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Screening of Pectinase Producing Bacteria and Their Efficiency in Biopulping of Paper Mulberry Bark

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ABSTRACT: Screening of pectinase producing bacteria and assessment of the effectiveness for biopulping of paper mulberry bark of the pectinase of the highest producer were carried out. Pectinolytic bacteria were initially screened from 6 identified and 118 unknown isolates. Twelve strains gave positive results, including 3 of *Erwinia carotovora* subsp. *carotovora*, 2 of *Erwinia chrysanthemi* and 7 of *Bacillus* sp.. Crude pectinases were prepared from the selected strains. Then, the activity of 3 pectinase types, namely polygalacturonase (PG), pectate lyase (PAL) and pectin lyase (PL), was investigated. The results showed the highest PG production from *E. chrysanthemi* strain N05 (342-11) isolated from onion and highest PAL and PL production from *Bacillus* sp. strain N10 isolated from paper mulberry bark. Both N05 and N10 possess similar optimum conditions at pH 10.0 and 35 °C, and were stable at pH 3-12 for 30 minutes and 20-40 °C for 24 h. Furthermore, the efficiency of *Bacillus* sp. strain N10 pectinase in biopulping of paper mulberry bark was studied. The bark was pretreated by soaking for 24 h in distilled water, 0.5% NaOH solution, or 1.0% NaOH solution or not pretreated. Then it was incubated with 20,000, 40,000, 100,000 and 200,000 units of enzyme solution and compared to water and NH_4Cl-NH_4OH buffer controls. Bark without any pretreatment, which was incubated with 100,000 unit of the enzyme had the maximum level of reducing sugar released, the softest pulp and fibers that were clearly separated when observed under the scanning electron microscope. Chemical composition analysis of the pulp showed slight increases in holocellulose 5%, alpha-cellulose (2%) and hemicellulose (3%) contents, whereas lignin content decreased by 50% when compared to the non-treated bark.

KEYWORDS: Pectinase, Bacteria, Biopulping, Paper mulberry bark.

INTRODUCTION

Pectinases are mixtures of several enzymes that act on pectic substances, which are structural polysaccharides in the middle lamella and primary cell wall of higher plants. Pectinases are important in processing fruits and vegetables and in wine production. Specifically, these enzymes are used for clarification of fruit juice, enzymatic fruit mash treatment, improvement of juice yield and color in wine production, improvement of extraction yield of oils from some plant tissues and maceration and liquefaction of plant tissue.

Handmade paper has been produced from paper mulberry in Thailand for over a hundred years. It is unique and represents the long history of Thai culture. However, modern technology has changed ways of life of people, including those in rural areas. Machinery and chemicals, commonly called new technology, are replacing the simple and environmentally friendly method of making handmade paper used in the past. Handmade paper production has become industrialized and mechanized. The products are consumed domestically and exported. In the conventional paper mulberry paper making process, 4 to 7% NaOH is used for pulping in order to soften the bark before the pulp is produced. This chemical is cheap, easy-to-buy and effectively produces pulp within a short period of time. However, the chemicals left in the effluent are very harmful and pollute the environment, especially when discharged into rivers or canals. Effluent from every step of the pulp and paper making process can be toxic to humans, animals and other living things around that area. To solve these

problems, some cleaner technologies have been developed to reduce or replace the use of chemicals.

Biopulping by microorganisms and/or enzymes is an alternative which interests many scientists, because the methods are environmentally friendly and have specific reactions which will produce high quality of pulp. Many kinds of enzymes are used in the pulp and paper industry, depending on the process. In biopulping there are many research reports on xylanase and ligninase. Pectinase has been reported mostly in producing fiber from flax, kenaf and a few annual fiber crops. Currently, high amounts and values of pectinases are imported to Thailand for many industries. As pectinases are useful in maceration of plant tissue, the idea of applying pectinase for production of high quality pulp and fiber was interesting. Thailand is a source of biodiversity, including many organisms that produce pectinases. The price of commercial food grade pectinases is around 3,800 Baht/l. Thus research and development of technology to produce enzymes, including pectinases has been initiated. This paper screened for bacteria producing pectinases. After that, the strains producing highest enzyme activities were selected, the optimum conditions for their activities and their stability were studied and the efficiency of the enzyme from one of the highest producers in biopulping of paper mulberry bark was preliminarily investigated.

