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Original Research Paper

Alkaline and thermostable polygalacturonase from *Streptomyces halstedii* ATCC 10897 with applications in waste waters

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ABSTRACT

Pectin degrading enzymes with polygalacturonase (PG) activity hydrolyze α-(1,4) glycosidic bonds of polysaccharides present in higher plants. In the current study one hundred bacterial strains were screened for extracellular PG activity using an inductive culture medium. Optimization of fermentation conditions for *Streptomyces halstedii* ATCC 10897 was conducted using experimental designs. The maximum enzymatic activity obtained was 3.489 U/mL and 98.0% of viscosity reduction after 12 h of fermentation using soy peptone as unique source of carbon and nitrogen. PG from *S. halstedii* ATCC 10897 showed high thermal stability, an approximate molecular weight of 48 kDa and its optimum conditions for catalytic reaction were 50 °C and pH 12.0. This study reveals that alkaline PG is a useful enzyme for depectinization in alkaline pulping mill and papermaking waste waters.

1. Introduction

Pectic substances are complex polysaccharides, its structure is composed by D-galacturonic acid molecules linked by α-(1,4) glycosidic bonds. Other molecules as galacturonan, rhamnogalacturonan, arabinan and methyl residues are integrated to the main chain to form partially esterified polymers with variable molecular weight. Pectic substances are present in the middle lamella of the primary cell wall and its function is to support and contribute to plant tissues firmness (Pedrolli et al., 2009). Polygalacturonases (PG) cleave glycosidic linkages by hydrolysis mechanism and its activity is classified into endopolygalacturonase (endoPG) and exopolygalacturonase (exoPG) according to the internal and external action, respectively (Gummadi et al., 2007; Jacob, 2009). Microbial PG are distributed among fungi, bacteria and yeast and often occur in different forms having molecular weights in the range of 30–80 kDa (Gummadi et al., 2007). Reports of bacterial PG have shown sizes of 44 kDa for *Bacillus halodurans* M29, 63 kDa for *S. erumpens* 7317 and 65 kDa for *Yersinia enterocolitica* (Jayani et al., 2010; Kar and Ray, 2011; Mei et al., 2013).

PG are employed in food industries for maceration, extraction and filtration of fruit juices and vegetable extracts, as well as for depectinization of effluents; however these applications are limited by acidic and neutral activity (Jayani et al., 2005). On industrial scale, fungal species, especially those from *Aspergillus*, are usually used for commercial applications.Unlike fungal PG, bacterial PG are active at alkaline pH and this feature is valuable for many environmental applications (Das et al., 2012; Hoondal et al., 2002; Rehman et al., 2012). Alkaline PG could be used in waste waters from pulping mill and papermaking process because of the elevated content of pectin, cellulose, hemicellulose and vegetable particles (Pokhrel and Viraraghavan, 2004).

The aim of this research was to screen different bacterial strains and select the one with the highest PG activity using a culture medium designed for the induction of target enzymes. The enzymatic activity from *S. halstedii* ATCC 10897 was enhanced by optimization of the fermentation conditions such as time, pH and culture medium composition. ExoPG and endoPG activities were characterized in terms of optimum pH and temperature.

In the present study, fermentation of *S. halstedii* ATCC 10897 resulted in highest productivity for PG production, with an elevated ability to degrade pectic substances producing oligogalacturonides and decreasing the viscosity of pectinaceous materials. The wide range of alkalophilic activity represents a versatile option to be used in different applications such as treatment of alkaline paper waste waters (Hoondal et al., 2002).

2. Materials and methods

2.1. Reagents and microorganisms

Culture medium compounds were obtained from Britania S.A. (Argentina) and polygalacturonic acid from citrus peel was

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purchased from Sigma S.A. (Brasil). Chemical reagents were purchased from Biopack S.A. (Argentina). Microorganisms were obtained from the collection of Laboratory of Sustainable Biotechnology (LIBioS) of National University of Quilmes (Argentina).

2.2. Screening of microorganisms for PG activity

One hundred of bacterial strains from the LIBioS collection were screened. Strains were conserved in sterile glycerol 20% at −130 °C. Bacterial cultures were grown for 16 h at temperature and growth liquid culture medium according to the standard for each genus. Composition of *Streptomyces* growth medium was glucose (4 g/L), yeast extract (4 g/L) and malt extract (10 g/L). Inducer medium containing citrus pectin (10 g/L), yeast extract (1 g/L), soy peptone (5 g/L), NaCl (2.5 g/L), $MgSO_4$ (0.5 g/L) and KH_2PO_4 (1.0 g/L) at pH 7.0 was inoculated in flasks (100 mL) with 10 mL of culture medium at 200 rpm. The initial cell concentration was 5×10^7 colony-forming units (CFU)/mL. Basal medium of *Streptomyces* contains yeast extract (3 g/L) and salts above mentioned. Periodically, 1 mL of broth was collected and centrifuged at 8000*g* for 10 min. Cell concentration was measured optically at 600 nm ($OD_{600\ nm}$) and cell-free supernatants were assayed for PG activity.

2.3. Polygalacturonase activity assays

PG assay was performed by reaction of culture supernatants with 1% (w/v) polygalacturonic acid solution in 0.1 M citrate-phosphate buffer at pH 7.0.

