Pitch Control of Recycled Whitewater from Papermaking by *Aspergillus oryzae*

Liangliang Sui,[a] Guanglei Zhao,[a,*] and Xiaofeng Li [b,**]

Whole cells of *Aspergillus oryzae* 35 were used as a biocatalyst for the degradation of glycerol trioleate, which largely contributes to pitch deposits in papermaking. Different types of inducers in culture media showed various effects on both the biomass and lipase activity of whole-cell biocatalysts. The cells of *A. oryzae* 35 cultured with Tween 80 showed higher catalytic activity than the others. The effects of several key factors on *A. oryzae* 35-catalyzed treatment of a glycerol trioleate-containing whitewater model were investigated, and the optimal pH value, reaction temperature, substrate concentration, and shaking speed were determined to be 7.5, 45 °C, 50%, and 200 rpm, respectively. Results from a practical treatment of whitewater resin sediments with *A. oryzae* showed that the mean particle size of the original whitewater was sharply reduced from 534 nm to 356 nm after biocatalytic degradation. *Aspergillus oryzae* whole-cells are newly promising biocatalysts for whitewater treatment in papermaking industries because of their cost-effectiveness, simple preparation, and environmental friendliness.

Keywords: Glycerol trioleate; Aspergillus oryzae; Pitch trouble; Whitewater; Degradation; Whole-cell; Biocatalyst

Contact information: a: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, China; b: Laboratory of Applied Biocatalysis, School of Light Industry & Food Science, South China University of Technology, Guangzhou 510640, China;
** Corresponding author: glzhao@scut.edu.cn*

INTRODUCTION

Whitewater recycling is an important step in the papermaking process that can save fresh water, decrease losses in fiber and chemical additives, save resources, and reduce production cost. It can also reduce the discharge of pollutants, thus benefiting the environment (He and He 2000). However, with recirculated whitewater being used more widely in the papermaking process, dissolved and colloidal substances (DCS) in chemical mechanical pulp have accumulated on the surface of equipment, resulting in considerable negative effects on the operation of machines and product quality (Yu and Deng 2004). These organic contaminants, sometimes called "pitch trouble," are mainly derived from the lipophilic extract of raw materials introduced by the pulping process (Chen 2004). In order to deal with this problem, various methods have been adopted in papermaking mills, including chemical and enzymatic treatments (Bobacka *et al.* 1999). In enzymatic methods, isolated hydrolases, especially lipases and esterases, are employed to degrade glycerol trioleate with high substrate specificity, thermostability, and alkali resistance, thus effectively reducing DCS accumulation (Hasan *et al.* 2006). For example, resinic material could be hydrolyzed into less viscous resin acids and alcohols by free lipase (Jaeger and Eggers 2002). Several trials in paper mills also confirmed that enzymatic methods were very effective and feasible for controlling the harmful composition of pitch in wood-

containing pulp. However, the high cost of these methods greatly limits their scale-up application in the paper-making industries.

Whole-cell biocatalysis utilizes enzymes naturally located in or exuded from the cells to catalyze reactions, which avoids the tedious separation and purification procedures for preparing an isolated enzyme and thus greatly reduces the cost of biocatalytic procedures. Besides, the whole-cell biocatalyst also possesses similar advantages to enzymes, such as high selectivity and mild reaction conditions. Hence, the use of microbial whole-cells instead of enzymes has brighter practical prospects (Toida *et al.* 1998). Among several microorganisms available, *Aspergillus oryzae* is a promising host because of its high protein productivity and capability in using cheap culture materials. The culture media components can support the production of several types of hydrolase and the expression of lipases on the cell wall, cell membrane, and cytoplasm of *Aspergillus oryzae*, which can usually be induced by fatty acids and their derivatives. The whole-cells from *Aspergillus oryzae* strains have been used as a new type of biocatalyst in several fields, such as biodiesel production (Adachi *et al.* 2013) and polymer degradation (Liu *et al.* 2012a). Until then, no research had been reported in the treatment of pitch troubles by a whole-cell biocatalyst in the paper- and pulp-making processes.

In this research, the catalytic capability of whole-cell biocatalyst from *A. oryzae* was explored for the treatment of circulated whitewater using a factory-simulation system. In addition, several key factors affecting the whole-cell mediated reaction were investigated. A comparative evaluation was also made between free/immobilized lipases and whole-cells.

