type strain, by 60% or more [16].

The wild-type strain, *T. viride* TL-124, which is isolated from stacks of wheat straw, grows faster than *T. reesei* QM9414 and the wild-type *Aspergillus fumigatus* TL51. In addition, *T. viride* TL-124 has considerable β-glucosidase production. Thus, *T. viride* TL-124 was selected for strain improvement to enhance cellulase production. The current study was initiated to apply the combined mutagenic effects of treatments with UV irradiation, low-energy ion beam implantation, APNEDP, and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to the production of cellulase in wild-type *T. viride* TL-124 to obtain an industrial strain with enhanced cellulase production.

This work represents the first application of APNEDP in eukaryotic microorganisms.

MATERIALS AND METHODS

Microorganism and culture media. The wild-type *T. viride* TL-124 was isolated from stacks of wheat straw and maintained on potato dextrose agar (PDA) [3] in test tubes at 28°C. Minimal medium (MM) [17] was used for cultivation of *T. viride*. For the preliminary selection of mutants, cellulose-congo red medium was used (g/l: KH_2PO_4 – 0.5, $MgSO_4 \cdot 7H_2O$ – 0.25, congo red – 0.2, cellulose – 1.88, and agar – 20, pH 7.0). The wheat straw used in medium preparation for solid-state fermentation was treated according to the method of Gao et al. [18]. Solid-state fermentation medium was prepared in a 250-ml Erlenmeyer flask containing 10 g wheat straw, 0.25 g $(NH_4)_2SO_4$, and 25 ml of distilled water. The media were autoclaved for 30 min at 121°C.

UV irradiation. Protoplasts of *T. viride* TL-124 were prepared as described by Pentila et al. [19]. The protoplast suspension (2 ml of 10^6 per ml) was exposed to UV irradiation according to the method of Rubinder et al. [20]. D-sorbitol (1 M) was used as the osmotic stabilizer. The growing colonies were transferred before sporulating on PDA slants, for further studies.

Low-energy ion beam implantation. Low-energy ion implantation was performed in mutated *T. viride* according to the methods of Su et al. and Gu et al. [14, 15], at the heavy ion implantation facility [21] of the Chinese Academy of Sciences, Institute of Plasma Physics (ASIPP). Nitrogen ions with energy of 15 keV were used at an implantation dose of $90 \times 2.6 \times 10^4$ icons/(s cm^2).

Atmospheric pressure, non-equilibrium discharge plasma (APNEDP). The spores of mutated *T. viride* were placed 2.0 mm downstream of the plasma torch nozzle exit and were treated with pure helium plasmas with Pin = 180 W and Q_{He} = 15.0 slpm. The corresponding gas temperature of the plasma jet was less than 40°C [16].

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) mutagenesis. The spores of mutated *T. viride* were treated with MNNG (Sigma, USA) according to the method of Chand et al. [13]. The treated sample was washed immediately with PBS buffer (pH 6.5), and spread on a MM plate containing 0.5% LiCl.

Fermentation. Different fermentation runs were conducted using the shake-flask method. To determine the effects of different carbon sources on cellulase production, mutant *T. viride* were grown in MM in which the 2% glucose was substituted with different carbon sources. To determine the effects of different nitrogen sources on cellulase production, wheat straw replaced glucose as the sole carbon source in MM, and the 0.5% $(NH_4)_2SO_4$ was substituted with different nitrogen sources. Finally, initial pH, growth temperature, and rotation speed were optimized via individual experiments.

Enzyme assay. Crude enzyme preparation was prepared according to the method of Latifian et al. [22]. The protein concentration of the crude enzyme preparation was determined by the Bradford assay using bovine serum albumin (BSA) fraction V (Sigma) as the standard. Filter paper activity (FPA) (total cellulase) and CMCase (endoglucanase), β-glucosidase, and cellobiohydrolase (exoglucanase) activities were determined according to the method of Ghose [23] with some modifications. The released sugar was measured by the dinitrosalicylic acid method [24]. The enzyme activity is expressed as U/mg of protein, where one unit (U) is defined as the amount of enzyme required to liberate 1 μmol of product per min at 50°C.