Materials and Methods

Bacterial strains were from two sources. The first one was kindly provided from the stock culture collection of Dr. Surang Suthirawut (Dept. of Microbiology, Fac. of Science, KU). The other source was de novo collection from soil, water, effluent from a pulp and paper mill, paper mulberry bark, vegetables and fruits, etc. Pure strains of bacterial cultures were carried out by the dilution plate method and streak plate method. The three screening methods carried out were: screening on modified crystal violet-pectate (CVP) medium[1], screening on medium for pectolytic bacteria (MP)[2] and screening by bacterial ability to produce soft rot symptom on potato[3]. The strains selected by the above mentioned screens were transferred onto nutrient agar and kept in liquid paraffin, in sterile distilled water and freeze drying[4]. The methods of culture collection varied according to the species of the bacteria and their further applications. Selected bacterial strains were identified by their basic morphology and biochemical properties according to Bergey's Manual of Systematic Bacteriology[5].

The procedures for preparation of crude pectinases were described below: Bacterial stock cultures were prepared in nutrient broth, incubated at ambient temperature for 48 h with shaking. The cultures were diluted to a turbidity yielding an absorbance at 660 nm between 0.05-0.1 and then the turbidity was measured again to make sure that all of the cultures had the same amount of cells. Bacterial cultures were transferred into Basal Medium pH 8.0 (specific medium broth)[6] and incubated at ambient temperature for 48 h with shaking. Crude enzyme pectinases were extracted by centrifugation at 10,000 rpm, 4 °C for 15 min. The supernatant obtained was crude pectinase. Crude pectinases produced by selected bacterial strains were investigated for the activities of 3 pectinases, namely, polygalacturonase (PG)[7], pectate lyase (PAL)[8] and pectin lyase (PL)[8].

One unit of polygalacturonase (PG) corresponds to the amount of enzyme which decreases the viscosity of 4.0 ml of 2% pectin solution by 50% within 10 min at pH 10.0 and 35 °C in ostwald viscometer[7].

One unit of pectate lyase (PAL) corresponds to the amount of enzyme which lyses a 0.4% sodium pectate solution and releases products with an absorbance of 0.2 at 235 nm within 10 min at pH 10.0 and 35 °C[8].

One unit of pectin lyase (PL) corresponds to the amount of enzyme which lyses a 0.4% pectin solution and releases products with an absorbance of 0.2 at 235 nm within 10 min at pH 10.0 and 35 °C[8].

The optimum pH and temperature for pectate lyase (PAL) activity and stability were studied. The optimum pH for the activity of PAL was investigated in 0.1 M acetate buffer (pH 3.0, 4.0 and 5.0), 0.1 M phosphate buffer (pH 5.0, 6.0, 7.0 and 8.0) and 0.1 M NH_4Cl-NH_4OH buffer (pH 8.0, 9.0, 10.0, 11.0 and 12.0). The optimum temperature for their activity was investigated at 20, 25, 30, 35, 37, 40, 45, 50, 55 and 60 °C at the optimum pH obtained previously. To study enzyme stability at different pH values, crude PAL enzyme was mixed with various buffer solutions in order to obtain various pH levels. The mixtures were then incubated at 4 °C under ambient temperature for 30 min and 24 h. After that, the pH was adjusted to the optimum value and the activity of PAL was assayed. Crude PAL at their optimum pH (from previous step) was incubated at various temperatures for 30 min and 24 h to test temperature stability. After that, the activity of PAL was measured at 35°C .