EndoPG was determined by measuring the viscosity (η) of the reaction mixture (1:9 v/v supernatant:substrate), incubated at 45 °C for 10 min in a rheometer (AR-G2/TA Instrument) at 24 °C and 300 s^{-1} shear rate. EndoPG activity was expressed as percentage of viscosity reduction (% η-reduction). ExoPG activity was measured using colorimetric method with 3′,5′-dinitrosalicylic acid reagent (Miller, 1959) which quantifies reducing groups released during the incubation of reaction mixture (1:4 v/v supernatant:substrate) at 45 °C for 20 min. D-galacturonic acid was used as standard. One unit of exoPG activity was defined as the amount of enzyme required to release 1 μmol of D-galacturonic acid per min under described conditions.

2.4. S. halstedii ATCC 10897 fermentation conditions and design of culture medium

The effect of pH in fermentation process was studied by varying the pH range from 3.0 to 10.0. *S. halstedii* ATCC 10897 cultures were harvested after 24 h and supernatants were analyzed for PG activity and cell concentration. Then, citrus pectin (0–12.5 g/L), soy peptone (0–21 g/L) and yeast extract (0–6 g/L) were evaluated to optimize culture medium constituents and obtain maximum enzymatic activity. The composition and concentration of salts were identical to that used in the screening medium. Response variables were cell growth ($OD_{600\ nm}$), exoPG (U/mL) and endoPG (% η-reduction) enzymatic activities. Other parameters such as temperature, orbital agitation rate and fermentation time were fixed at 20 °C, 200 rpm and 24 h, respectively. Experimental designs were replicated and the results were statistically analyzed with the software Statgraphics Centurion XV. Analysis of variance and multiple comparison test (LSD) were performed considering a level of confidence at 95% ($p < 0.05$). Finally, fermentation kinetic was evaluated to determine the time of maximum enzymatic activity and productivity. Under the optimized conditions, exoPG (U/mL) and endoPG (% η-reduction) were measured periodically for 24 h and results were compared with fermentation on screening medium.

2.5. Effect of temperature and pH on PG activity

The effect of temperature on exoPG and endoPG activities was determined at pH 7.0, incubating reaction mixtures at different temperatures (5–80 °C). Enzymatic activities were assayed at optimal temperature as a function of pH with 0.1 M citrate-phosphate buffer (pH 5.0–8.0) and 0.1 M glycine–NaOH buffer (pH 9.0–12.0). Thermal stability of enzymes was assayed by preincubation of supernatant without substrate at different temperatures (30–70 °C) and aliquots were taken for PG activity assay at regular intervals.

2.6. Application in pulping mill waste water

Effluents of pulping mill and paper process result at high pH with elevated content of colloidal organics with pectinaceous material in an alkaline environment. Pulp waste waters with a viscosity of 0.025 Pa · s were treated with the enzymatic product in a relation of 1:9 enzyme:waste water, at different temperatures (24–50 °C) and viscosity was monitored in a rheometer (AR-G2/TA Instrument) at 300 s^{-1} shear rate.

2.7. PG molecular weight

PG was partially purified from supernatant by salt precipitation method with 60% ammonium sulfate saturation. The mixture was incubated at 4 °C for 8 h, centrifuged at 10,000*g* for 30 min and precipitates were solubilized in glycine–NaOH buffer. Desalting and concentration was performed in VivaspinTM ultrafiltration devices (10,000 MWCO). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 12% was used for molecular weight estimation with BSA as standard (Laemmli, 1970).

3. Results and discussion

3.1. Selection of strain with PG activity

On the basis of previous studies for PG production, a culture medium containing citrus pectin (10 g/L) was formulated and tested to induce PG secretion (Ahlawat et al., 2009; Beg et al., 2000a; Jacob et al., 2008b; Jayani et al., 2010; Kapoor and Kuhad, 2002; Kapoor et al., 2000; Kar and Ray, 2011; Rehman et al., 2012; Soares et al., 1999). *Bacillus sp.* was the control strain, resulting in 0.403 U/mL and 15.9% viscosity reduction under standard conditions. Afterwards, more than 100 strains from several bacterial genus as *Aeromonas*, *Bacillus*, *Citrobacter*, *Erwinia*, *Lactobacillus*, *Pseudomonas*, *Serratia*, *Streptomyces* and *Xanthomonas* were screened for PG activity using batch fermentation. Results showed that 92 strains were able to grow on this medium and these strains were tested for polygalacturonic acid degradation at pH 7.0 (Table 1). Among the evaluated bacteria, 12 strains from *Streptomyces*, *Bacillus*, *Serratia*, *Citrobacter* and *Enterococcus* genus showed PG activity higher than 0.2 U/mL and the maximum viscosity reduction achieved was 95.2% (Table 2).

Five *Streptomyces* strains (ST1, ST2, ST3, ST4 and ST5) showed more elevated PG activity than *Bacillus sp.* Strain ST1 identified as *Streptomyces halstedii* ATCC 10897, exhibited a considerable activity (0.551 U/mL) and viscosity reduction (94.2% η-reduction) at only 24 h of fermentation. Table 3 compares this study with fermentation times of maximum PG activity from previously reported microorganisms.

