Journal of Global Biosciences ISSN 2320-1355
Vol. 3(1), 2014, pp. 354-360 http://mutagens.co.in

IMPROVEMENT OF L-LACTIC ACID PRODUCTION FROM ORANGE PEELS IN MIXED CULTURE SYSTEM

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Abstract

Orange peels were used in a 7-l fermentor to directly form L-lactic acid in the mixed culture system. The synthesis of pectinase and cellulase using *Aspergillus niger* GH-06 was enhanced significantly by inoculation of *Lactobacillus casei* G-02 at 16 h of culture with addition of phytic acid at 3 g/l, which activities reached 108.6 and 17.8 U/ml in 72 h, respectively, over 2-folds higher than that of the original culture using single strain. In the following simultaneous saccharification and fermentation procedure, the L-lactic acid concentration of 116.3 g/l was obtained from orange peels in 36 h with high conversion efficiency of 90.3%.

Key words: *Aspergillus niger*; lactic acid; *Lactobacillus casei*; orange peels; mixed culture system.

Date of Online: 28-02-2014

INTRODUCTION

Recently, much work has been done to improve the production of L-lactic acid from inexpensive raw substrate in a cheap and energy-saving process using genetically modified organisms and multi-stage fermentation system (1-11). Citrus fruits are among the most important fruits grown and consumed all over the world. Oranges, which alone account for about 55% of the global citrus fruit production , are composed of orange peel with 50% of the total fruit weight. Orange peels, being rich in fermentable sugars, such as, glucose, fructose, and sucrose, along with insoluble polysaccharides cellulose and pectin (12), has been proposed for many years as a possible substrate for ethanol production (13-17). However, there have been few studies on lactic acid production from orange peels.

Orange peels contains nearly 40% (w/w) of insoluble carbohydrates (Basis on dry matter), which was composed of 40% (w/w) of the cellulose and 60% (w/w) of the pectin (15). Therefore, the conversion of carbohydrates in citrus peel requires the synchronously enzymatic hydrolysis by cellulase and pectinase. A number of bacterial and fungal strains produce cellulase and pectinase. Due to its GRAS property, multi-enzyme system, and extensive culture requirement, *Aspergillus niger* is the most common and preferred microorganism (18,19). It was reported that the fermentation activity of the yeast was inhibited significantly by peel oil in the orange peels (20); therefore, to improve the lactic acid production from orange peels, it is necessary it simultaneous enhancing the cellulase and pectinase activities and decreasing the inhibitions from peel oil. In this article, *A. niger* GH-06 and *Lactobacillus casei* G-02 were co-cultured in the submerged cultivation system for enzymes production, then additional mashed fresh oranges peels and its flour were directly convert to lactic acid in the following simultaneous saccharification and fermentation process.

MATERIALS AND METHODS

Microorganisms and Media

Lactobacillus casei G-02, deposited in the China Center of Type Culture Collection (CCTCC) as CCTCC M 208232, were described elsewhere (9). *Aspergillus niger* GH-06, a novel producer of cellulase and pectinase, was isolated from the soil and subjected to mutagenesis with UV, ^{60}Co, and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as reported previously (21). The identification of this strain was performed according to the descriptions of Dai (22). It was deposited in the Culture and Information Center of Industrial Microorganism of China University (CICIM-CU) at Jiang Nan university as CICIM F0045(T), and maintained on slants of potato agar and subcultured every month. The basal medium used for the production of cellulase and pectinase contained (g/L): wheat bran 20, orange peels flour 20, adjusted to pH=6.0 with 0.1 M of HCl or NaOH.

Mandarin oranges were procured from a local supermarket in bulk, and the fresh peels were removed, mashed directly and sterilized at 121 °C for 15 min. Dry-matter content of the fresh oranges peels was 25.8 %. To prepare flour, the oranges peels were dried to a constant weight in a forced air-flow oven at 70 °C for 48 h, and then ground using an electric mill. The resulting flour contained 9.1 % (w/w) of moisture and 63.6 % (w/w) of total sugar. Wheat bran was obtained from local source and milled commercially. All chemicals were purchased from Sigma Chemical Co., Missour, USA.

