Appl Biochem Biotechnol (2008) 149:287–293
DOI 10.1007/s12010-007-8096-9

Potential Application of Alkaline Pectinase from *Bacillus subtilis* SS in Pulp and Paper Industry

Sonia Ahlawat · R. P. Mandhan · Saurabh Sudha Dhiman · Rakesh Kumar · Jitender Sharma

Received: 28 September 2007 / Accepted: 8 November 2007 /
Published online: 5 December 2007

Abstract Pectinase production from *Bacillus subtilis* SS was optimized under solid-state fermentation (5,943 U/g of dry bacterial bran). The pectinase produced was stable in neutral to alkaline pH range at 70 °C; therefore, the suitability of this pectinase in pulp and paper industry was investigated. The enzyme pretreatment process was optimized, and a pectinase dose of 5 IU/g of oven-dried pulp (10% consistency) at pH 9.5 temperature 70 °C after 150 min of treatment gave the best pretreatment to the pulp. An increase of 4.3% in brightness along with an increase of 14.8 and 65.3% in whiteness and fluorescence, respectively, whereas a 15% decrease in the yellowness of the pretreated pulp were observed. There was a 5.85% reduction in kappa number and 6.1% reduction in permanganate number along with a reduction in the chemical oxygen demand value. Significant characteristics showed by pectinase open new possibilities of application of this cellulase-free enzyme in the pulp and paper industry by reducing the negative environmental impact of chemicals apart from improving the properties of paper.

Keywords Cellulase-free alkaline pectinase · *Bacillus subtilis* · Solid-state fermentation · Pulp and paper

Introduction

Over the recent few years, there has been a tremendous increase in the awareness regarding the effects of pollution, and public pressure has influenced both industry and government. One such area is the pulp and paper industry where the quantities of raw materials processed are huge, as well as the use of naturally hazardous chemicals are also large [1]. With the advancement of biotechnology; enzymes have found their way into many new industrial processes. Demand is increasing to replace some traditional processes such as chemical bleaching with biotechnological processes involving microorganisms and

S. Ahlawat · R. P. Mandhan · S. S. Dhiman · R. Kumar · J. Sharma (✉)
Department of Biotechnology, Kurukshetra University, Kurukshetra 136119, India
e-mail: jksharmakuk@rediffmail.com

enzymes such as pectinases [2], xylanases [3], and cellulases [4], which not only provide an economically viable alternative but are also more environmental friendly [5].

During the production of paper, pulping is the step in which cellulose fibers are broken apart and most of the lignin is removed. The residual lignin is then removed by a multistep bleaching process [4]. Viikari et al. [6] discussed the effect of pulping on the hemicellulose component of the plant biomass subjected to kraft pulping. Biological bleaching is carried out using lignolytic [7] or by using hemicellulolytic enzymes [8]. Once this layer of hemicellulose is removed, the lignin layer is easily available for the degradative action of the lignolytic enzymes [9]. As a result, reduced amounts of chlorinated compounds of lignin are discharged as effluent, causing less environmental pollution and damage.

No report is published so far on the efficacy of pectinase pretreatment for biobleaching. With the advancement of biotechnology and increased reliance of pulp and paper industries on the use of microorganisms and their enzymes for biobleaching and papermaking, the use of enzymes other than xylanase and ligninase, such as pectinase, mannanase, and galactosidase is increasing in the pulp and paper industries in many countries [4, 10]. During papermaking, pectins can depolymerize polymers of galacturonic acids and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching [5, 11]. The Japanese published patent application 2-1118191 by Jujo Paper discloses treating mechanical pulp with pectinase to degrade pectins on the fibers thus weakening the bond between lignin and cellulose and further refining the pulp before bleaching. It has been found that bleached or alkaline-treated pulps contain a substantial amount of harmful pectins. By incorporating pectinase in the bleached or alkaline-treated pulp, such harmful pectins in the aqueous phase of the pulp are degraded and thus rendered harmless to the papermaking process.