RESULTS AND DICUSSION

Mutagenesis. The wild-type *T. viride* TL-124 strain was subjected to successive mutagenic treatments using UV irradiation, low-energy ion beam implantation, APNEDP, and MNNG. The preliminary selection of mutants was based on the diameter of the clearing zones surrounding the colonies grown on cellulose-congo red medium [9, 25]. After each mutagenic treatment, the cellulase activity of the clones that displayed the largest clearing zones was assessed under solid-state fermentation, and the most promising strain was subjected to the next mutagenic treatment. After four mutagenic steps, more than 3000 mutant colonies were produced, and about 1000 mutants were assayed for cellulase activity. Mutant N879 was selected because it had the highest total cellulase production.

Cellulase activity assay. The assayed protein concentrations indicated obvious differences among the mutants of *T. viride*. The protein concentration in the crude enzyme preparation from the mutants ranged from 0.021 to 0.064 mg/ml, compared with 0.052 mg/ml in the preparations from the wild-type strain (Fig. 1). These results indicate that the mutations caused changes in protein production or secretion and are in agreement with a report by Prabavathy et al. [26]. Various factors such as the presence of non-protein components in solutions or non-cellulase proteins in the preparations may interfere with the determination of protein concentration [9, 27, 28].

Solid-state fermentation is an attractive process for economically producing cellulase because of its low capital costs for equipment and low operational costs. Thus, the selected mutants of *T. viride* were evaluated for production of extracellular FPA under solid-state fermentation, using the economical raw material wheat straw as substrate (Fig. 2a). All of the selected mutants showed a slight difference in FPA compared with the wild-type strain. The differences in activity did not allow for a direct comparison of volumetric activities, and the immediate alternative for comparing the data was the activity based on the protein concentration (U/mg) [7]. The comparison of the activities based on protein concentration revealed obvious differences between the mutants and the wild-type strain. On the basis of protein concentration, the FPA of the mutants ranged from 1.53 to 5.33 U/mg, compared with 2.24 U/mg for the wild-type strain

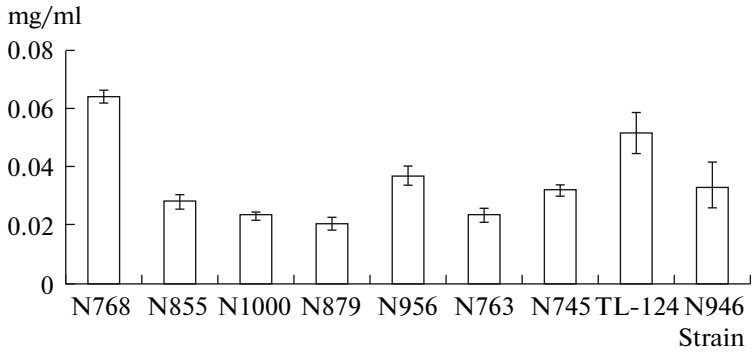

Fig. 1. Protein concentrations (mg/ml) of the crude enzyme preparations from the *T. viride* mutant strains compared with the wild-type strain, *T. viride* TL-124. Data were determined from a minimum of three replicates. Error bars represent the standard deviation of each data point.

(Fig. 2b). The maximum cellulase activity (U/mg) was observed with mutant strain N879, which produced 2.38-fold FPA and 2.61-fold CMCase, 2.18-fold β-glucosidase, and 2.27-fold cellobiohydrolase activities, relative to the respective activities in the wild-type strain (Table). The mutant strain N879 also produced higher FPA and CMCase and cellobiohydrolase activities, compared with *T. reesei* QM9414 and *A. fumigatus* TL51 (Table). Notably, the mutant N879 produced β-glucosidase at a level that was about 3-fold that of *T. reesei* QM9414 and approximately equal to that produced by *A. fumigatus* TL51 (Table).

Here, we used *T. reesei* strain QM9414 as a standard for comparing the cellulase production of the best mutant obtained in this study. It should be noted that the *T. viride* mutant strain N879 also produced high levels of extracellular β-glucosidase (29.22 U/mg), compared with *T. reesei* QM9414 (9.59 U/mg), when grown in wheat straw (Table). This was improved further under controlled culture conditions and genetic approaches. In addition, the specific FPA and CMCase and cellobiohydrolase activities in strain N879 were 1.92-, 2.46-, and 1.60-fold the respective enzyme activities in the standard strain, *T. reesei* QM9414 (Table). These results indicate the effectiveness of low-energy ion beam implantation and APNEDP in producing useful *T. viride* mutants. These also demonstrate that *T. viride* mutant N879 has potential for development as a cellulase production system.