Cultivation

To prepare the inocula of *A. niger* GH-06, aliquots (50 ml) of each medium were dispensed in 250 ml Erlenmeyer flasks, incubated at 30 °C under shaking conditions (140 rpm), and then inoculated into a 7-l jar fermentor (KF-7 l; Korea Fermentor Co., Inchon, South Korea) with a working volume of 4 liters. The aeration rate was 4.5 liters/min, and the agitation speed was controlled at 140 rpm. During the culture process, mycelium was periodically withdrawn and centrifuged at 5000×*g* and 4 °C for 5 min. The supernatant was used for the analysis of the enzyme activity.

Effect of the additional phytic acid and inoculated *Lactobacillus casei* G-02 on enzymes production *A. niger* GH-06

In order to determine the effect of the addition of phytic acid to the medium on the production of cellulase and pectinase using *A. nige*r GH-06, phytic acid was added to the basal medium at the concentration from 1 g/L to 5 g/L, and the medium without phytic acid was used as a control. During the process, mycelia were periodically withdrawn and centrifuged at 5000 rpm and 4 °C for 5 min. Supernatant was used for the analysis of the activities of extracellular cellulase and pectinase.

Previous study found that the production of glucoamylase can be ceased by the glucose in the culture medium (23), therefore, to enhance the enzyme activity, a 5 ml of *Lactobacillus casei* G-02 cell suspension (with cell count of 10^8/ml) from the slant was inoculated at each stage of culture to the enzyme-producing medium to exhaust the residual glucose. The culture without the inoculation of *Lactobacillus casei* G-02 was used as a control.

Effect of the fermentation condition on L-lactic acid production by *Lactobacillus casei* G-02

In this process, to economically produce lactic acid in short period of time, the mashed fresh orange peels were used firstly as the substrate. To determine the optimum initial mashed fresh orange peels concentration for L-lactic acid production, the fermentation were carried out in the medium with stepwise addition of 5 to 40 g mashed fresh orange peels into 100 ml mixed culture of *A. niger* GH-06 and *Lactobacillus casei* G-02. The fermentations were run at 30 °C for 36 h in the 500-ml conical flasks containing same inoculation volume (20%), enzyme activity (40 U/ml).

In order to study the effect of enzyme activity and inoculation volume on L-lactic acid production, the submerged culture of *A. niger* GH-06 was grown aerobically for 60 h, and then filtrated by 8-folds gauze to remove the fungal mycelia. The filtrated culture was diluted appropriately and used for L-lactic acid fermentation tests. The fermentations were carried out in the media containing pectinase activities (10 to 80 U/ml), inoculation volume (5 to 30%), and mashed fresh orange peels (350 g/l) to investigate the effect of the enzyme activity and inoculation volume on the L-lactic acid production, and the culture without the enzyme activity was used as a control.

Fermentation tests for determining the maximum concentration of L-lactic acid obtained by *Lactobacillus casei* G-02 were carried out in fed-batch culture with sequential feeding with mashed fresh orange peels. The 100 mL of mycelium-free culture supernatant, the whole liquid culture, and the mycelium disrupted whole liquid culture (ultrasonically at 4°C for 200 cycles of 5 s) of *A. niger* GH-06 were supplemented with 30 to 50 g of mashed fresh orange peels respectively, and the three

were compared as a fermentation medium. The initial pH was 5.0, and the pH was not controlled during the fermentation.

L-lactic acid fermentation from orange peel by simultaneous saccharification and fermentation procedure

The saccharification and fermentation of orange peels proceeded simultaneous within the 7-l fermentor containing 4 l medium, which was composed of pectinase activity and inoculation volume of 40 U/ml and 20%, respectively. In this fed-batch fermentation, the 4 L of mycelium disrupted whole liquid culture of *A. niger* GH-06 were supplemented with 1800 g of mashed fresh orange peel at the beginning of the fermentation, and then mass of 400 g of the flour was added after 16 h of fermentation. All of the above fermentations were carried out at 30°C for 36 h. The medium was agitated at the beginning of the fermentation to submerge the solid matter in the liquid, and then incubated without agitation at 30 °C. During the fermentation process, liquid culture was periodically withdrawn and used for the analysis of L-lactic acid and total sugar.