The application of an alkali- and thermostable cellulase-free pectinase for large-scale use in pulp biotechnology requires efforts that are aimed at process optimization, simplification, and cost reduction. Pretreatment of paper pulp requires the use of cellulase-free pectinase, as cellulase destroy the structure of cellulose and thus diminish the quality of pulp. In the present study, we are reporting the production of alkali-thermostable and cellulase-free pectinase by *Bacillus subtilis* SS using cheap agricultural residues under solid-state fermentation (SSF) and its suitability for the pulp and paper industry. We pay particular attention to the optimization of various parameters such as enzyme dose, pH, retention time, and temperature using pectinase with a view to find their role in the effective biobleaching process.

Materials and Methods

Microorganism and Culture Conditions

The bacterial strain used in the present investigation was isolated from a sanitary landfill and identified as *B. subtilis* SS by IMTECH, Chandigarh, India, and has been given MTCC accession no. 8509. For pectinase production in SSF, to 10 g of wheat bran, 20 ml mineral salt solution (g/l: $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.5; pH 7.0) was added and after autoclaving inoculated with 10% (v/w) inoculum of an overnight culture and incubated in a humidified incubator at 37 °C for 72 h. Thereafter, enzyme was extracted twice with 10 mM glycine phosphate buffer pH 9.5 (100 ml for 10 g of substrate). The enzyme extract was centrifuged at 10,000×*g* for 30 min at 4 °C, and the clear supernatant was used as a crude enzyme. Further, different parameters such as incubation period, moistening agent, moisture level, inoculum size, and effect of additives were optimized for pectinase production under SSF.

Pectinase and Cellulase Assays

Pectinase and cellulase activities were determined by the dinitrosalicylic acid method [12]. Pectinase and cellulase assays were performed in the same way as described in our previous studies [13]. One unit of pectinase or cellulase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to galacturonic acid or glucose per minute. All the experiments were carried out independently in triplicates, and the results presented here are the mean of the three. Pectinase activity in SSF was expressed as U/g of dry substrate.

Optimization of Various Parameters for Pretreatment of Pulp

Kraft pulp used for pretreatment was obtained from Ballarpur Industries, Yamunanagar, Haryana, India, and pulp used in this process was a mixture of six different trees namely, eucalyptus rulla, small vaneer, bamboo, poplar, eucalyptus, and debarka bamboo hardwood. Different parameters such as enzyme dosage, pH, retention time, and temperature were optimized by carrying out the enzymatic treatment at 10% (w/v) pulp consistency in transparent plastic bags. Optimization of enzyme dose and retention time was performed by treating moistened unbleached pulp with varying doses of pectinase, ranging between 5 and 12.5 IU/g for variable time intervals from 60 to 180 min. For optimization of pH and temperature, experiments were conducted at different pH values ranging from 7 to 10 and at temperature varying from 55 to 70 °C. After pretreatment, the pulp samples were washed with distilled water, filtered and suction dried, and stored in plastic bags for the later determination of various properties. All the experiments were carried out in triplicates.

Pulp Properties

The enzyme-treated pulp was washed, and hand sheets were prepared under standardized pressure and air dried in a room with standardized light, humidity, and temperature, according to Technical Association of Pulp and Paper Industry (TAPPI) standard methods [14]. The brightness of the of the hand sheets was measured as %ISO (International Organization of Standardization) by reflectance at 457 nm with ISO Colourtech, USA, according to TAPPI protocol (T-452 om-87). The Kappa number, which estimates the lignin content, was determined by the reaction of pulp samples with acidified potassium permanganate (TAPPI method T236 cm-85), and permanganate number was estimated by reaction of pretreated pulp with acidified permanganate solution by using starch and potassium iodide as an indicator. The yellowness, whiteness, and fluorescence of pretreated pulp were also evaluated by ISO Colourtech at 457 nm (T 1216).