It is worth mentioning that most studied bacterial sources of PG activity for industrial applications are *Bacillus* species such as *B. pumillus*, *B. subtilis*, *B. alcalophillus*, *B. halodurans*, *B. megaterium* and *B. licheniformis*, and all these microorganisms were isolated from vegetal material and soil samples (Ahlawat et al., 2008; Mei et al., 2013; Rehman et al., 2012; Sharma and Satyanarayana, 2006). Thus,

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Table 1
Screening of strains for polygalacturonic acid degradation. The positive response is expressed regarding to the reducing sugars released by hydrolysis.

Positive		Negative			
Genus	Strains	Genus	Strains	Genus	Strains
Bacillus	6	*Achronobacter*	1	*Flavobacterium*	2
		Aeromonas	6	*Geobacillus*	3
Citrobacter	3	*Agrobacterium*	1	*Klebsiella*	2
		Arthrobacter	2	*Lactobacillus*	5
Enterococcus	3	*Brevibacterium*	2	*Lactococcus*	1
		Cellulomonas	2	*Leuconoctoc*	2
Serratia	3	*Chromobacterium*	1	*Micrococcus*	1
		Corynobacterium	1	*Pediococcus*	1
Streptomyces	16	*Escherichia*	1	*Proteus*	3
		Enterobacter	3	*Pseudomonas*	3
		Erwinia	3	*Thermonospora*	2
		Psychrophilic strains[NI]	9	*Weibsella*	1
				Xanthomonas	3
Total of tested strains: 92					

[NI] : unidentified species

Table 2
Enzymatic activity and culture conditions of the screened strains with highest PG activity.

Strain code	Enzymatic activity[a]	
	ExoPG (U/mL)	EndoPG (% viscosity reduction)
BA1 (*Bacillus sp.*)	0.403	15.9
ST1	0.551	94.2
ST2	0.534	93.3
ST3	0.476	95.2
ST4	0.433	94.2
ST5	0.431	93.4
ST6	0.365	6.9
BA2	0.301	61.1
SE1	0.202	20.1
BA3	0.230	10.9
CI1	0.222	10.8
EN1	0.209	7.9

[a] Values are mean of 3 replicates. Standard error was less than 12%. Screening culture medium with citrus pectin (10 g/L), initial pH 7.0 and agitation rate 200 rpm.

Table 3
Time of fermentation for maximum PG activity by different microorganisms.

Microorganisms	Fermentation time (h)	References
S.halstedii ATCC 10897	24	This study
B. pumillus dcsr1	34	Sharma and Satyanarayana (2006)
Streptomyces sp. QG-11-3	36	Beg et al. (2000a,b)
S. erumpens MTCC 7317	36	Kar and Ray (2011)
B. licheniformis KIBGE IB-21	48	Rehman et al. (2012)
B. sphaericus MTCC 7542	72	Jayani et al. (2010)
S. lydicus	96	Jacob et al. (2008a,b)

this work represents an important finding about *S. halstedii* ATCC 10897 as a novel microorganism with elevated extracellular PG activity.

3.2. Culture conditions for PG activity from S. halstedii ATCC 10897

Growth factors and culture conditions for microbial enzyme production are important in industrial bioprocesses, which could promote the metabolism in order to obtain high yield of enzymes for different applications. Based on the nutrients reported in screening culture medium, a new one specific to *S. halstedii* ATCC 10897 was developed by sequential experimental designs considering variables such as pH values, citrus pectin, yeast extract and soy peptone concentration.

The pH of the medium plays an important role in features like cell concentration and enzyme production. To study the effect of initial pH on PG production and biomass of *S. halstedii* ATCC 10897, culture was grown on the medium having different pH values (3.0–10.0). Interestingly, high biomass concentration (Fig. 1a) and PG activity (Fig. 1b and c) were found at a broad pH range. The pH levels between 5.0 and 8.0 were the most favorable for growth (3.51 ± 0.11 $OD_{600\ nm}$) and PG activity (0.546 ± 0.01 U/mL and $94.1 \pm 1.6\%$ η-reduction) without significant differences (LSD test) suggesting a direct relation between growth and PG production. At pH 9.0, exoPG and endoPG activities decreased 32% and 10%, respectively (Fig. 1b and c). Any variation of extreme acidic ($pH < 5.0$) or alkaline ($pH > 9.0$) pH level resulted in a lack of biomass and PG activity. The selected pH for further studies was 8.0 because of the potential alkaline PG activity for industrial applications. In contrast to other reports, *S. halstedii* ATCC 10897 resulted in high adaptability to a broad pH range (5.0–9.0).

Complex carbon sources, agro industrial wastes and pectin in fermentation medium have been reported as substrates that induces PG activity in some microorganisms (Beg et al., 2000a; Kapoor and Kuhad, 2002; Rehman et al., 2012). Screening medium containing 10 g/L of citrus pectin was modified by varying the citrus pectin concentration between 0 and 12.5 g/L and the initial pH was adjusted at 8.0 after sterilization.

