ISSN 1330-9862
(FTB-1684)

original scientific paper

Influence of Mode of Fermentation on Production of Polygalacturonase by a Novel Strain of *Streptomyces lydicus*

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Received: November 30, 2005
Accepted: March 22, 2006

Summary

Production of different pectinolytic enzymes was attempted using the actinomycete strain *Streptomyces lydicus* in submerged fermentation. Polygalacturonase and pectin lyase activities were detected in the culture supernatant, but the strain was not able to produce pectin esterase. Polygalacturonase production was studied in submerged, slurry-state and solid-state fermentation systems. All the experiments were carried out under static and shaking conditions. Solid-state fermentation under static condition was found to be promising. Various agroindustrial residues were tried as substrates for solid-state fermentation. Wheat bran was proved to be the best substrate.

Key words: *Streptomyces lydicus*, polygalacturonase, pectin lyase, pectin esterase, submerged fermentation, slurry-state fermentation, solid-state fermentation

Introduction

Pectic substances, present in the primary cell wall and middle lamella of higher plants, contribute to the firmness and structure of plant tissues (*1*). Different pectinolytic enzymes are involved in the breakdown of pectin and are widely distributed in higher plants and microorganisms. They are important for plants as they help in cell wall extension and fruit softening. They have a role in maintaining ecological balance by causing decomposition of plant materials.

According to the reaction mechanisms, pectinases can be classified as esterases, eliminative depolymerases (lyases) and hydrolytic depolymerases (polygalacturonases). Pectin esterase (E.C. 3.1.1.11) catalyses the hydrolysis of methylated carboxylic ester groups in pectin into pectic acid and methanol. Pectin lyases (E.C. 4.2.2.10) cleave α-(1,4)-glycosidic linkages by transelimination, which results in galacturonide with a double bond between C-4 and C-5 at the non-reducing end. Polygalacturonases are involved in the hydrolysis of α-(1,4)-glycosidic linkages in homogalacturonans. Endo-polygalacturonases (E.C. 3.2.1.15) bring about random hydrolysis of the polymer, whereas exo-polygalacturonases (E.C. 3.2.1.67) act sequentially from the non-reducing end.

The commercial preparations of pectinases are produced mainly from fungi, especially *Aspergillus niger* (*2*). Microbial pectinases account for 25 % of the global food enzyme sale (*3*). Applications of pectinases include fruit juice extraction and clarification, wine processing, oil extraction, coffee and tea leaf fermentation, retting and degumming of fibers, *etc*. (*4*).

Pectinases are produced by submerged (SmF) and solid-state fermentation systems (SSF) (*5–8*), whereas slurry-state fermentation (SLSF) has rarely been reported (*9*). Studies have been conducted on comparative production of pectinases in systems of SmF and SSF (*10*). SSF has several advantages over SmF system such as higher concentration of products, less effluent generation, requirement for simple equipments, *etc*. (*11*). Reports are very few on the comparison of SmF, SSF and SLSF for the production of pectinases. The present study involves the detection of different pectinolytic activities of a novel strain of *Streptomyces lydicus* and the compari-

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son of polygalacturonase production in the three different modes of fermentation. Effect of various agroindustrial residues on polygalacturonase production in SSF is also studied.

Materials and Methods

Microorganism

The actinomycete strain used in the present study was isolated earlier in this laboratory from mangrove sediment, collected from Cochin, India and was identified as *Streptomyces lydicus* (MTCC 7505) by IMTECH, Chandigarh, India. The culture was maintained on starch casein agar slants at 4 °C.

Preparation of inoculum

The medium used for inoculum preparation contained (in g/L): KH_2PO_4 2, K_2HPO_4 2, $(NH_4)_2SO_4$ 2, yeast extract 3 and pectin (SRL, India) 5 (*12*). The pH of the medium was adjusted to 7.0 using 1 M NaOH. The strain was grown for 24 h in 250-mL Erlenmeyer flask containing 100 mL of liquid medium. Adequate aeration was provided by agitation at 175 rpm at 30 °C. The inoculum contained $2.5 \cdot 10^4$ CFU/mL.

Submerged fermentation

The composition of fermentation medium was similar to the medium used for the preparation of inoculum where pectin was replaced by wheat bran. Sterilization was performed at 1.05 kg/cm^2 for 15 min. Fermentation was carried out in 250-mL Erlenmeyer flask containing 100 mL of growth medium with 10 % inoculum at 30 °C, under static and shaking (175 rpm) conditions for 5 days. Samples were collected at regular intervals and centrifuged at 10 000 rpm for 20 min at 4 °C. The culture supernatant was used as the enzyme source.