The efficiency of crude pectate lyase enzyme in biopulping of paper mulberry bark was investigated. Paper mulberry bark was pretreated by soaking for 24 h in (1) distilled water, (2) 0.5% NaOH, and (3) 1.0% NaOH, and (4) non-soaked bark was used as control. After that, all of four pretreatments were incubated with PAL at concentrations of 20,000, 40,000, 100,000 and 200,000 units and compared with bark samples soaked in distilled water and NH_4Cl-NH_4OH buffer. The treated bark samples were then analysed for reducing sugar in the effluent after biopulping process[9],

fiber by visual and sensory estimation, morphology of fiber by observation under the Scanning Electron Microscope (SEM) and chemical properties of paper mulberry pulp[10].

Results and Discussion

From stock cultures there were 6 bacterial strains which were capable of producing pectinases. They were 3 strains of *Erwinia carotovora*, 2 strains of *Erwinia chrysanthemi*, and 1 strain of *Bacillus* sp. Eighty-four samples were collected from various sites and screened on nutrient agar to obtain from 118 bacterial isolates, as shown in Table 1.

Table 1. Number of bacterial colonies observed on nutrient agar derived from various sample sources.

Sources	Number of Sample	Number of isolated colonies
soils	12	19
effluent	17	19
wood bark	12	23
vegetables	15	26
fruits	18	20
ready-to-eat	10	11
Total	84	118

Only bacterial strains that showed positive results on CVP medium, MP medium and soft rot symptom on potato were selected for further study. There were a total of 12 strains, as shown in Table 2. The twelve selected bacterial strains were maintained by two methods. For short-term preservation, the bacterial cultures were inoculated on nutrient agar. For long-term preservation, the bacterial cultures were preserved in liquid paraffin or sterile distilled water or freeze dried. Bacterial codes N01- N03, N04 - N05 and N06 - N12 were identified as *E. carotovora* subsp. *carotovora*, *E. chrysanthemi* and *Bacillus* sp., respectively.

The crude enzymes from the 12 bacterial strains had different properties. Bacterial strain N05 showed the highest PG activity of 135.14 unit/ml, while bacterial strain N08 had the lowest PG activity at 1.06 unit/ml, as shown in Figure 1(a). Bacterial strain N10 showed the highest PAL activity at 16.07 unit/ml, while bacterial strain N11 showed the lowest PAL activity at 2.64 unit/ml, as shown in Figure 1(b). Bacterial strain N10 showed the highest PL activity at 4.48 unit/ml, while bacterial strain No12 showed the lowest PL activity at 0.60 unit/ml, as shown in Figure 1(c). Bacterial strains producing the highest amount of all these three pectinases were selected to study the optimum conditions and stability of their enzymes.

Table 2. Selected pectinase producing bacterial strains.

Code No.	Bacterial strains	Sample sources
N 01	*E. carotovora* subsp. *carotovora* (144-2)	radish
N 02	*E. carotovora* subsp. *carotovora* (486-7)	cauliflower
N 03	*E. carotovora* subsp. *carotovora* (N7129)	Japanese radish
N 04	*E. chrysanthemi* (329-21)	bean sprouts
N 05	*E. chrysanthemi* (342-11)	onion
N 06	unknown	soil from Khon Kaen
N 07	unknown	soil from Talingchun
N 08	unknown	effluent-paper mill
N 09	unknown	pine bark
N 10	unknown	paper mulberry bark
N 11	unknown	guinea-pepper
N 12	unknown	sweet potato