Results showed high levels of PG activity at values up to 10 g/L of citrus pectin and a further increase in concentration resulted in a partial loss of enzyme activity (Fig. 2). Compared to maximum response, at 12.5 g/L of citrus pectin a relative activity of 24 for exoPG (Fig. 2a) and 66% for endoPG (Fig. 2b) was detected. These results suggest that an excess of citrus pectin could have an antagonistic effect on PG production and a similar behavior was reported by Jayani et al. (2010). Furthermore, it was observed that citrus pectin was not the main PG inducer because of the high enzymatic activity found in absence of pectin (0.534 U/mL and 69.3% η-reduction).

Other main components of the culture medium were yeast extract and soy peptone and they could play an important role in bacterial metabolism and enhancement of PG production. Therefore, a factorial experimental design was developed to determine the effect of these substrates on PG activity by *S. halstedii* ATCC 10897. Table 4 depicts evaluated conditions of the full factorial experimental design 3^2 and their effects on PG activity. Culture conditions of treatment 7 were the most favorable which resulted in 0.544 U/mL and 86.4% η-reduction. These results showed a considerable variation in PG activity due to soy peptone (p-value = 0.0001). Yeast extract levels did not have a significant effect on enzyme production (p-value > 0.1). Both PG activities raised with the increase of soy peptone concentration and yeast extract did not have any effect on PG activity. This fact could be better observed in Fig. 3 (Pareto chart of effects), where the absolute effect of the variables is represented.

The experimental data was statistically analyzed and modeled to identify the effects of studied factors on response variables (Table 4 and Fig. 3). The correlation factors (R^2) obtained for exoPG and endoPG activities were higher than 0.95 which means that adjusted models explain 99.386 and 95.645% of the variation observed, respectively. Since the interaction between both of the independent variables (*AB*) had a negative significant effect, the culture medium optimization was followed by another experimental design without yeast extract. Higher levels of soy peptone were evaluated in the culture medium and it was observed that PG

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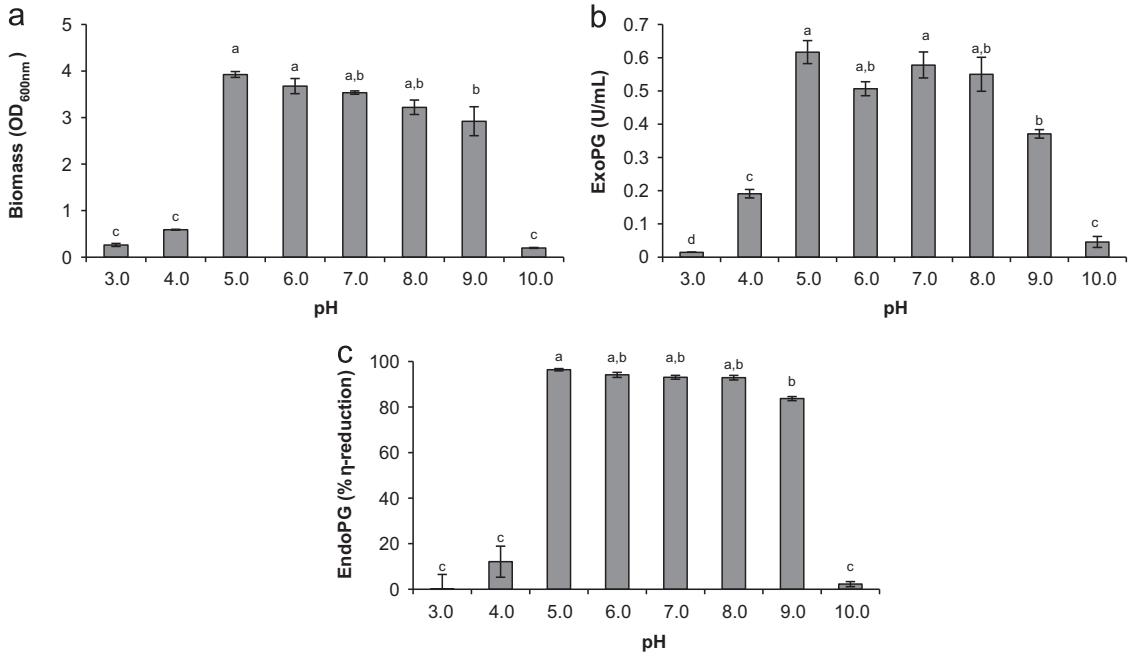

Fig. 1. Effect of initial pH of media on (a) growth, (b) exoPG activity and (c) endoPG activity by *S. halstedii* ATCC 10897. No significant differences among treatments named with the same letter (LSD test, p-value < 0.05) were observed.