Solid-state fermentation

Wheat bran was used as the solid substrate for SSF. A mass of 10 g of substrate was added to 250-mL Erlenmeyer flasks and moistened with 5 mL of salt solution containing (in g/L): KH_2PO_4 4, K_2HPO_4 4 and $(NH_4)_2SO_4$ 4. The pH of the solution was adjusted to 7.0. The medium was sterilized at 1.05 kg/cm^2 for 45 min, inoculated with 5 mL of broth culture and incubated at 30 °C, under static and shaking (175 rpm) conditions for 4 days. The final moisture content of the flask was 50 %.

Slurry-state fermentation

Wheat bran was the substrate for SLSF too. A mass of 10 g of substrate was added to 250-mL Erlenmeyer flask and moistened with 45 mL of distilled water and 5 mL of salt solution containing (in g/L): KH_2PO_4 4, K_2HPO_4 4 and $(NH_4)_2SO_4$ 4. The pH of the solution was adjusted to 7.0. The medium was sterilized at 1.05 kg/cm^2 for 45 min, inoculated with 5 mL of broth culture and incubated at 30 °C, under static and shaking (175 rpm) conditions for 4 days. The final moisture content of the flask was 85 %.

Enzyme extraction

A suitable amount of the fermented matter of SLSF and SSF was thoroughly mixed with 10 mL of distilled water by keeping the flasks on a rotary shaker for 1 h at 200 rpm. The mixture was filtered through muslin cloth and the filtrate was centrifuged at 10 000 rpm for 20 min at 4 °C. The supernatant was taken for analysis.

Selection of substrate for SSF

Various agroindustrial residues, coconut oil cake (COC), cotton seed oil cake (CSOC), ground nut oil cake (GOC), sesame oil cake (SOC), rice bran (RB) and wheat bran (WB), were tried as substrates for SSF. All the substrates were used after drying.

Enzyme assays

Polygalacturonase (PG) activity was determined according to Honda *et al*. (*13*). Reaction mixture containing 0.5 mL of suitably diluted enzyme was incubated with 0.5 mL of 0.5 % polygalacturonic acid (SIGMA) in 100 mM McIlvaine buffer (pH=7.0) for 1 h at 45 °C. The reaction was arrested by adding 2 mL of 100 mM borate buffer (pH=9.0) followed by 1 mL of 1 % cyanoacetamide. The mixture was immersed in a boiling water bath for 10 min, cooled and read against a suitable blank at 276 nm using a spectrophotometer (Shimadzu, UV-2401). One unit (U) of PG was defined as 1 μmol of galacturonic acid released/(mL·min).

Pectin lyase (PL) activity was estimated by monitoring the increase in absorbance at 235 nm due to the formation of double conjugate bond of the unsaturated uronide formed during the reaction. Culture supernatant (0.3 mL) was mixed with 3 mL of 1 % citric pectin (SIGMA) in 100 mM McIlvaine buffer (pH=7.0) and 2.7 mL of 100 mM McIlvaine buffer (pH=7.0) and incubated for 1 h at 45 °C and 150 rpm. To stop the reaction, one mL of the above mixture was transferred to 5 mL of 0.5 M HCl and read against a suitable blank at 235 nm. One unit (U) of enzyme was defined as the amount of enzyme required to liberate 1 μmol of unsaturated galacturonide/(mL·min). The molar absorption coefficient used was 5500 $M^{-1} \cdot cm^{-1}$ (*14*).

The amount of pectin esterase (PE) was determined by titrimetric method (*10*). A volume of 2 mL of enzyme was added to 10 mL of 0.5 % pectin (SIGMA) in 0.1 M NaCl, the pH was adjusted to 6.0 with 0.1 M NaOH and the mixture was incubated at 45 °C for 1 h. The amount of carboxyl groups released was determined by titration with 0.02 M NaOH. One unit (U) of PE was defined as the amount of enzyme releasing one milliequivalent of ester hydrolysed/(mL·min).

Enzyme yield was expressed as specific activity (U/mg protein).

Total soluble protein and dry mass

Total soluble protein in the culture filtrate was estimated according to Lowry's method (*15*) using bovine serum albumin as the standard. A known mass of the fermented matter of SSF and SLSF was dried in oven until constant mass and dry mass was calculated.

Results and Discussion

Pectinolytic enzyme profile

Pectinolytic enzyme profile of *Streptomyces lydicus* was examined under submerged fermentation for 4 days on a rotary shaker at 175 rpm and 30 °C. Different pectinolytic activities exhibited by the culture were PG and PL. PE production was not detected in the culture supernatant. The time course study was conducted in SmF to monitor the production pattern of PG and PL. Fig. 1 illustrates the time course of PG in SmF.