Results and Discussion

Highest titer of pectinase was obtained using wheat bran as substrate (3,711 U/g of dry bacterial bran [DBB]) when the culture was incubated at 37 °C for 72 h. Wheat bran is a complete nutritious feed for microorganisms having all the ingredients and remains loose even under moist conditions thereby providing good aeration and large surface area [15, 16], which can be used by microbes for growth and metabolic activity [17]. Pectinase yield further increased (4,763 U/g of DBB) when wheat bran was moistened with tap water (Cl^- 0.08%, Ca^{++}, Mg^{++} 0.5%, HCO_3^- 0.4%) at a substrate-to-moisturizing agent ratio of 1:2.5 (w/v). An inoculum level of 15% (5,119 U/g of DBB) was optimized for pectinase production. When inoculum

size of 20% was used, enzyme production declined (4,901 U/g of DBB), which could be due to competition for nutrients among the bacterial population, as observed in *Thermomucor indicae seudaticae* [18]. Addition of yeast extract resulted in maximum increase in pectinase activity (5,943 U/g of DBB), while supplementation of glucose resulted catabolite repression in wheat bran (2,476 U/g of DBB). Similarly, pectinase production by *A. niger* on pectin supplemented with glucose repressed the enzyme [15].

Optimization of Various Parameters for Enzyme Controlled Treatment

No cellulase activity was detected in the pectinase produced from *B. subtilis* at different pH and enzyme dilutions, which make it suitable for application in the pulp and paper industry. Various parameters such as enzyme dosage, retention time, pH, and temperature were optimized to obtain the same %ISO brightness as it was achieved through the conventional chemical bleaching process. It is simple to adjust the pH in industries but difficult and expensive to control the temperature because of the cost of cooling. Therefore, to make the large-scale operations more simple and cost effective, the enzymes used should have higher pH and temperature stability [1]. Pectinase extracted from *B. subtilis* showed stability over broad range of pH 7–10 and temperature range of 55–70 °C. A retention time of 150 min and enzyme dosage of 5 IU/g of dry pulp was found suitable for pretreatment after optimization. Enzyme dose and retention time beyond this value did not enhance the efficiency of pretreatment. However, maximum enzyme efficiency was obtained at pH 9.5 and at a temperature of 70 °C. High activity and stability of the enzymes at neutral and alkaline pH are important prerequisite for the effect of kraft pulps. Moreover, the pH and temperature optima of commercially available enzymes range from 3 to 8 and from 30 to 75 °C, respectively [19]. Using optimized parameters and careful blending of pulp and enzyme preparation, a biobleaching process can be developed that will enable the production of pulps with less chlorine consumption.

Pretreatment of Kraft Pulp with Enzyme

To obtain the best results with enzymatic pretreatment, parameters like enzyme dosage, retention time, pH, temperature, and pulp consistency must be optimized to obtain effective dispersion of enzyme [4]. The maximum pretreatment efficiency of cellulase-free pectinase was observed when pulp (50 g oven dried) with 10% consistency was treated with an enzyme dose of 5 IU/g at 70 °C and at pH 9.5 after 150 min of incubation under optimized conditions in transparent plastic bags. There was a 5.85% reduction in kappa number and 6.1% reduction in permanganate number. After enzymatic treatment of unbleached kraft pulp, a 4.3% increase in brightness was obtained with respect to the control (Fig. 1) Montiel et al. [20] reported the reduction in pulp kappa number by 0.7 U and an enhancement in pulp brightness by 1.7%ISO after enzymatic treatment followed by alkaline extraction by using endo-β-mannanase produced by *Streptomyces ipomoea* CECT 3341. Pectinase treatment caused an increase of 14.8 and 65.3% in whiteness and fluorescence, respectively, and a decrease of 15% in yellowness of the pulp was observed as compared to the untreated control pulp (Fig. 2). Earlier, this pectinase enzyme from *B. subtilis* SS in combination with xylanase increased the whiteness and fluorescence by 20 and 84%, respectively [13]. The enzymatic treatment of unbleached pulp efficiently reduced the chemical oxygen demand (COD; %) value of the effluent (14 kg/ton) up to 12.5% as compared to the control (16 kg/ton). The reduction in COD value is highly appreciable in terms of reduction in environment pollution, which is caused because of the release of waste effluents from the