Analytical methods

The cellulase activity and pectinase activity were assayed as described previously (18, 24). One unit (U) of enzyme activity was defined as the amount of enzyme (in 1 ml), which liberates 1 µmol of reducing sugar equivalent from sodium carboxymethyl cellulose (cellulase) or pectin (pectinase) per min. Reducing sugars were estimated with 3,5-dinitrosalicylic acid (DNS), using glucose as standard. Total reducing sugar was assayed by the same method after acid hydrolysis (adjusted to pH=1.0 with sulfuric acid and heated for 30 min at 100 °C). The pH was measured by pH-meter. L-lactic acid was determined by SBA-40C immobilized enzyme biosensor. All the experiments were repeated three times, and the values are expressed as the means of duplicate measurements on three independent samples. The data were analyzed by SAS software (USA).

RESULTS AND DISCUSSIONS

Enzymes production in the mixed culture

In the media added phytic acid at the concentration of 3 g/L, extracellular enzyme activities increased from 29.4 and 5.8 U/mL in the basal medium to 60.7 and 12.1 U/mL for pectinase and cellulase, respectively. Both pectinase and cellulase activities were enhanced over 2-fold more than those in the basal medium after 3 days of submerged culture (Fig. 1A). It was also found that when the *Lactobacillus casei* G-02 was inoculated after 10 h of the culture, the enzyme activities were all higher than that in the control; and only in the case of *Lactobacillus casei* G-02 inoculation at 16 h of the culture, maximum pectinase activity of 126.8 U/ml was obtained, which was over 2-fold higher than that of the control (Fig. 1B). Likewise, the cellulase was enhanced from 12.1 U/ml to 17.8 U/ml. These results were consistent with our recent report that the co-cultured system may attribute to the synergetic effect between the both strains (9).

L-lactic acid production from orange peels in the simultaneous saccharification and fermentation process

Maximum L-lactic acid productivity of 2.3 g/l/h was obtained in the medium with pectinase activity of 40 U/ml, meanwhile, the higher the inoculation volume, the higher the lactic acid productivity. Nevertheless, the L-lactic acid productivity increased indistinctively when the inoculation volume was enhanced up to 20% of the medium. Increasing the mashed fresh orange peels concentration resulted in an enhanced L-lactic acid concentration; however, the productivity and conversion efficiency decreased dramatically when the mashed fresh orange peels concentration was over 350 g/l. Because the fermentable sugar concentration was 5.6 g/L in medium with this concentration of mashed fresh orange peels, therefore, the inhibition effect mainly resulted from the orange peel oil in the fresh orange peels as reported recently by Mark (20). Nevertheless, as shown in Figure 2, the inhibition was weakened in the medium with the mycelium disrupted whole liquid culture, and maximum lactic acid productivity was obtained in the medium with 450 g/l of mashed fresh orange peels. These results were consistent with our previous report that the fungal mycelia were responsible for the enhanced growth and fermentation activities of producer strain (25).

To convert orange peels to L-lactic acid in the fed-batch culture economically, optimum fermentation conditions were used to maximize the lactic acid productivity. As shown in Figure 3, the saccharification speed of the orange peels just being equal to the consumption speed of the

Lactobacillus casei G-02 which weakened the sugar repression significantly (26). Then high L-lactic acid concentration of 116.3g/l was obtained in 36 h, and the conversion efficiency of total sugar to L-lactic acid was 90.3% of the theoretical lactic acid yield.

For the first time, *A. niger* GH-06 and *Lactobacillus casei* G-02 were co-cultured to enhance the cellulase and pectinase synthesis for L-lactic acid fermentation from orange peels. It was found that the addition of phytic acid and inoculation of *Lactobacillus casei* G-02 influenced the enzyme production significantly. The maximum enzymes activities were obtained in the medium containing 3 g/L of phytic acid with the inoculation of *Lactobacillus casei* G-02 at 16 h of culture, which mainly due to the synergetic effect between *A. niger* GH-06 and *Lactobacillus casei* G-02.

As previous studies have found that orange peel oil repressed fermentation activity of the yeast dramatically, maximum L-lactic acid productively was obtained with mashed fresh orange peels at the concentration of 450 g/L in the simultaneous saccharification and fermentation process in the mycelium disrupted whole liquid culture. Because the extracellular inhibition effects from orange peel oil and sugar were all weakened, maximum L-lactic acid concentration of 116.3 g/l was obtained after 36 h in the fed-batch fermentation without additional nutrients, and the conversion efficiency of total sugar to L-lactic acid was 90.3% of the theoretical yield. This present type of fermentation process might make orange peels a promising raw material for L-lactic acid production.