EXPERIMENTAL

Materials

Aspergillus oryzae was purchased from the microorganism research institution of the Chinese Academy of Sciences. Glycerol trioleate was purchased from Jingchun Reagent Co., Ltd. (Shanghai, China). Polyvinyl alcohol was obtained from Runjie Chemical Reagent Co., Ltd. (Shanghai, China). Olive oil was obtained from National Pharmaceutical Reagent Co., Ltd. (Shanghai, China). Novozym435 was obtained from Novozymes (China) investment Co., Ltd. (Guangzhou, China). Purified lipase was obtained from Mingyao Trading Co., Ltd. (Guangzhou, China). Whitewater samples were provided by a Chinese Paper Mill producing alkaline peroxide-bleached mechanical pulp. All reagents were obtained commercially and were of analytical grade.

Methods

Preparation of the whole-cell biocatalyst

The strains were maintained on potato-dextrose-agar slants at 28 °C for 3 days. The spores from the slants were dispersed in 50 mL sterile water. Then a 3%(v/v) spore suspension (1×10^8/L) was inoculated into a culture medium containing ($g.L^{-1}$) $(NH_4)_2SO_4$ 5.0, K_2HPO_4 1.0, $MgSO_4\cdot7H_2O$ 0.2, Tween 80/glucose 5.0, and yeast extracts 1.0. Namely, 3 mL of suspension was added to 100 mL of culture medium. To obtain the whole cell biocatalyst, the cultivation was then carried out in 500-mL flasks containing 100 mL culture media in a rotary shaker at 30 °C and 180 rpm for 3 days. The fungal cells were harvested by filtration to remove the supernatant, washed twice with distilled water, freeze-dried at 30 °C for 24 h, and then stored at 4 °C (Feng *et al.* 2015).

Assays of lipase activity of whole-cell biocatalyst

The lipase activity of the whole-cell biocatalyst was assayed by measuring the amount of free fatty acid content liberated from the hydrolysis of olive oil (Abramic *et al.* 1999).

Preparation of white water simulant

A certain amount of glycerol trioleate was mixed with 4% polyvinyl alcohol (PVA) solution at a volume ratio of 3:1. Then the mixture was dispersed by a high-speed dispersion machine twice (each time for 3 min, at intervals of 5 min).

General procedure for whole cell-treated glycerol trioleate solutions

Lyophilized powder (0.3 g) of *Aspergillus oryzae* was added into a 30-mL mixture of glycerol trioleate emulsion and phosphate buffer (1:1, v/v). The reaction was then carried out at a certain temperature for 3 h with vigorous shaking. Samples were taken out and measured every 5 min, and the concentration of product oleic acid was determined according to the following equation (Eq. 1),

$$C\ (\text{mol.L}^{-1}) = (V_1 - V_2) \times 0.005 \times 1000 \div V_0, \quad (1)$$

where C is the oleic acid concentration; V_1 (mL) is the NaOH solution of the sample consumption; V_2 (mL) is the NaOH solution of the blank consumption; and V_0 (mL) is the volume of the samples.

Glycerol trioleate degradation by Aspergillus oryzae, free and immobilized lipase

Fifteen mL of glycerol trioleate emulsion was mixed with 15 mL of phosphate buffer. The reactions were initiated by the addition of 0.5 g of lyophilized powder of *Aspergillus oryzae*, immobilized Novozym435, or purified lipase and kept for 3 hours at a certain temperature (45 °C for the cells and immobilized enzyme, 50 °C for the purified lipase) with vigorous shaking.

Practical treatment of whitewater resin sediments with Aspergillus oryzae

To investigate the practical effect of industrial whitewater after treatment with *A. oryzae*, 0.1 g lyophilized powder of lipase-expressing cells was added into 5 mL industrial whitewater and the reaction was conducted at 45 °C for 3 h. Then, the mixture was centrifuged at 3000 rpm to remove the biocatalyst, and the size of the deposit was determined by a laser particle analyzer.