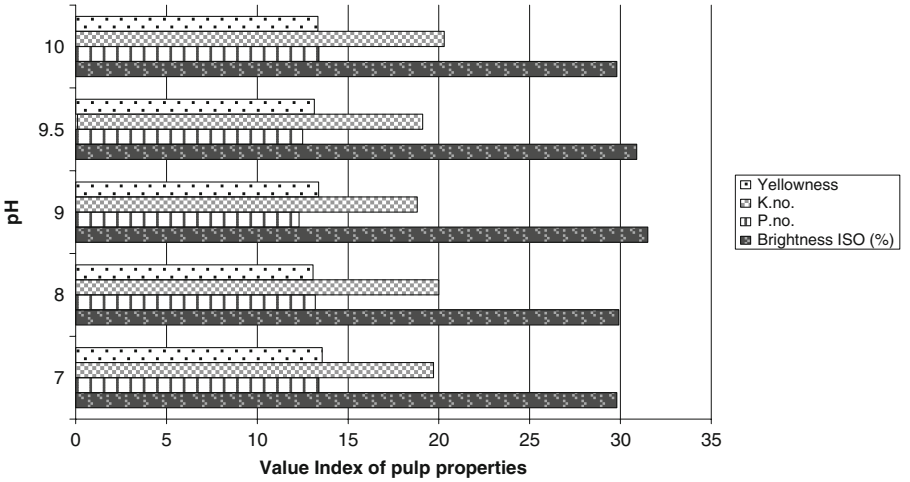

Fig. 1 Effect of different pH on yellowness, K. no., P. no., and brightness ISO (%) by pretreatment of pulp. All these experiments were performed at constant enzyme dose of 5 IU/g and 10% pulp consistency, at temperature 70 °C and at retention time of 150 min

paper industry into the surroundings. Significant properties showed by pectinase open new possibilities of application of this enzyme in the pulp and paper industry. Earlier, Beg et al. [21] reported the overall bleach boosting of eucalyptus kraft pulp with alkaline pectinase from *Streptomyces* sp. QG-11-3 in combination with xylanase from the same organism for biobleaching. The application of xylanases for improvement in pulp bleaching has been reported by several workers by using xylanase from *Streptomyces cuspidosporus* [22], *Bacillus licheniformis* [23], *Streptomyces* sp. strain S38 [24], and *Bacillus pumilus* [25], but

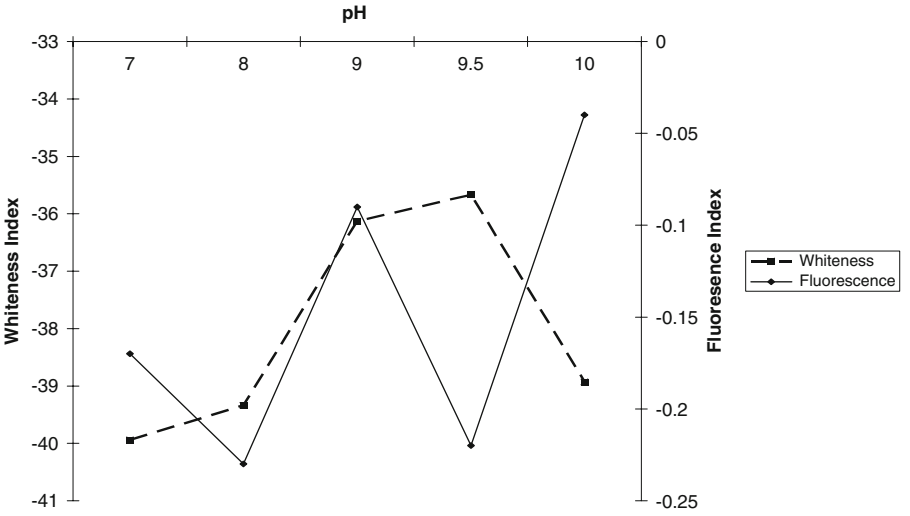

Fig. 2 Effect of different pH on whiteness and yellowness by pretreatment of pulp. All these experiments were performed at constant enzyme dose of 5 IU/g and 10% pulp consistency, at temperature 70 °C ad at retention time of 150 min