Fermentation. Cellulase production depended on the nature of the carbon and nitrogen sources in the culture medium. Several carbon and nitrogen sources were tested for their effect on the FPA of *T. viride* mutant strain N879. The optimal carbon source for cellulase production was α-lactose (Fig. 3a). According to previous studies, this may be attributable to the inducible nature of *T. viride* cellulases, which would indicate that α-lactose was a good inducer of cellulase enzymes in the mutant strain N879, consistent with a previous report [8]. Compared with α-lactose, cellulose and wheat straw showed lesser effects on cellulase production. It may be that α-lactose is metabolized faster and utilized more quickly than the long insoluble chains covalently bound to molecules such as cellulose and wheat straw, which are too large to be transported through cell walls. Wheat straw, a raw natural material, has a more complex structure than cellulose, which may explain why the effect of cellulose on cellulase production was greater than that of wheat straw.

Based on the nitrogen source comparison, nitrate and peptone were the best inorganic and organic nitrogen sources, respectively (Fig. 3b). The total cellulase produc-

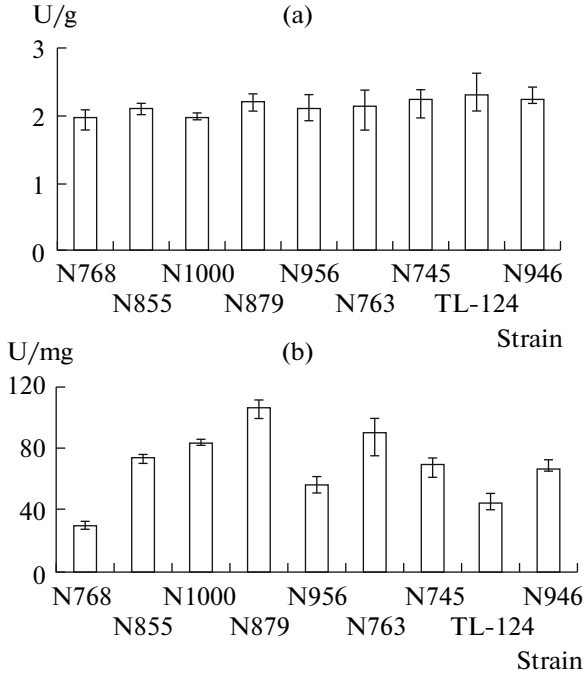

Fig. 2. FPA production in wild-type and mutant *T. viride* strains under solid-state fermentation. (a) FPA expressed as U/g dry weight. (b) FPA expressed as U/mg protein. Data were determined from a minimum of three replicates. Error bars represent the standard deviation of each data point.

Cellulase activities of the mutated *T. viride* and wild type strain under solid-state fermentation*

Strain	CMCase, U/mg protein	Cellobiohydrolase, U/mg protein	β-glucosidase, U/mg protein	FPA, U/mg protein
T. viride TL-124	867.35	26.81	13.39	44.75
T. viride N879	2261.54	60.90	29.22	106.60
T. reesei QM9414	919.11	38.03	9.59	55.43
A. fumigatus TL51	1233.00	43.75	36.89	84.28

* Data were determined from a minimum of three replicates.

tion was affected less significantly by changes in the nitrogen source than by changes in the carbon source.

The fermentation conditions were optimized with monofactorial experiments. The optimal initial pH was 5.5 (Fig. 4a), and the mutant N879 produced the maximum total cellulase at a growth temperature of 30°C (Fig. 4b). *T. viride* N879 showed highest FPA at the speed of rotation was 160 rpm (5.84 U/mg protein), compared with the condition of 120 rpm (4.86 U/mg protein) and 200 rpm (4.00 U/mg protein) (Fig. 4c). Morewver, total cellulase production in strain N879 was lower at the condition of stationary (3.84 U/mg protein) compared with any rotation condition tested in this study (Fig. 4c). This observation may be explained by the fact that shaking alters the metabolism of the fungus, which may increase cellulase produc-