RESULTS AND DISCUSSION

Effect of Inducers on the Catalytic Activity of *Aspergillus oryzae* Strains

The catalytic activity of a whole-cell biocatalyst greatly depends on the culture conditions, since the enzymes are synthesized when the cells are growing. In this research, a typical inducer (glucose or Tween 80) was added into the culture media, respectively, and their effects on two different *Aspergillus* strains, *Aspergillus oryzae* 34 and *Aspergillus oryzae* 35, were investigated.

Table 1 shows that the cells cultured under different inducer-containing media showed quite different values of biomass concentration and lipase activity. *A. oryzae* 35 induced by Tween 80 showed the highest lipase activity (up to 106.32 U.g^{-1}), which meets

the necessary required treatment of whitewater pitch deposits in papermaking. Tween 80 is one type of triglyceride derivative that can sustainedly release oleic acid, which acts as an inducer for lipase production in filamentous fungi (Hama *et al.* 2006).

Table 1. Effect of Different Inducers on Biomass and Lipase Activities of the Cells from *A. oryzae* Strains

Inducer	Strains	Biomass[a] (10^{-3}g.mL^{-1})	Lipase Activity (U.g^{-1})
Glucose	*A. oryzae*35	1.43±0.1	21.0±2
Glucose	*A. oryzae*34	1.74±0.1	55.0±3
Tween 80	*A. oryzae*35	2±0.2	106.9±5
Tween 80	*A. oryzae*34	2±0.2	47.0±3
[a] dry weight of the cells			

Effect of Reaction Temperature on Degradation of Glycerol Trioleate Catalyzed by *Aspergillus oryzae*

Temperature plays an important role in a biocatalytic reaction and affects not only the thermodynamics equilibrium but also the enzymatic activities. Generally, the temperature of whitewater in a paper mill ranges from 50 to 60 °C. Hence, the effect of reaction temperatures in a range of 45 to 65 °C on the degradation of a simulated whitewater was tested. As illustrated in Fig. 1, glycerol trioleate was rapidly degraded within 10 min at 45 °C, giving oleic acids up to nearly 10 mmol.L^{-1}. When the reaction time was further increased to 3 h, oleic acid concentration was increased slowly. When the reaction temperature was increased from 45 to 65 °C, the concentration of the reaction product decreased from 20.0 mmol.L^{-1} to 7.5 mmol.L^{-1}, indicating the partial thermal inactivation of the enzyme in the cells. The results also confirmed that the whole-cells may be employed even under high temperatures in whitewater recycling.

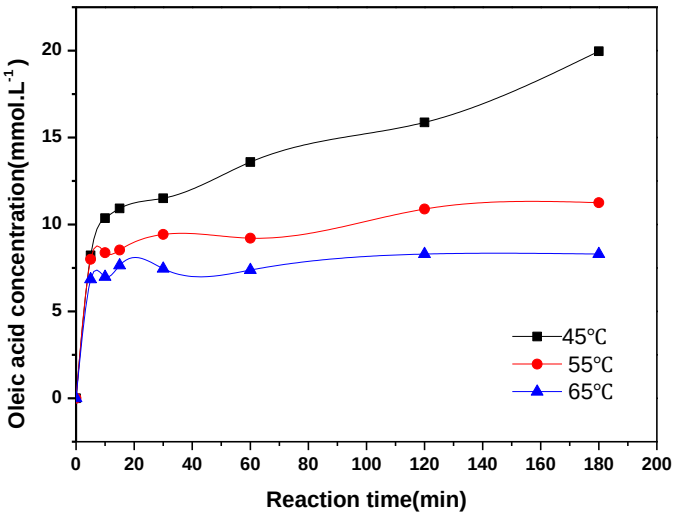

Fig. 1. Effect of reaction temperature on the decomposition of glycerol trioleate with *A. oryzae* 35 whole-cell biocatalyst in buffer pH 7.5

Effect of Reaction pH on the Degradation of Glycerol Trioleate by *Aspergillus oryzae*

For the whole-cell catalyzed process, pH is one of the dominant factors influencing the reaction (Ko *et al.* 2012), which may affect the enzymes located on the cells, and also influence the morphology of the cells. Figure 2 shows the influence of pH on the reaction system of the degradation of glycerol trioleate emulsion by *Aspergillus oryzae*. The cells showed much lower activity when the pH was changed from 7.5 to higher values (*e. g.*, 8.5 and 9.5). At pH 7.5, oleic acid concentration increased, and it reached the highest value (25 mmol.L^{-1}) when the reaction time was increased to 2 h. After 2 h, the oleic acid concentration increased slowly, mainly because of the decrease in the contacting interface between catalyst and substrate caused by substrate consumption.