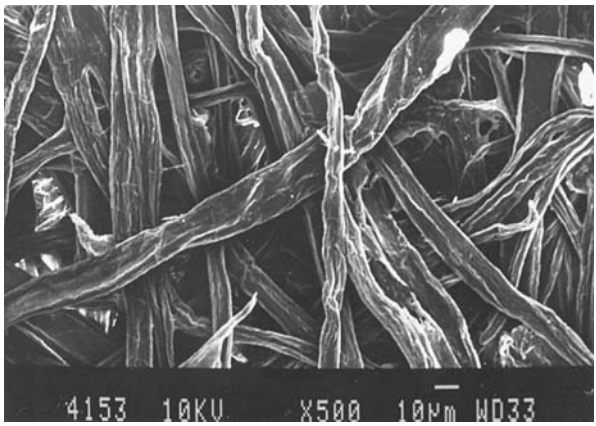

Fig. 3 Scanning electron micrograph of unbleached (control) eucalyptus kraft pulp (×500)

there is little information available about application of pectinase in the pulp and paper industry.

Scanning electron microscopic studies revealed that pectinase from *B. subtilis* introduced greater porosity, swelling up, and separation of pulp microfibrils and pulp fibers (Fig. 3) compared to smooth surfaces of untreated pulp (Fig. 4). When the pulp fibers were subjected to enzymatic treatment, swelling, separation, and loss in compactness in the pulp fibers were observed. The enzymatic treatment of pulp renders the pulp fibers more accessible to the chemical bleaching agents, thereby reducing the requirement of chlorine and chlorine compounds in the subsequent bleaching process [26].

Conclusion

Improvement in process economics and realistic cost estimates are the important factors that play a major role in the commercial success of any technology. Currently, in the paper industry, research is directed toward the discovery of enzymes that are more robust with respect to pH and temperature kinetics. Pectinase produced from *B. subtilis* SS is cellulase-free and has wide range of thermal and pH stability, which are well suited for the pulp and

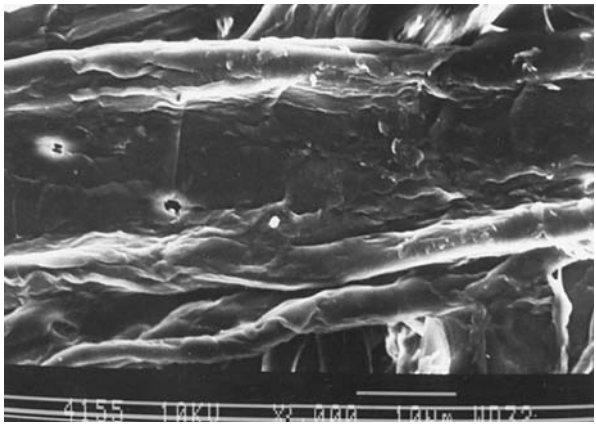

Fig. 4 Scanning electron micrograph of eucalyptus kraft pulp after pretreatment (×2,000)

paper industry. The cost-effective agricultural residue wheat bran is used under SSF to achieve higher pectinase yield, thereby reducing the cost of enzyme production, thus facilitating the adaptation of this environment-friendly technology in the paper industry. Pretreatment of pulp with pectinase enhanced the desired properties of pulp to a significant level. Efforts are continuing to explore the potential of pectinase in the prebleaching as well as in bleaching stages to reduce the chlorine/chlorine dioxide requirement in papermaking.

Acknowledgment The authors thank Ballarpur Industries (BILT), Haryana, India, for providing kraft pulp and laboratory facilities. Sonia Ahlawat greatly acknowledges the financial assistance provided by Kurukshetra University, Kurukshetra, in the form of University Research Scholarship during the course of the investigation.