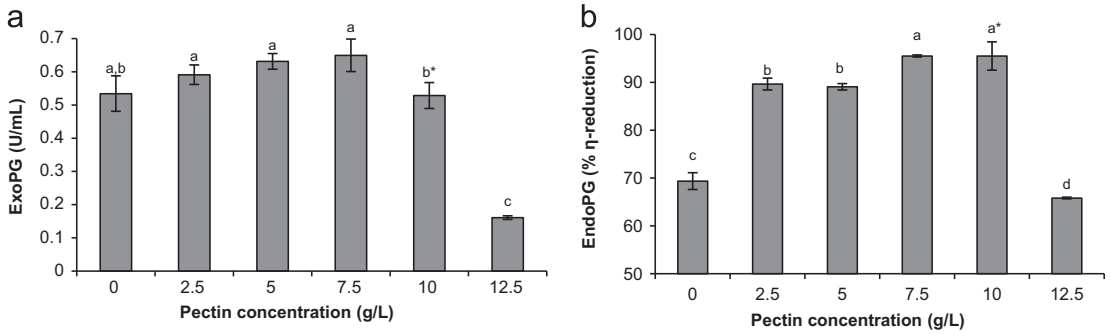

Fig. 2. Effect of citrus pectin concentration on (a) exoPG and (b) endoPG production by *S. halstedii* ATCC 10897. No significant differences between treatments named with the same letter (LSD test, p-value < 0.05) were observed.

Table 4
Matrix of full factorial experimental design 3^2 for PG activity by *S. halstedii* ATCC 10897 cultivated during 24 h at 28 °C, 200 rpm with addition of soy peptone (A) and yeast extract (B) as studied factors. Initial pH was adjusted at 8.0.

Treatment	Factors (g/L)		Response variable[a]	
	Soy peptone (A)	Yeast extract (B)	ExoPG (U/mL)	EndoPG (% η-reduction)
1	0	0	0.017	9.0
2	0	3	0.077	39.3
3	0	6	0.118	37.6
4	3	0	0.373	76.6
5	3	3	0.370	69.6
6	3	6	0.410	65.3
7	6	0	0.544	86.4
8	6	3	0.473	83.4
9	6	6	0.414	78.9
P-value ExoPG	**0.000**	0.116	$R^2=0.993$	$R^2=0.956$
P-value EndoPG	**0.000**	0.497	Significance level 95%	

[a] Values are mean of 3 replicates. Standard error was less than 8%.

activity (Fig. 4a and b) rise with increasing concentrations of this substrate detecting the maximum activity at 15 g/L (1.052 U/mL and 95.0% η-reduction). As more content of soy peptone did not affect enzyme activity, culture medium with 15 g/L was selected for further characterization of *S. halstedii* ATCC 10897 PG activity. The use of soy peptone as main substrate might be suitable for PG production because it represents a vegetable source with high content of amino acids and carbohydrates.

Soy peptone contains vitamins, minerals and amino acids and it also contains some soy sugars at concentrations up to 3 g/L. These compounds are essential for proper metabolism thus facilitating bacterial growth and enzymatic activity by *S. halstedii* ATCC 10897. In this context, some major constituents as glycine and D-galacturonic acid were evaluated and it was found that they promote enzyme production at concentrations of 3 g/L in culture medium. The increase in enzymatic activity was 7.28-fold when basal medium were enriched with D-galacturonic acid. Beg et al. (2000b) suggested that production of xylanase and pectinase is not only stimulated by their own substrates as xylan or oligogalacturonides but also by the undefined nutrients contained in both peptone and yeast extract. Furthermore, Kapoor and Kuhad (2002) reported that the amino acids that improved PG activity by *Bacillus sp.* MG-cp-2 under submerged fermentation were glycine, serine, alanine, arginine and glutamic acid. Amino acids mentioned are contained at high percentage in soy peptone used in the present study. It is likely that these compounds stimulate PG production by *S. halstedii* ATCC 10897.

Table 5 shows a comparison among the three culture media used in this study and it provides evidence of increased PG activity

with soy peptone as unique and very profitable substrate. Results showed that optimized medium duplicate exoPG activity in comparison to screening medium, and endoPG activity reached almost 100% of viscosity reduction of the pectin solution. Also, there was no evidence of PG activity in the inoculum medium and this may be explained by the high concentration of monosaccharides which could repress catabolic production of PG (Beg et al., 2000a; Kapoor et al., 2000; Rehman et al., 2012). Pectin is the most reported source that induces PG activity and it is enhanced by supplying nitrogen from organic sources as yeast extract, peptone, casein, tryptone, beef extract and malt extract (Jacob et al., 2008a; Kar and Ray, 2011; Rehman et al., 2012). In this study, only soy peptone has been used for growth and PG production.

Enzyme production in the optimized culture medium was supported by SDS-PAGE (Fig. 5). After purification and concentration of culture supernatants, a single band was found at 48 kDa which proved an approximation of the molecular weight of studied PG.

3.3. Fermentation kinetic

S. halstedii ATCC 10897 was grown in the optimized medium. ExoPG and endoPG activities were measured by intervals and were compared with the initial screening medium (Fig. 6). A rapid increase in exoPG activity (Fig. 6a) was observed and the maximum activity was reached at 12 h of culture (1.076 U/mL) in contrast to screening medium in which the increase in enzymatic

Table 5
Growth and PG activities by *S. halstedii* ATCC 10897 in three different culture media used in the sequential experimental design.

Parameters	Culture media		
	Inoculum	Screening	Optimized[b]
ExoPG[a](U/mL)	0.012	0.551	1.052
EndoPG[a](% η-reduction)	1.59	94.28	96.12
Biomass[a]($OD_{600\ nm}$)	3.95	3.26	4.45

[a] Values are mean of 3 replicates. Standard error was less than 10%.
[b] Fermentation conditions were pH 8.0 at 28 °C and 200 rpm during 24 h.