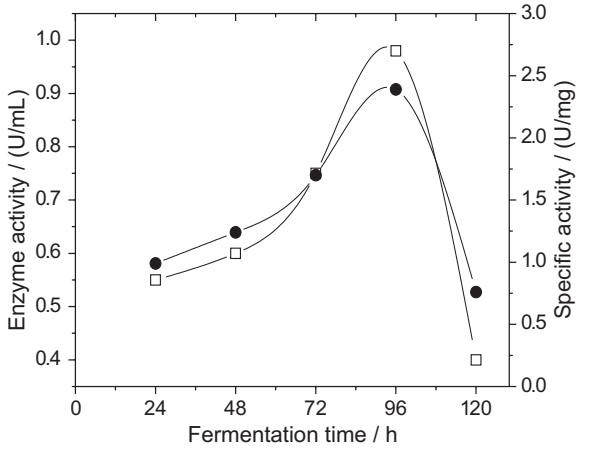

Fig. 1. Time course of polygalacturonase in SmF. (□) Enzyme activity (U/mL), (●) specific activity (U/mg protein)

PG titer was maximized after 96 h of incubation in SmF. The highest enzyme activity recorded was 0.98 U/mL with a specific activity of 2.39 U/mg. Though the optimum incubation time was 96 h, specific activities were 51 and 71 % at 48 and 72 h, respectively. The time course of pectin lyase in SmF is given in Fig. 2.

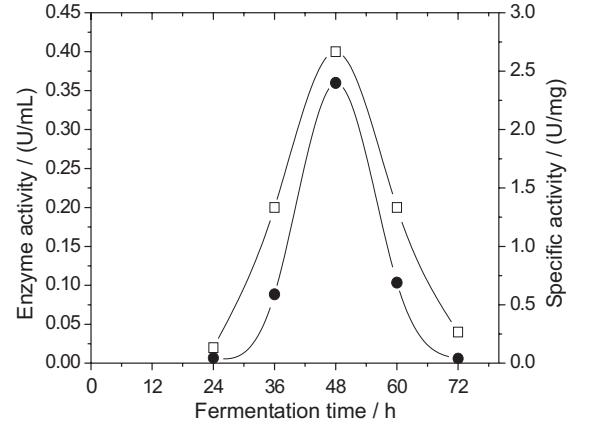

Fig. 2. Time course of pectin lyase in SmF. (□) Enzyme activity (U/mL), (●) specific activity (U/mg protein)

Pectin lyase activity reached its peak after 48 h of incubation and then decreased. The highest enzyme titer recorded was 0.4 U/mL having a specific activity of 2.4 U/mg. Since there was no significant production of PL when compared to earlier reports (*16*), further studies were focused on production of polygalacturonase only.

Influence of mode of fermentation

Influence of mode of fermentation on the production of PG was examined using SSF, SmF and SLSF, each under static and shaking conditions. PG activity was recorded only in SSF and SmF. SLSF was devoid of PG activity.

The highest enzyme yield was registered with SSF under static condition. The activity was 108.5 U/g with a specific activity of 5.43 U/mg. When agitation had been provided in SSF, the specific activity was decreased by 60 %. The least activity was observed in SmF with agitation.

The specific activity in SSF (static) was twofold when compared to SmF (shaking). PE and PG production by *Aspergillus niger* was about four and six times higher, respectively, in SSF than those observed in the SmF system (*10*). The time course of PG in SSF (static) is given in Fig. 3.

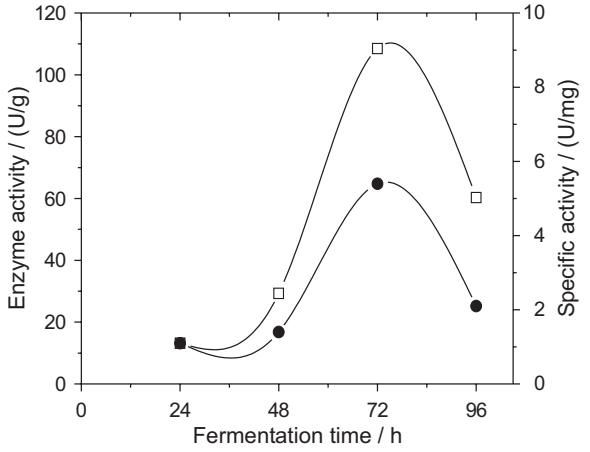

Fig. 3. Time course of polygalacturonase in SSF. (□) Enzyme activity (U/g), (●) specific activity (U/mg protein)

Incubation period for maximum enzyme titer was 72 h in SSF, while the same was 96 h in SmF. In *Aspergillus niger* maximum production of PE and PL was after 48 h in SmF, whereas in SSF system, higher values were achieved in shorter time (24 h) (*10*). There are physiological differences between SSF and SmF, which in turn affect enzyme production in each fermentation system. SSF includes the growth and metabolism of microorganisms in the absence of or near-absence of any free flowing water. Microbial growth and product formation occur at or near the surface of the solid substrate particle. Such a system, being closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites (*17*).