References

1. Srinivasan, M. C., & Rele, M. V. (1999). *Current Science*, *77*, 137–142.
2. Bruhlmann, F., Leupin, M., Erismann, K. H., & Fiechter, A. (2000). *Journal of Biotechnology*, *76*, 43–50.
3. Beg, Q., Bhushan, B., Kapoor, M., & Hoondal, G. S. (2000). *Enzyme and Microbial Technology*, *27*, 459–466.
4. Bajpai, P. (1999). *Biotechnology Progress*, *15*, 147–157.
5. Viikari, L., Tenkanen, M., & Suurnakki, A. (2001). *Biotechnology* (2nd ed.). New York: Wiley.
6. Viikari, L., Ranua, M., Kantelinen, A., Sundquist, & Linko, M. (1986) In: Proceedings of the Third International Conference on Biotechnology for the pulp and paper industry, Stockholm, pp 67–69.
7. Onysko, K. A. (1993). *Biotechnology Advances*, *11*, 179–198.
8. Gubtiz, G. M., Lisching, T., Stebbing, D., & Saddler, J. N. (1997). *Biotechnology Letters*, *19*, 491–495.
9. Seelenfreund, D., & Vicuna, R. (1990). *Journal of Industrial Microbiology*, *5*, 17–24.
10. Kirk, T. K., & Jefferies, T. W. (1996). In T. W. Jeffereies, & L. Viikari (Eds.) *Enzymes for pulp and paper processing. ACS symposium series* pp. 1–14. Washington DC: American Chemical Society.
11. Reid, I., & Ricard, M. (2000). *Enzyme and Microbial Technology*, *26*, 115–123.
12. Miller, G. L. (1959). *Analitical Chemistry*, *31*, 426–428.
13. Ahlawat, S., Battan, B., Dhiman, S. S., Sharma, J., & Mandhan, R. P. (2007). *Journal of Industrial Microbiology and Biotechnology*, *34*(12), 763–770.
14. TAPPI Test Methods (1996). *Technical association of the pulp and paper industry.* Atlanta, GA: TAPPI.
15. Babu, K. R., & Satyanarayana, T. (1995). *Proceedings of Biochemistry*, *30*, 5–9.
16. Feniksova, R. V., Tikhomrova, A. S., & Rakhleeva, B. E. (1960). *Microbiologica*, *29*, 745–748.
17. Pandey, A., Selvakumar, P., Carlos, R. S., & Poonam, N. (1999). *Current Science*, *77*, 1153–1169.
18. Kaur, P., & Satyanarayana, T. (2004). *World Journal of Microbiology and Biotechnology*, *20*, 419–425.
19. Viikari, L., Kantelinen, A., Sundquist, J., & Linko, M. (1994). *FEMS Microbiology Reviews*, *13*, 335–350.
20. Montiel, M. D., Hernandez, M., & Rodriguez, J. (2002). *Applied Microbiology and Biotechnology*, *58*, 67–72.
21. Beg, Q. K., Kapoor, M., Mahajan, L., & Hoondal, G. S. (2001). *Research Bulletin of the Panjab University Science*, *51*, 71–78.
22. Maheshwari, M. U., & Chandra, T. S. (2000). *World Journal of Microbiology & Biotechnology*, *16*, 257–263.
23. Damiano, V. B., Bocchini, D. A., Gomes, E., & Da Siva, R. (2003). *World Journal of Microbiology & Biotechnology*, *19*, 139–144.
24. Georis, J., Giannotta, F., De Buyls, E., Granie, R. B., & Frere, J. M. (2000). *Enzyme and Microbial Technology*, *26*, 178–186.
25. Battan, B., Sharma, J., Dhiman, S. D., & Kuhad, R. C. (2007). *Enzyme and Microbial Technology*, *41*, 733–739.
26. Beg, Q. K., Bhushan, B., Kapoor, M., & Hoondal, G. S. (2000). *Enzyme and Microbial Technology*, *27*, 459–466.