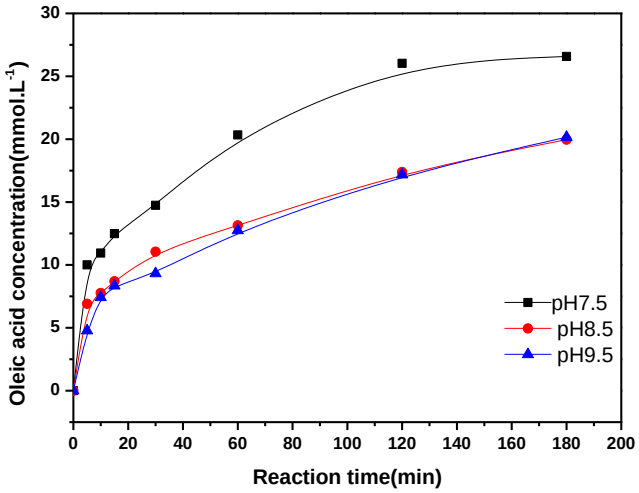

Fig. 2. Effect of reaction pH on the degradation of glycerol trioleate with *A. oryzae* 35 whole-cell biocatalyst at 45 °C

Effect of Substrate Concentration on the Degradation of Glycerol Trioleate by *Aspergillus oryzae*

Enzymes inside cells are in a naturally protected environment, and they are often more stable than isolated enzymes (Li *et al.* 2008; De Carvalho 2011). However, when whole cells are used as catalysts, the substrates must transfer into the cells to reach the active center of the enzymes, which decreases the overall reaction rate compared with the cases using purified enzymes. The effect of substrate concentration on the catalytic behaviors of the lyophilized whole-cells of *Aspergillus oryzae* in the degradation of glycerol trioleate is shown in Fig. 3.

In a whitewater stimulant system with a glycerol trioleate concentration of 16%, the product concentration increased significantly with increasing reaction time, and the maximum concentration of oleic acid reached 20 mmol.L^{-1}. When the substrate concentration was increased from 16% to 84%, the initial reaction rate increased significantly and the maximum concentration of oleic acid at 3 h increased to 50 mmol.L^{-1}. Hence, a high substrate concentration could raise the catalytic efficiency of whole cells of *Aspergillus oryzae* due to the relatively higher possibility of collision between substances and enzymes. The existence of an appropriate amount of water in this reaction system can form the hydration shell around the enzyme molecule, which maintains the stability and flexibility of the enzymatic structure (Iyer and Ananthanarayan 2008).

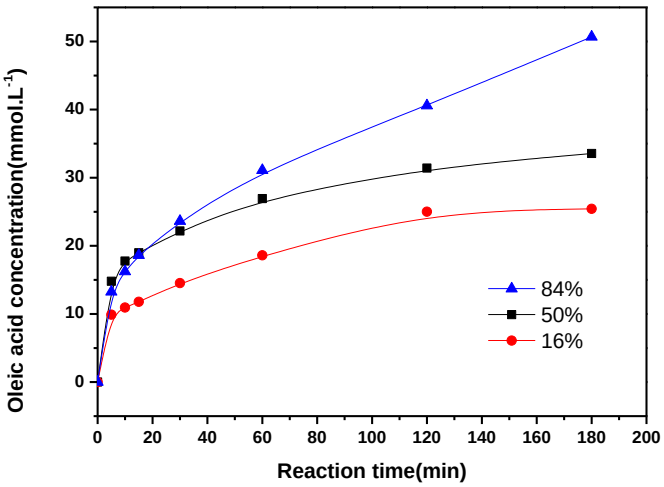

Fig. 3. Effect of substrate concentration on the degradation of glycerol trioleate with *A. oryzae* 35 whole cell biocatalyst at 45 °C

Effect of Shaking Speed on the Degradation of Glycerol Trioleate by *Aspergillus oryzae*

The rate of conversion was consistent with catalytic efficiency. The highest catalytic efficiency of the biocatalytic reaction was achieved at the optimal shaking speed, since suitable shaking may lead to sufficient contact between substrate and biocatalyst (Wang and Zhan 2001). Figure 4 shows only a small effect of shaking speed on the behavior of the whole-cell biocatalyst within the range of 200 to 600 rpm, indicating that the lipases were barely disturbed with shaking speed.