ACKNOWLEDGMENTS

We would like to thank the Financial support from the Program of Introducing Talents of Discipline to Universities (111 Project) (111-2-06), the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT, IRT0532), the Priority Academic Program Development of Jiangsu Higher Education Institutions, the Fundamental Research Funds for the Central Universities (JUSRP211A26), the Suqian Science Technology Funded Project (H201214).

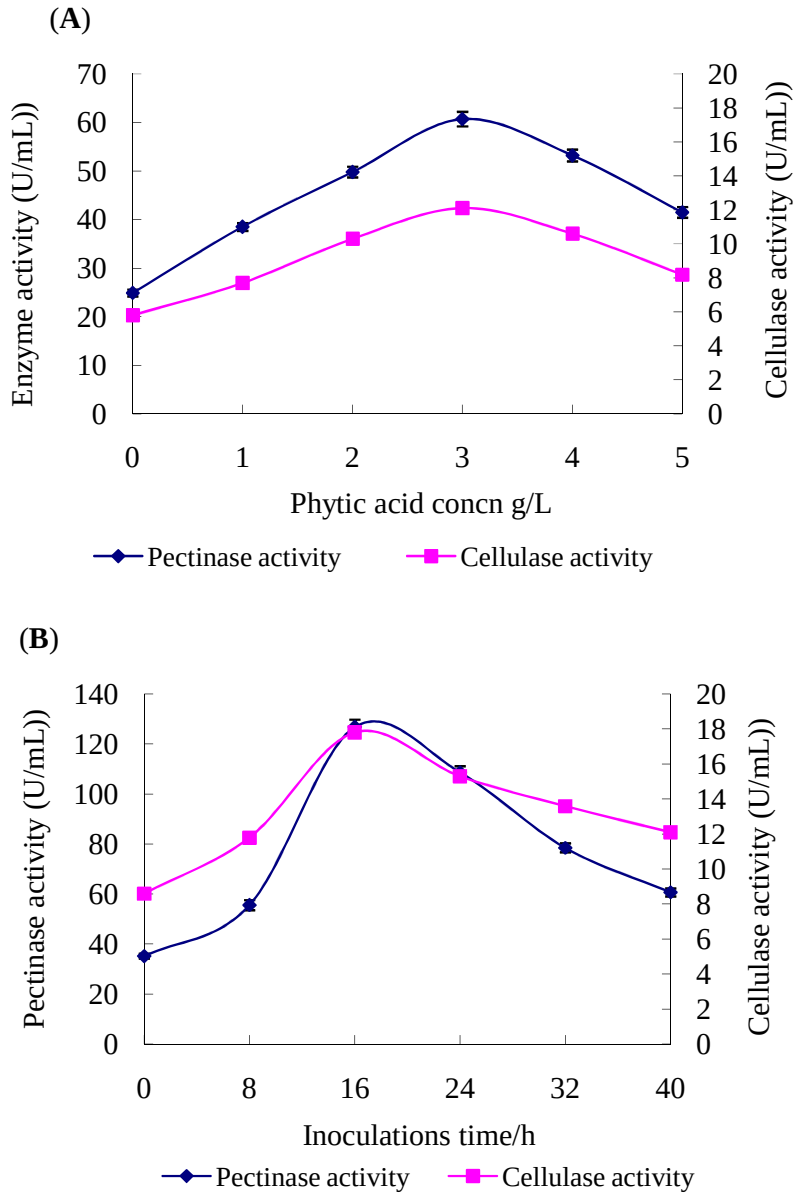

Fig. 1. Effect of additional phytic acid (A) and inoculated *Lactobacillus c*asei G-02 (B) in the basal medium on the pectinase and cellulase production of *A. niger* GH-06.