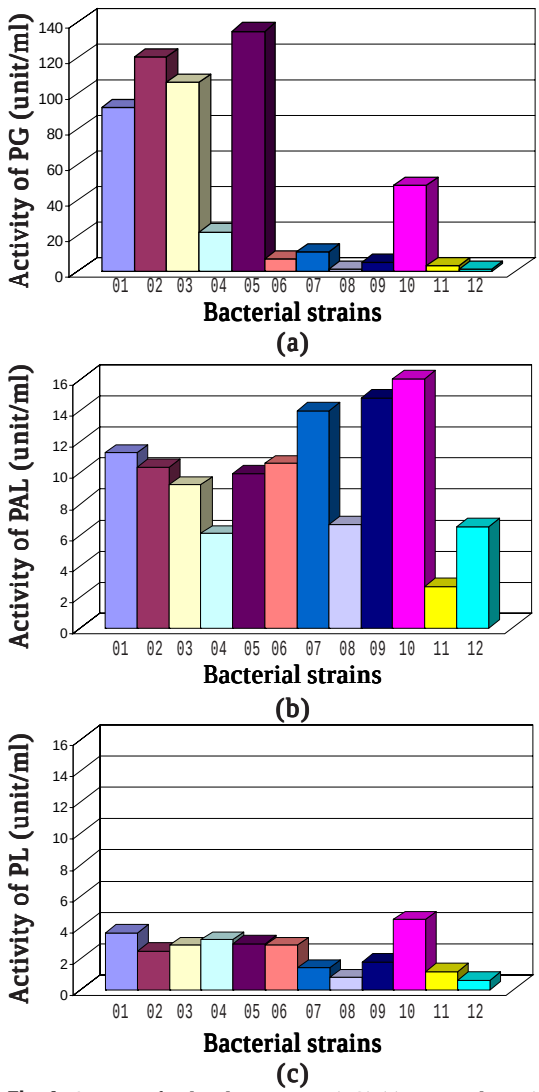

Fig 1. Activity of polygalacturonase (PG) (a), Pectate lyase (PAL) (b) and Pectin lyase (PL) (c) in unit/ml from bacterial strains N01-N12 when grown on basal medium with shaking at ambient temperature for 48 h.

Bacterial strains N05 and N10 were selected for crude enzyme preparation. Only pectate lyase (PAL) activity was assayed to determine the optimum conditions for their activities. PAL was selected for this study because of its tolerance in alkaline conditions,[11] which can reduce contamination from other microorganisms[12]. Moreover, Poonpairoj[13] applied PG in biopulping of paper mulberry bark and reported that the pulp had low tensile strength, could be easily torn, and the brightness of the pulp was slightly increased. Thus, we investigated the role of PAL in biopulping of paper mulberry bark.

PAL from bacterial strains N05 and N10 had the optimum pH at 10.0. They were stable at pH in the range of 3.0-12.0 and at 20-40 °C for 30 min and 24 h. The optimum temperature for PAL activity was at 35 °C and the range of temperature for high PAL activity was 20-40 °C.

The efficiency of crude pectate lyase enzyme for biopulping of paper mulberry bark was studied. Reducing sugars in the effluent obtained after soaking the pulp with various enzyme concentrations were analysed by the Nelson-Somogyi Method[9]. It was found that the effluent of the bark that was not soaked as pre-treatment and was soaked in 100,000 units of PAL showed the highest amount of reducing sugar, 7.21 mg/ml. The reason that reducing sugar from pre-treatments 1, 2 and 3 were lower than that of pre-treatment 4 where the bark was not soaked might be because after pre-treatment the bark samples were washed with water resulting in washing out the reducing sugar from those pre-treatments as well. Another reason might be that in all these pre-treatments the barks were soaked in alkaline solution and the residue from the alkaline solution might interfere with the activity of crude pectinase.

Results from visual and sensory estimation revealed that the texture of the pulp obtained from bark without pre-treatment soaked in 100,000 units of PAL was the softest and showed best separation of fiber, followed by that treated with enzyme at 200,000, 20,000 and 40,000 units, respectively, as shown in Figure 2.