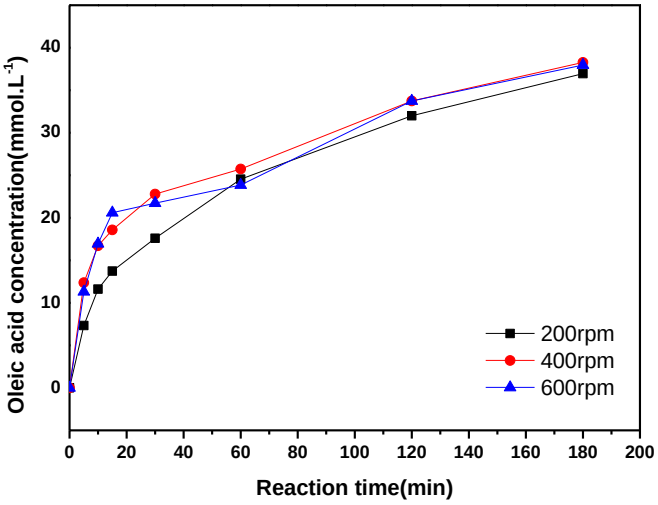

Fig. 4. Effect of shaking speed on the decomposition of glycerol trioleate with *A. oryzae* 35 whole-cell biocatalyst at 45 °C

To further study the potential of the whole-cell biocatalyst in control of pitch trouble in papermaking processes, its catalytic activity was investigated and compared with free lipase and immobilized lipase. The relative activity of each catalyst was determined by measuring the quantity of oleic acid degraded from the substrate.

Table 2 shows that the catalytic activity of the whole-cell biocatalyst was similar to that of the immobilized lipase; however, it was much less than that when free lipase was used. The whole-cell biocatalyst exhibited good acclimatization to the reaction system, performing the same as immobilized lipase in terms of its capacity to hydrolyze glycerol trioleate under the conditions above. The system is hardly reusable for free lipase, while easier for whole-cell biocatalyst or immobilized lipase. Usually, free enzymes are extracted from whole cells by separation and purification. Considering its low cost and simple operability, whole-cell biocatalyst is a promising option for the large-scale application of whitewater treatment.

Table 2. Biocatalytic Degradation of Glycerol Trioleate by *Aspergillus oryzae* and Free and Immobilized Lipases

Catalyst	V_0[a] ($mmol.L^{-1}.min^{-1}$)	Oleic Acid Concentration[b] ($mmol.L^{-1}$)
A. oryzae	13.57	67.8
Immobilized lipases	15.06	75
Free lipase	24.5	180

[a] initial reaction rate
[b] maximum concentration of oleic acid

Each reaction was carried out under the optimum conditions for the biocatalyst used (*Aspergillus oryzae*: pH 7.5, 45 °C, 200 rpm; free lipase: pH 7.5, 50 °C, 200 rpm; immobilized lipase: pH 8.5, 45 °C, 200 rpm).

Practical Treatment of Whitewater Resin Sediments with *Aspergillus oryzae*

High contents of glycerol trioleate in whitewater can form large particles, causing pitch problems (Fisher and Messnerk 1992). The particle size distribution of whitewater can indirectly reflect the degradation of glycerol trioleate and stickies detachment of the particle surfaces mixing into whitewater in production. Hence, the particle sizes of the original whitewater from a local mill and the whole-cell treated whitewater were determined and compared.

As depicted in Fig. 5, the particle size of the original whitewater was mainly distributed in the range of 50 to 6000 nm, and the mean particle size of whitewater was determined to be 534 nm. After treatment with *A. oryzae*, the proportions between 50 nm and 6000 nm changed noticeably. The mean particle size of whitewater was sharply reduced to 356 nm.