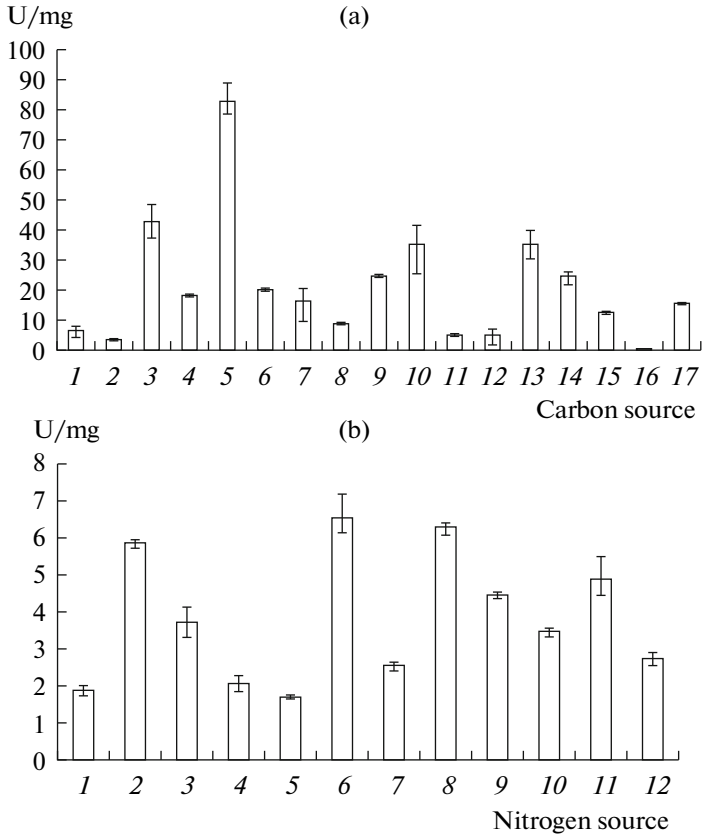

Fig. 3. Effects of the carbon (a) and nitrogen (b) sources on FPA production in mutant *T. viride* N879. (a) *1* – D-glucose; *2* – mannitol; *3* – maltose; *4* – cellulose; *5* – α-lactose; *6* – D-sorbitol; *7* – D-(+)-cellobiose; *8* – CMC; *9* – L-(-)-sorbose; *10* – D-mannose; *11* – wheat straw; *12* – D-galactose; *13* – D-(-)-fructose; *14* – starch soluble; *15* – sucrose; *16* – tween-80; *17* – glycerol. (b) *1* – urea; *2* – NH_4NO_3; *3* – $NH_4H_2PO_4$; *4* – yeast extract; *5* – beef extract; *6* – $Ca(NO_3)_2$; *7* – $(NH_4)_2HPO_4$; *8* – $NaNO_3$; *9* – KNO_3; *10* – peptone; *11* – $(NH_4)_2SO_4$; *12* – NH_4Ac. Data were determined from a minimum of three replicates. Error bars represent the standard deviation of each data point.

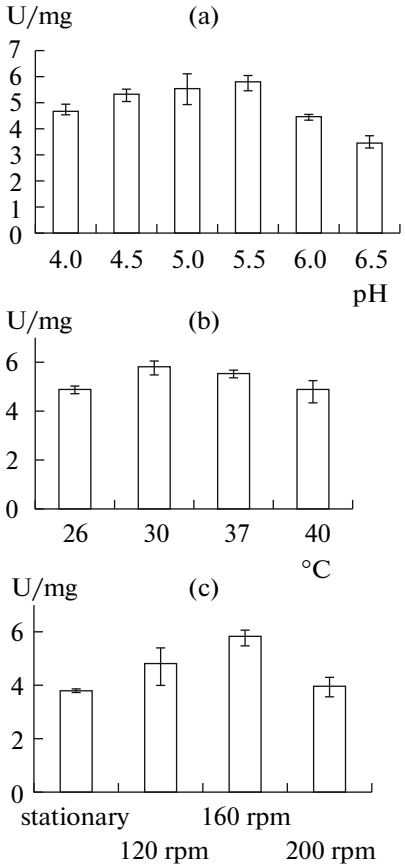

Fig. 4. Effect of initial pH (a), culture temperature (b) and rotation speed (c) on FPA production (U/mg protein) in mutant *T. viride* N879. Data were determined from a minimum of three replicates. Error bars represent the standard deviation of each data point.

tion. These fermentation conditions provide the foundation for future utilization the *T. viride* mutant N879.

ACKNOWLEDGMENT

This research was supported by the National Key Technology R&D Program of China (Grant No. 2006BAD07A01).