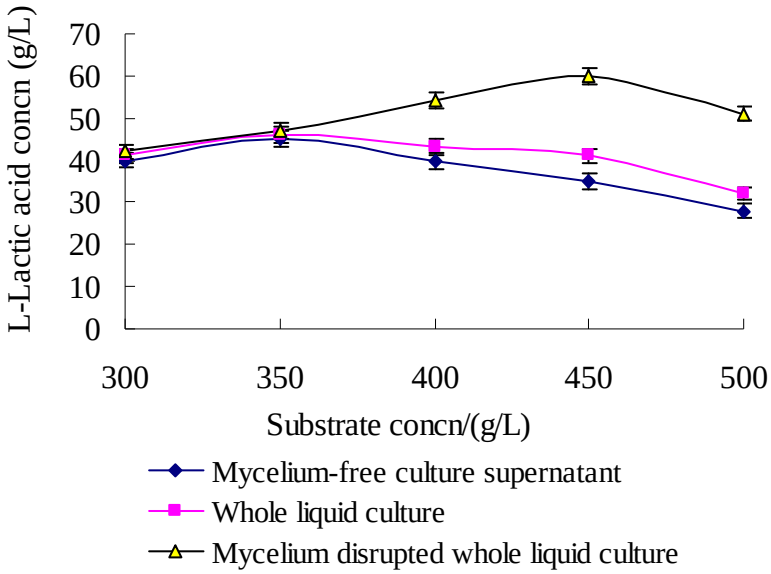

Fig. 2. L-lactic acid production by *Lactobacillus casei* G-02 in batch fermentation with sequential addition of mashed fresh orange peels. Whole liquid culture, mycelium disrupted whole liquid culture, or culture supernatant of *A. niger* GH-06 were used as medium, respectively.

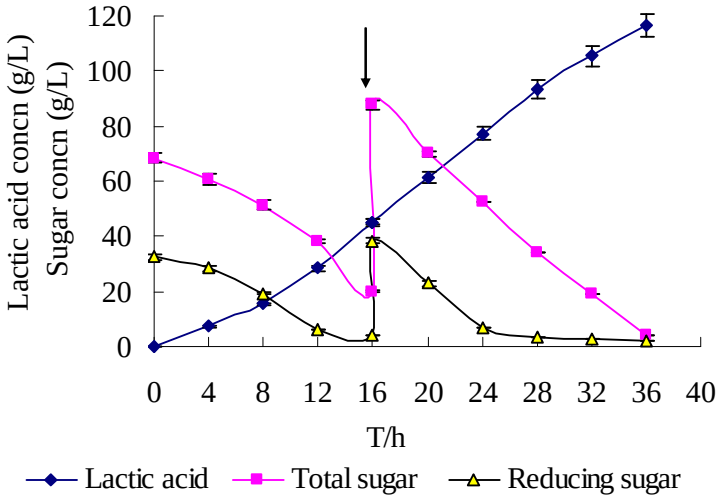

Fig. 3. L-Lactic acid production from range peels in fed-batch culture. The range peels flour of 400 g was added to 4 l of the mixed culture at 16 h (the arrow) of the fermentation.

REFERENCES

1. Watanabe, M., Makino, M., Kaku, N., Koyama, M., Nakamura, K., Sasano, K.: Fermentative L-(+)-lactic acid production from non-sterilized rice washing drainage containing rice bran by a newly isolated lactic acid bacteria without any additions of nutrients, J. Biosci. Bioeng., 115, 449-452 (2013).
2. Ye, L. D., Zhou, X. D., Bin Hudari, M. S., Li, Z., Wu, J. C.: Highly efficient production of L-lactic acid from xylose by newly isolated *Bacillus coagulans* C106, Biores. Technol., 132, 38-44 (2013).
3. Tang, Y., Bu, L. X., He, J., Jiang, J. X.: L(+)-Lactic acid production from furfural residues and corn kernels with treated yeast as nutrients, European Food Research Technol., 236, 365-371 (2013).
4. Shi, Z. M., Wei, P. L., Zhu, X. C., Cai, J., Huang, L., Xu, Z. N.: Efficient production of L-lactic acid from hydrolysate of Jerusalem artichoke with immobilized cells of *Lactococcus lactis* in fibrous bed bioreactors, Enzyme Microbial. Technol., 51, 263-268 (2012).