It can be inferred that the whole-cell biocatalyst can have a significant effect on the removal of resin sediments. A possible reason is that most resin sediments are connected with ester bonds, which can be cleaved and decomposed into fatty acids and glycerol with a lower viscosity (Liu *et al.* 2012b). The formation of resin sediments was lowered when bigger particles were degraded into smaller ones. Since large particles are more easily deposited on exposed parts of the paper machine, the reduction in particle size should decrease the incidence of equipment failure or occlusion of flow passages in the forming fabric or press-felts.

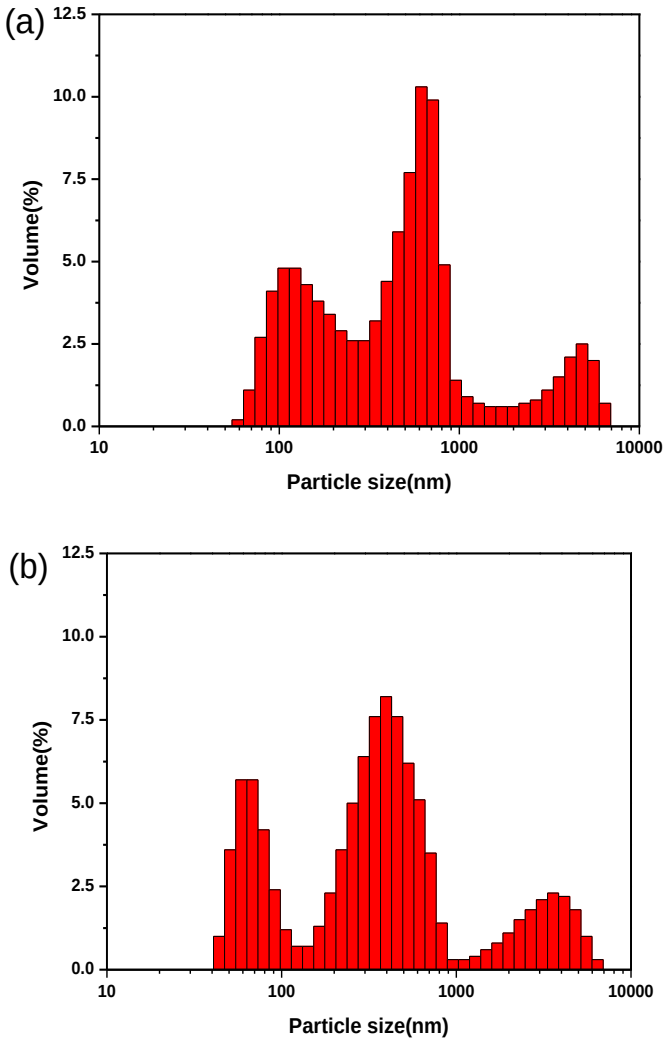

Fig. 5. Particle size distribution of (a) original whitewater; and (b) whitewater treated with *A. oryzae* at 45 °C

CONCLUSIONS

1. *A. oryzae* whole-cell biocatalyst was shown to be an excellent alternative catalyst for the degradation of resin that caused pitch trouble in the papermaking process.
2. This kind of biocatalyst offered significant advantages over conventional chemical treatment and costs less than the enzymatic process.
3. The preparation of a biocatalyst is a facile procedure requiring no complex purification process, and the prepared whole-cells can be directly used as lipase containers. Improvements in whole-cell biocatalyst performances should be performed in order to increase their reactivity as well as resistance and stability to deactivation caused by complex operational circumstances.

ACKNOWLEDGMENTS

The authors are grateful to the National Natural Science Foundation of China (No. 31270636), the Natural Science Foundation of Guangdong Province (No. S2013010012486), and the Program for New Century Excellent Talents in University (NCET-12-0192) for financial support.

REFERENCES CITED

Abramic, M., Lescic, I., Korica, T., Vitale, L., Saenger, W., and Pigac, J. (1999). "Purification and properties of extracellular lipase from *Streptomyces rimosus*," *Enzyme Microbial Technology* 25(6), 522-529. DOI: 10.1016/S0141-0229(99)00077-0

Adachi, D., Koh, F., Hama, S., Ogino, C., and Kondo, A. (2013). "A robust whole-cell biocatalyst that introduces a thermo- and solvent-tolerant lipase into *Aspergillus oryzae* cells: Characterization and application to enzymatic biodiesel production," *Enzyme and Microbial Technology* 52(6–7), 331-335. DOI: 10.1016/j.enzmictec.2013.03.005

Bobacka, V., Kreutzman, N., and Eklund, D. (1999). "The use of a fixative in combination with cationic starch in peroxide-bleached TMP," *Journal of Pulp and Paper Science* 25(3), 100-104.