Morphological study under a scanning electron microscope (SEM) revealed no clear difference of fiber separation among the 4 pre-treatments. However, after soaking the pretreated bark in various concentrations of enzyme, the fiber of the bark showed better separation. It was found that soaking the bark in 100,000 units of PAL gave the best fiber separation, followed by treatments with 200,000, 40,000 and 20,000 units of PAL, respectively, as shown in Figure 3. The bark that was pretreated with 0.5 and 1% NaOH showed the damage of fiber when compared to those treated with enzyme pectinase. Therefore, treatment with enzyme will offer high quality fiber and better quality of paper as end product.

An analysis of the chemical composition of the paper mulberry pulp without pretreatment incubated with 100,000 units of PAL showed increases of 5% in holocellulose, 2% in alpha cellulose and 3% in hemicellulose, while lignin decreased 50% when compared to dry bark without any treatment.

Fig 2. Paper mulberry pulp without pretreatment treated with crude PAL at various concentrations.

Conclusion

Pectinase producing bacteria identified in this study included 3 isolates of *Erwinia carotovora* sub sp. *carotovora*, 2 of *Erwinia chrysanthemi* and 7 of *Bacillus* sp. They are different in their ability to produce PG, PAL and PL. The results obtained here will be useful for further research to produce pectinases for domestic consumption for many industries. Moreover, treatment of paper mulberry bark with crude pectinase suggested the application of pectinases to produce high quality fiber from wood bark.

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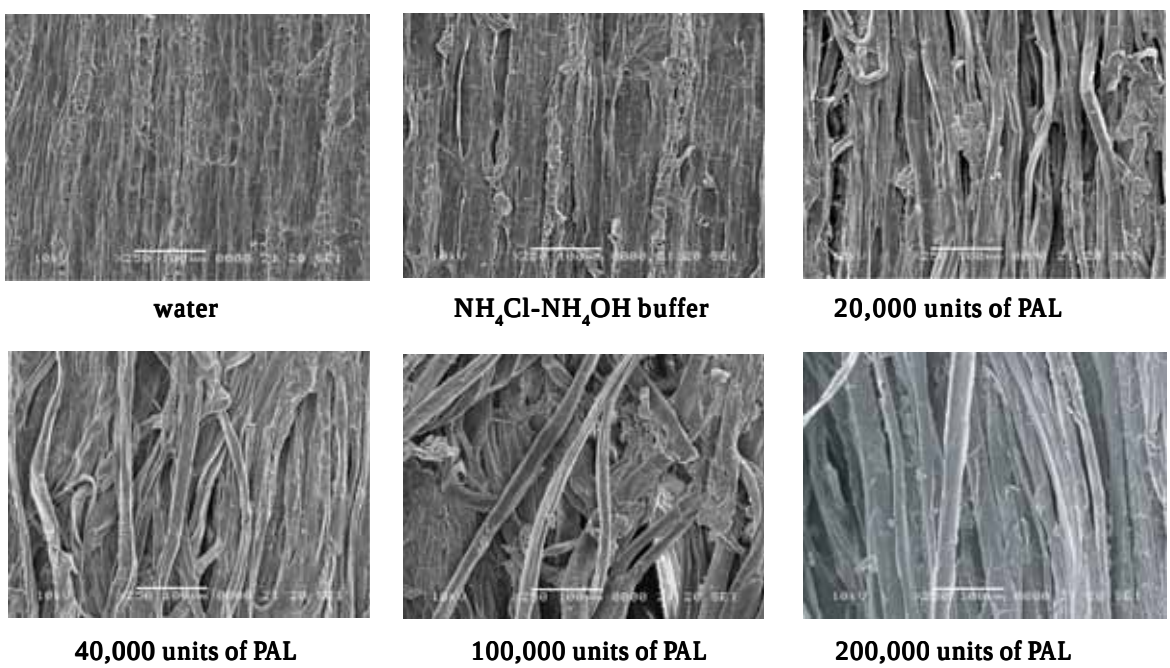

Fig 3. Paper mulberry pulp without pretreatments, followed by incubation with various concentrations of PAL observed under SEM 250X.