5. Taskin, M., Esim, N., Ortucu, S.: Efficient production of L-lactic acid from chicken feather protein hydrolysate and sugar beet molasses by the newly isolated *Rhizopus oryzae* TS-61, Food Bioproducts Processing, 90, 773-779 (2012).
6. Phrueksawan, P., Kulpreecha, S., Sooksai, S., Thongchul, N.: Direct fermentation of L(+)-lactic acid from cassava pulp by solid state culture of *Rhizopus oryzae*. Bioprocess Biosystems Engineering, 35, 1429-1436 (2012).
7. Djukic-Vukovic, A. P., Mojovic, L. V., Vukasinovic-Sekulic, M. S., Rakin, M. B., Nikolic, S. B., Pejin, J. D., Bulatovic, M. L.: Effect of different fermentation parameters on L-lactic acid production from liquid distillery stillage, Food Chem., 134, 1038-1043 (2012).
8. Gao, M.T., Kaneko, M., Hirata, M., Toorisaka, E., Hano, T.: Utilization of rice bran as nutrient source for fermentative lactic acid production. Bioresour Technol., 99, 3659-3664 (2008).
9. Ge X.Y., Qian H.□Zhang W.G.: Improvement of L-lactic acid production from Jerusalem artichoke tubers by mixed culture of *Aspergillus niger* and *Lactobacillus sp*., Bioresour Technol., 100, 1872–1874 (2009).
10. Maas, R.H.W., Bakker, R.R., Jansen, M.L.A., Visser, D., De Jong, E., Eggink, G., Weusthuis, R.A.: Lactic acid production from lime-treated wheat straw by Bacillus coagulans, neutralization of acid by fed-batch addition of alkaline substrate, Appl. Microbiol. Biotechnol., 78, 751-758 (2008).
11. Romani, A.; Yanez, R., Garrote, G.; Alonso, J.L.: SSF production of lactic acid from cellulosic biosludges, Bioresour Technol., 99, 4247-4254 (2008).
12. Grohmann, K; Cameron, R. G.; Buslig, B. S.: Fractionation and pretreatment of orange peel by dilute acid hydrolysis, Bioresour Technol., 54, 129–141 (1995).
13. Grohman, K.; Baldwin, E. A.; Buslig, B. S.: Production of ethanol from enzymatically hydrolysed orange peel by the yeast *Saccharomyces cerevisiae*, Appl. Biochem. Biotechnol., 45/46, 315–327 (1994).
14. Grohman, K.; Cameron, R. G.; Buslig, B. S.: Fermentation of orange peel hydrosylates by ethanologenic *Escherichia coli*, Appl. Biochem. Biotechnol., 57/58, 383–388 (1996).
15. Harinder S.O., Praveen V.V., Ronald L.M., Lavudi S., Jithma P.A.: Ethanol Production from Orange Peels: Two-Stage Hydrolysis and Fermentation Studies Using Optimized Parameters through Experimental Design, J. Agric. Food Chem., 58, 3422–3429 (2010).
16. Oberoi, H.S., Vadlani P.V., Madl R.L., Saida L., Abeykoon J.P.: Ethanol production from orange peels: two-stage hydrolysis and fermentation studies using optimized parameters through experimental design, J. Agric. Food Chem., 58, 3422-3429 (2010)
17. Wilkins, M. R.; Widmer, W.W.; Grohmann, K.: Simultaneous scahharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol, Process Biochem., 42, 1614–1619 (2007).
18. Patil, S.R., Dayanand, A.: Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state condition, Bioresour. Technol., 97, 2054-2058 (2006).
19. Sharma, A., Khare, S.K., Gupta, M.N.: Hydrolysis of rice hull by crosslinked *Aspergillus niger* cellulase, Bioresour. Technol., 78: 281-284 (2001).
20. Mark, R.W.: Effect of orange peel oil on ethanol production by *Zymomonas mobilis*. Biomass Bioenergy, 33, 538-541 (2009).
21. Skowronek, M., Fiedurek, J.: Selection of biochemical mutants of *Aspergillus niger* resistant to some abiotic stresses with increased inulinase production, J. Appl. Microbiol., 95, 686–691 (2003).
22. Dai, F.L.: Characteristics and Identification of Fungi, Science Press, Beijing. pp. 94–96 (1987).
23. Rajoka, M.I., Yasmeen, A.: Inuction, and production studies of a novel glucoamylase of *Aspergillus niger*, World J. Microbiol. Biotechnol., 21, 179-187 (2005).
24. Yu, X.B., Yun, H.S. Koo, Y.M.: Production of cellulase by *Trichoderma reesei* Rut C30 in wheat bran-containing media, J. Microbiol. Biotechnol., 8, 208–213 (1998).
25. Ge, X.Y., Zhang, W.G.: A shortcut to the production of high ethanol concentration from Jerusalem artichoke tubers, Food Technol. Biotechnl., 43, 241-246 (2005).
26. Atiyeh, H., Duvnjak, Z.: Production of fructose and ethanol from cane molasses using *Saccharomyces cerevisiae* ATCC 36858, Acta Biotechnol., 23, 37–48 (2003).