Chen, J. X. (2004). "Source, damage and monitoring of dissolved matters of whitewater," *Paper and Papermaking* 4, 11-15.

De Carvalho, C. C. C. R. (2011). "Enzymatic and whole cell catalysis: Finding new strategies for old processes," *Biotechnology Advances* 29(1), 75-83. DOI: 10.1016/j.biotechadv.2010.09.001

Feng, G., H. Wu, Li, X., Lai, F., Zhao, G., Yang, M., and Liu, H. (2015). "A new, efficient and highly-regioselective approach to synthesis of 6-O-propionyl-d-glucose by using whole-cell biocatalysts," *Biochemical Engineering Journal* 95, 56-61.

Fisher, K., and Messnerk, K. (1992). "Reducing troublesome pitch mills by lipolytic enzymes," *Tappi* 75(2), 130.

Hama, S., Tamalampudi, S., Fukumizu, T., Miurab, K., Yamajib, H., Kondob, A., and Fukuda, H. (2006). "Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production," *Journal of Bioscience and Bioengineering* 101(4), 328-333. DOI: 10.1263/jbb.101.328

Hasan, F., Shah, A. A., and Hameed, A. (2006). "Industrial applications of microbial lipases," *Enzyme and Microbial Technology* 39(2), 235-251. DOI: 10.1016/j.enzmictec.2005.10.016

He, B. H., and He, Y. Y. (2000). "Clean production and closed recycling of water in papermaking system—one of the researches on closed water systems in papermaking and 'zero effluent'," *China Pulp & Paper* 5, 43-47.

Iyer, P. V., and Ananthanarayan, L. (2008). "Enzyme stability and stabilization – Aqueous and non-aqueous environment," *Process Biochem.* 43, 1019-1032. DOI: 10.1016/j.procbio.2008.06.004

Jaeger, K. E., and Eggers, T. (2002). "Lipases for biotechnology," *Current Opinion in Biotechnology* 13, 390-397. DOI: 10.1016/S0958-1669(02)00341-5

Ko, H-.J., Bang, W-.G., Kim, K. H., and Choi, I-.G. (2012). "Production of p-acetaminophenol by whole-cell catalysis using *Escherichia coli* overexpressing bacterial aryl acylamidase," *Biotechnology Letters* 34(4), 677-682. DOI: 10.1007/s10529-011-0811-5

Li, N., Zong, M., and Ma, D. (2008). "Regioselective acylation of nucleosides catalyzed by," *European Journal of Organic Chemistry* 32, 5375-5378. DOI: 10.1002/ejoc.200800780

Liu, H. W., Xu, X. L., and Li, X. F. (2012a). "Research on intracellular lipase production of *Aspergillus oryzae* 35 induced by Tween 80," *Modern Food Science* 8, 911-914.

Liu, K., Zhao, G., He, B., Chen, L., and Huang, L. (2012b). "Immobilization of pectinase and lipase on macroporous resin coated with chitosan for treatment of whitewater from papermaking," *Bioresource Technology* 123, 616-619. DOI: 10.1016/j.biortech.2012.07.074

Toida, J., Arikawa, Y., Kondou, K., Fukuzawa, M., and Sekiguchi, J. (1998). "Purification and characterization of Triacylglycerol lipase from *Aspergillus oryzae*," *Bioscience Biotechnology and Biochemistry* 62(4), 759-763. DOI: 10.1271/bbb.62.759

Wang, X., and Zhan, H. (2001). "Technology progress of pitch control by biological methods," *China Pulp & Paper* 6, 58-63.

Yu, F., and Deng, Y. (2004). "The build of dissolved solids in closed white water systems," *Tappi* 3(8), 17-24.

Article submitted: June 24, 2015; Peer review completed: August 13, 2015; Revised version received: August 18, 2015; Accepted: August 29, 2015; Published: September 8, 2015.
DOI: 10.15376/biores.10.4.7232-7241