REFERENCES

1. Jarvis, M., *Science*, 2003, vol. 426, no. 6967, pp. 611–612.
2. Zhang, Y.H.P. and Lynd L.R., *Biotechnol. Bioeng.*, 2004, vol. 88, no. 7, pp. 797–824.
3. Adsul, M.G., Bastawde, K.B., Varma, A.J., and Gokhale, D.V., *Bioresource Technol.*, 2007, vol. 98, no. 7, pp. 1467–1473.
4. Gomes, I., Gomes, J., Steiner, W., and Esterbauer, H., *Appl. Microbiol. Biotechnol.*, 1992, vol. 36, no. 5, pp. 701–707.
5. Henrissat, B., *Cellulose*, 1994, vol. 1, no. 3, pp. 169–196.
6. Zhang,Y.H.P., Himmel, M.E., and Mielenz, J.R., *Biotechnol. Adv.*, 2006, vol. 24, no. 5, pp. 452–481.
7. Martins, L.F., Kolling, D., and Camassola M., Dillon A.J.P., and Ramos, L.P., *Bioresource Technol.*, 2008, vol. 99, no. 5, pp. 1417–1424.
8. Ahamed, A. and Vermette, P., *Biochem. Eng. J.*, 2008, vol. 40, pp. 399–407.
9. Zaldivar, J., Nielsen, J., and Olsson, L., *Appl. Microbiol. Biotechnol.*, 2001, vol. 56, no. 1–2, pp. 17–34.
10. Anwar, M.N., Suto, M., and Tomita, F., *Appl. Microbiol. Biotechnol.*, 1996, vol. 45, no., pp. 684–647.
11. Szengyel, Z., Zacchi, G., Varga, A., and Reczey, K., *Appl. Biochem. Biotechnol.*, 2000, vol. 84–86, no. 1–9, pp. 679–691.
12. Xia, L.M. and Shen, X.L., *Bioresource Technol.*, 2004, vol. 91, no. 3, pp. 259–262.
13. Chand, P., Aruna, A., Maqsood, A.M., and Rao, L.V., *J. Appl. Microbiol.,* 2005, vol. 98, no. 2, pp. 318–323.
14. Su, C.X., Zhou W., Fan, Y.H., Wang, L., Zhao, S.G., and Yu, Z.L., *J. Ind. Microbiol. Biotechnol.*, 2006, vol. 33, no. 12, pp. 1037–1042.
15. Gu, S.B., Yao, J.M., Yuan, Q.P., Xue, P.J., Zheng, Z.M., Wang, L., and Yu, Z.L., *Appl. Microbiol. Biotechnol.*, 2006, vol. 72, no. 3, pp. 456–461.
16. Wang, L.Y., Huang, Z.L., Li, G., Zhao, H.X., Xing, X.H., Sun, W.T., Li, H.P., Gou, Z.X., and Bao, C.Y., *J. Appl. Microbiol.*, 2010, vol. 108, no. 3, pp. 851–858.
17. Tao, L., Dong, H.J., Chen, X., Chen, S.F., and Wang, T.H., *Appl. Microbiol. Biotechnol.*, 2008, vol. 80, no. 4, pp. 573–578.
18. Gao, J.M., Weng, H.B., Zhu, D.H., Yuan, M.X., Guan, F.X., and Xi, Y., *Bioresour Technol.*, 2008, vol. 99, no. 16, pp. 7623–7629.
19. Penttila., M, Nevalainen., H., Ratto, M., Salminen, E., and Knowles, J., *Gene*, 1987, vol. 61, no. 2, pp. 155–164.
20. Rubinder, K., Chadha, B.S., Sing, N., Saini, H.S., and Singh, S., *J. Ind. Microbiol. Biotechnol.*, 2002, vol. 29, no. 2, pp. 70–74.
21. Zengliang, Yu. et al., Patent No. ZL93103361.6, 2000, People's Republic of China.
22. Latifian, M., Hamidi-Esfahani, Z., and Barzegar, M., *Bioresource Technol.*, 2007, vol. 98, no. 18, pp. 3634–3637.
23. Ghose, T.K., *Pure Appl. Chem.*, 1987, vol. 59, no. 2, pp. 257–268.
24. Miller, G.L., *Anal. Chem*, 1959, vol. 31, no. 3, pp. 426–428.
25. Teather, R.M. and Wood P.J., *Appl. Environ. Microb.*, 1982, vol. 43, no. 4, pp. 777–780.
26. Prabavathy, V.R., Mathivanan, N., Sagadevan, E., Murugesan, K., and Lalithakumari, D., *Enzyme Microb. Technol.*, 2006, vol. 38, no. 5, pp. 719–723.
27. Adney, W.S., Mohagheghi, A., Thomas S.R., and Himmel, M., *Am. Chem. Soc.*, Washington, 1995, pp. 256–271.
28. Zaia, D.A.M., Zaia, C.T.B.V., and Lichtig, J., *Quim Nova*, 1998, vol. 21, no. 6, pp. 787–793.