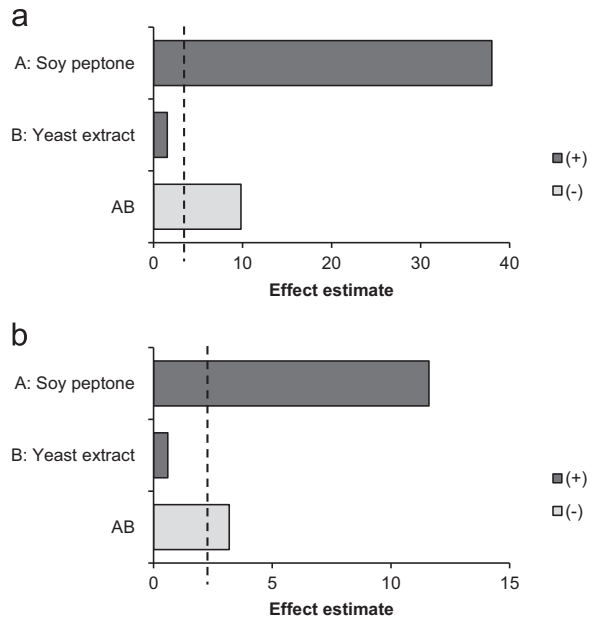

Fig. 3. Pareto chart of effects (absolute value) of the full factorial design 3^2 on (a) exoPG and (b) endoPG activity. Dashed line indicates the significance level (α=0.05).

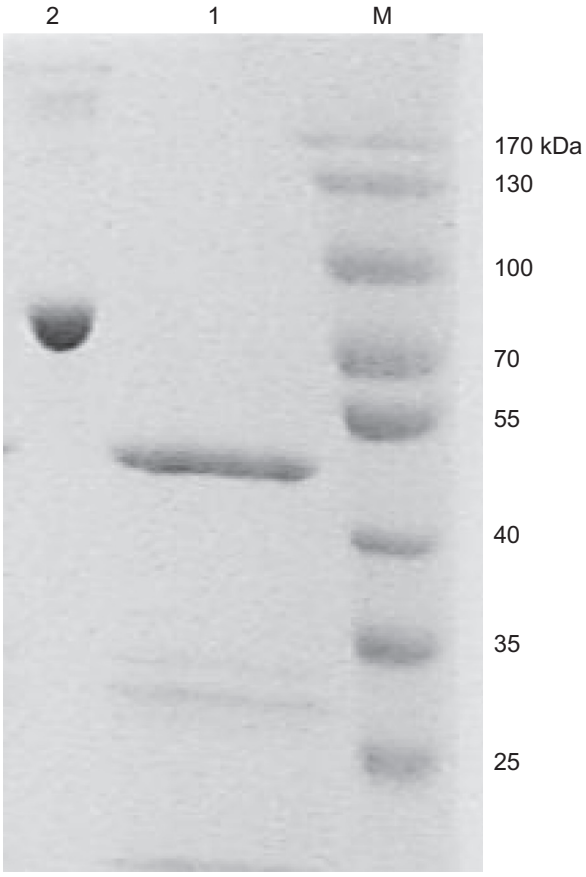

Fig. 5. SDS-PAGE analysis of *S. halstedii* ATCC 10897 PG. Lane M: protein marker. Lane 1: partially purified PG. Lane 2: BSA.

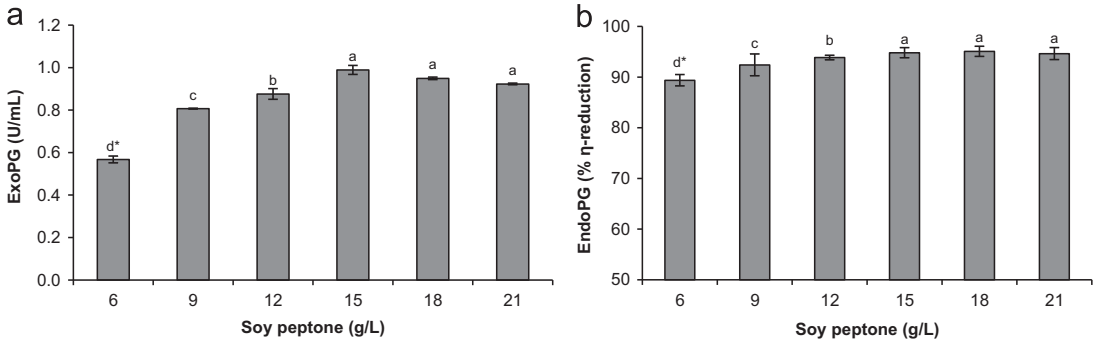

Fig. 4. Effect of soy peptone concentration on (a) exoPG and (b) endoPG activities by *S. halstedii* ATCC 10897. No significant differences among treatments named with the same letter (LSD test, p-value < 0.05) were observed.