SLSF was not suitable for PG production. In practice, SLSF system resembles SSF except in the high moisture content. Very high moisture content has negative consequences on growth, as porosity of the medium and oxygen diffusion in the solid material is reduced (*18*).

In SmF and SSF, static fermentation was found to be better than shaking mode. This observation may be attributed to the fact that actinomycetes are filamentous in nature and agitation may break their mycelia.

Based on the above results SSF was selected as the mode of fermentation for further experiments to enhance PG yield.

Substrate selection for SSF

The ideal solid substrate is one that provides all the necessary nutrients for the microorganism. Thus the selection of an appropriate solid substrate plays an important role in the development of an efficient SSF process (*19*). Agroindustrial residues are generally considered as the best substrate for SSF processes. The selection of a substrate for enzyme production in an SSF process depends on several factors like cost and availability of the substrate, and thus involves screening of several agroindustrial residues. The solid substrate not only provides nutrients for microbial growth but also serves as a support for the cells (*20*). Substrate selection for SSF was carried out using various agroindustrial residues (Fig. 4).

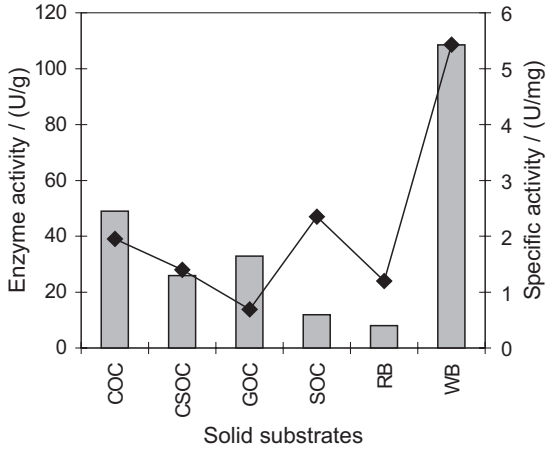

Fig. 4. Substrate selection for SSF. (▮) Enzyme activity (U/g), (—◆—) specific activity (U/mg protein), COC – coconut oil cake, CSOC – cotton seed oil cake, GOC – ground nut oil cake, SOC – sesame oil cake, RB – rice bran,WB – wheat bran

Among the different substrates tried, wheat bran was found to be the best for PG production. The result is significant since wheat bran is produced worldwide in enormous quantities as an important by-product of the cereal industry. Enzyme production using wheat bran will make the process economical.

Native PAGE of the enzyme obtained from both SmF and SSF was performed to compare the pattern of protein expression in the two different modes of fermentation (Fig. 5).

Production of pectinases from actinomycetes is seldom reported (*5,21*). In the present investigation, it was found that the filamentous bacteria are a promising source for the same. Further optimization studies for PG production by *Streptomyces lydicus* are going on.

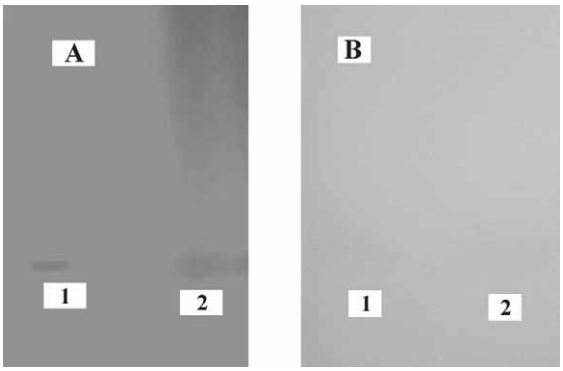

Fig. 5. Native PAGE of *S. lydicus* crude polygalacturonase. A: crude extract stained with Coomassie Brilliant Blue; B: zymogram stained with 1 % cetrimide; 1 and 2: SmF and SSF, respectively

Conclusions

The novel strain of *Streptomyces lydicus* produced significant titer of PG and traces of PL in submerged fermentation. The strain could not produce PE. PG production reached its peak after 96 h of incubation, while PL activity reached its maximum after 48 h. Further studies were focused on PG and among the three modes of fermentation tried, the best mode of fermentation for PG production was SSF under static condition. The highest enzyme production was obtained after 72 h in SSF. The specific activity in SSF registered twofold increase when compared to SmF. Shorter period of incubation and enhanced enzyme production favoured SSF over SmF. PG production was not recorded in SLSF. The best substrate for PG production by SSF was wheat bran.

Acknowledgements

The authors thank the Director of the Regional Research Laboratory, Trivandrum, for providing laboratory facilities. Financial support provided by Council of Scientific and Industrial Research, New Delhi (India), for carrying out this study is duly acknowledged.

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