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activity was evident after 14 h and the highest activity was at 24 h (0.545 U/mL). This kinetic profile confirmed that optimized medium duplicates the activity rapidly which implies an augmented productivity. Regarding endoPG activity (Fig. 6b), a sustainable increase was observed since the beginning of fermentation in both cultures. There is a difference between the time of the maximum activity being 12 and 18 h in optimized and screening medium, respectively. After reaching the top enzymatic activity, in both of the evaluated medium, PG activity remained constant. This behavior results of interest because it suggests high enzyme stability and also that the interaction with other components in the medium did not affect the activity. A different behavior was reported in other studies, where 50% activity loss was observed after the maximum reached at 36 h with *Streptomyces erumpens* MTCC 7317 (Kar and Ray, 2011) and at 48 h with *Bacillus licheniformis* KIBGE IB-21 (Rehman et al., 2012).

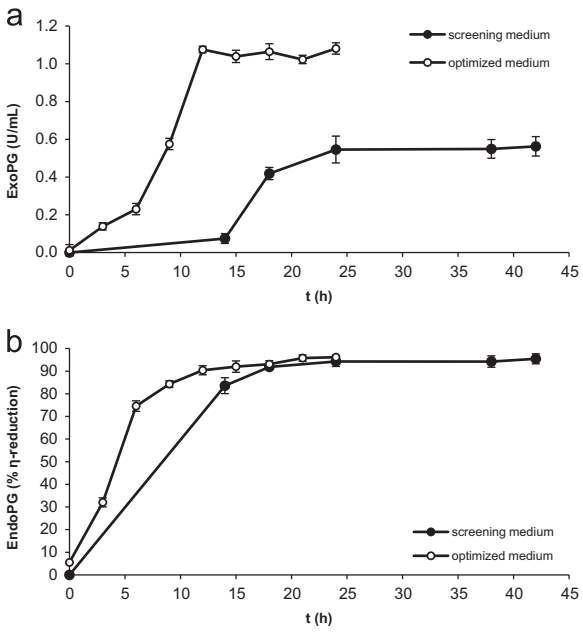

Fig. 6. Kinetic of enzyme production (a) exoPG and (b) endoPG by *S. halstedii* ATCC 10897 in screening and optimized culture medium. Initial culture conditions were: pH at 8.0, 28 °C and 200 rpm.

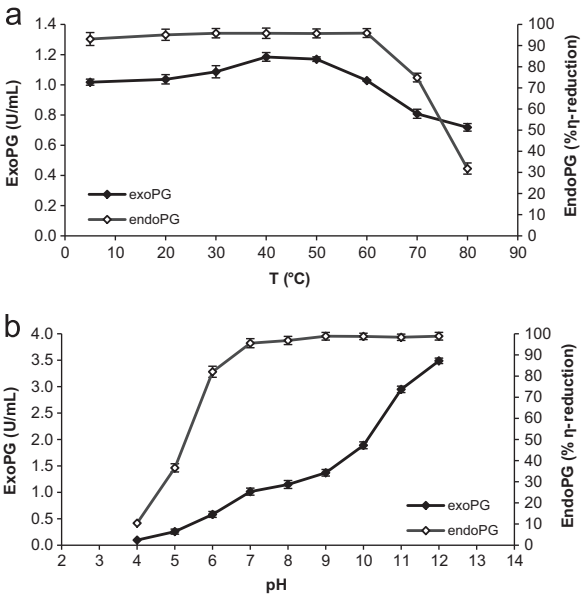

Fig. 7. Characterization of exoPG and endoPG activity. (a) Effect of temperature at pH 7.0 and (b) effect of pH at 50 °C.

3.4. Effect of temperature and pH on PG activity

The effect of temperature and pH on PG activity is shown in Fig. 7. ExoPG and endoPG from *S. halstedii* ATCC 10897 exhibited good activity in a broad temperature range from 5 to 50 °C (Fig. 7a). At 60 °C endoPG was completely active but exoPG lost 15% of its maximum activity. At 80 °C enzymes retained 60 and 35% of exoPG and endoPG activity, respectively. The effect of pH on enzymatic activity was evaluated at 50 °C. As shown in Fig. 7b, PG exhibited elevated endoPG activity from pH 7.0 to 12.0 (98.0% η-reduction). ExoPG activity showed the maximum value at pH 12.0 (3.489 U/mL) and at pH 13.0 reaction mixture was not completely soluble. Results reported in the present study showed an extremely high alkalophilic enzymatic activity and it resulted in 3.3-fold up of initial assay conditions.

Thermal stability of PG was analyzed in terms of residual activity after exposure of supernatants at temperatures between 30 and 70 °C (Fig. 8). At 30 and 40 °C enzymes were completely active for 144 h. ExoPG exhibited 50% of residual activity after 528 and 360 h at 30 and 40 °C, respectively (Fig. 8a). EndoPG retained more than 60% of its activity at the end of the evaluation (Fig. 8b). After 4 h, both PG retained more than 80% of the activity at 50 °C, whereas denaturation of enzymes was observed at 60 and 70 °C; but this behavior at higher temperatures may respond to increasing kinetic energy and enzymes probably denature due to changes in their native structure. Finally, thermal stability found in this study results applicable to waste water treatment. Effluents of pulping mill and papermaking process are disposed at temperatures not higher than 50 °C. Fig. 9 shows the effect of enzyme product of *S. halstedii* ATCC 10897 on the waste water and it resulted in strong action in viscosity reduction as product of colloidal material degradation. Viscosity reduction achieved after 15 min of enzymatic reaction were 85.2%, 90.1% and 97.6% at 30, 40 and 50 °C, respectively. Reaction at room temperature (24 °C) also proved great impact with a viscosity reduction of 82%.

Most of the microbial PG studies in literature are mesophilic and act in acidic pH because main source are fungal species (Jayani et al., 2005). PG reported with enzymatic activity at alkaline pH are produced by *Bacillus licheniformis* and act at pH 10.0 but further variation result in 90% of activity loss. In addition, its optimum temperature is 45 °C but its thermal stability is weak and retains 50% of activity up to 72 h at 30 and 40 °C (Rehman et al., 2013). Reports of pectin degrading enzymes from *Streptomyces* showed that optimum pH is between 6.0 and 7.0 but more alkalophilic reactions caused important reduction of enzymatic activity (Jacob et al., 2008a; Kar and Ray, 2011).Overall, results described in this work exceed the range of action up to pH 12.0 and cold and mesophilic reactions (5–50 °C) in extreme alkaline environment could also be implemented with PG from *S. halstedii* ATCC 10897.

4. Conclusions

In the present study, by means of a proposed method of screening, *Streptomyces halstedii* ATCC 10897 was identified as a great PG producer among several strains from different genus analyzed. Enzymatic activity was enhanced by optimizing fermentation process and enzyme assay conditions. Through sequential experimental designs maximum activity was found after 12 h of culture, initial pH 8.0 and soy peptone as the main substrate which induces PG and provides both carbon and nitrogen source. In addition, this is the first report where pectin is not used as inducer.

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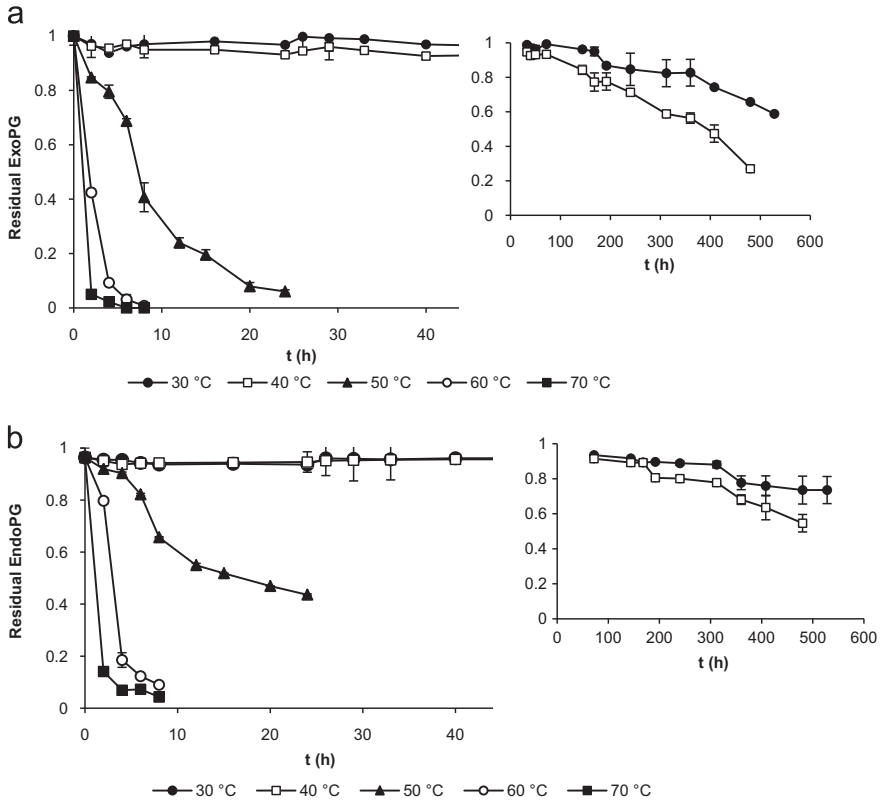

Fig. 8. Thermal PG stability. (a) Residual exoPG activity and (b) residual endoPG activity.

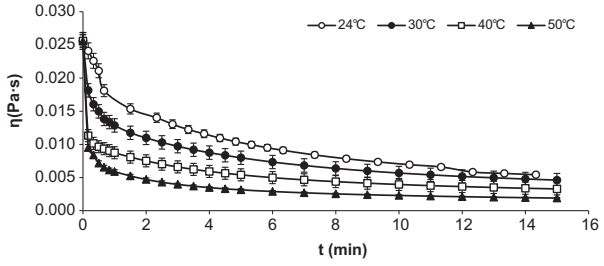

Fig. 9. Effect of PG activity in treatment of waste water from papermaking process at different temperatures.

Regarding to bioprocess design, substrates in culture medium have important impact in production costs.

The fermentation process showed an elevated productivity, with higher activity at alkaline environments and suitable thermal stability. Optimum assay conditions were at pH 12.0 and 50 °C. Approximate molecular weight by SDS-PAGE was 48 kDa. The novel extremely alkaline PG described in this work was applied successfully in treatment of pulping and papermaking waste waters.

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