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A Thermotolerant Xylan-Degrading Enzyme Is Produced by *Streptomyces malaysiensis* AMT-3 Using by-Products From the Food Industry

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HIGHLIGHTS

- A thermo-tolerant xylanase from *Streptomyces*.

Abstract: This study evaluated the production of endoxylanases by Streptomyces malaysiensis AMT-3 in submerged fermentation using by-products of the food industry at 28ºC. In shake-flasks experiments, the highest endoxylanase activity of 45.8 U.mL-1 was observed within 6 days in a medium containing (w/v) 2.5% wheat bran and 1.2% corn steep liquor. The same culture conditions were used to evaluate the enzyme production in a 2 L stirred tank reactor under different agitation (300, 450 and 600 rev.min-1) and aeration (30 and 60 L.h-1) conditions. The use of 450 rev.min-1 coupled to an aeration of 90 L.h-1 resulted on 81.3 U.mL-1 endoxylanase activity within 5 days. The effect of temperature and pH on endoxylanase activity and stability showed the highest activity at 60 ºC and pH 6.0. Zymography showed the presence of three xylanolytic bands with molecular masses of 690, 180 and 142 kDa. The results showed that the thermotolerant actinobacterial endoxylanase can be produced in high titers using by-product of the food industry.

Keywords: *Streptomyces malaysiensis* AMT-3; thermotolerant endoxylanase; wheat bran; corn steep liquor.

INTRODUCTION

Xylan is an heterogeneous carbohydrate polymer of xylose molecules, consisting of an homopolymeric backbone of β-1,4 linked D-xylopyranose units and short chain branches of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues [1,2,3]. It represents the major component of hemicelluloses found in plants, and plays a major role in holding the cell walls together [4]. Hemicellulose, together with cellulose and lignin are major components of the lignocellulosic biomass, which is the most abundant and renewable organic material on Earth [5]. However the lignocellulosic biomass, available as by-products from agriculture, forestry

and food industries, is largely unused and remains as waste. Nevertheless burning for energy generation has been used in some cases such as for the sugarcane biomass. The task of processing through economical biotechnological routes remains a challenge to be met [6]. Low cost efficient enzymes, such as xylanases, are one of the challenges to be faced as these enzymes, besides cellulases are of paramount importance for lignocelluloses breakdown, helping in the recovery of fermentable sugars [7,8]. Complete hydrolysis of xylan requires different xylanolytic enzymes such as endoxylanase, β-xylosidase, α-arabinofuranosidase, acetyl esterase, and α-glucoronidase [3]. Endoxylanases (β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidases (β-1,4-D-xyloside xylohydrolase; EC 3.2.1.37) are the main constituents of this enzymes hydrolytic system. Since endoxylanases randomly cleave the xylan backbone, the reaction products are diverse, which may include xylose, xylobiose, xylooligomers, heterodisaccharides of xylose and glucose and their positional isomers [5]. Endoxylanases are produced by many mesophilic microorganisms, and have already been found in bacteria, such as Bacillus and Clostridium species [3,4,9], in several species of Streptomyces [10–12], and in fungi such as Thermomyces, Aspergillus and Trichoderma [6,13,14].

The use of substrates containing by-products agro-industrial xylan, such as Brewers' spent grain (BSG) and wheat bran (WB) for endoxylanase production are more economical options. Brewers' spent grain is the most abundant brewing by-product, and presents 30% hemicellulose, 15% cellulose, 30% lignin and 15% proteins [15]. Wheat bran is also an abundantly available by-product of the wheat milling industry, usually accounting for 14 to 19% of the wheat grain. Its major constituents are non-starch polysaccharides (about 46%) including mainly glucuronoarabinoxylans. It also presents cellulose, a mixed linked glucan β-D-1-3 and β-D-1-4, starch (10–20%), proteins (15–22%), lignin (4–8%) and some minor constituents [16].

Streptomyces malaysiensis AMT-3 was isolated from Brazilian cerrado soil as an endoxylanase-producing strain [10,11] with enzyme levels that were compared favourably to reported data. So, the aim of the present work was to optimize endoxylanase production by Streptomyces malaysiensis, using WB or BSG as low cost xylan-containing substrates, coupled to the use of yeast extract (YE) or corn steep liquor (CSL) as a main nitrogen source.

MATERIAL AND METHODS

Microorganism propagation and maintenance

Streptomyces malaysiensis AMT-3, a non-antibiotic producer strain, was grown on agar plates containing (g.L−1): malt extract, 10.0; yeast extract, 4.0; glucose, 4.0 and agar, 15.0 at 28 ºC for 10 days. Spore suspension was maintained in 20% (v/v) glycerol at –20 ºC [17].

Fermentation conditions and culture sampling

The actinobacterial strain was cultivated in 250-mL Erlenmeyer flasks containing 50 mL of the culture medium (pH 7.0) which was inoculated with 50 μL of a spore suspension (4×109 spores·mL-1) obtained from a 12-days culture in malt extract–yeast extract agar [18]. Cells were cultivated in a salt mineral medium containing (g.L-1): KH_2PO_4, 9.0; K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 0.2; CaCl2, 0.05; $MnSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001 added of different concentrations of WB and BSG, as main substrate, and YE and CSL, as supplement substrate. A combination of the different carbon and nitrogen sources was performed in order to optimize the production of *endoxylanase* by *S. malaysiensis* AMT-3. WB and BSG at 0.5 and 2.5% (w/v), were separately combined to either YE or CSL at 0.1 and 1.2% to study the enzyme production in 16 shake-flasks fermentation runs (Table 1).

Table 1. Media composition used in the different submerged fermentation conditions

Medium	Nitrogen Sources		Carbon Sources	
	Yeast Extract (YE) % (w/v)	Corn Steep Liquor (CSL) % (w/v)	Wheat Bran (WB) % (w/v)	Brewer´s Spent Grain (BSG) % (w/v)
1	0.1	-	0.5	-
2	0.1	-	2.5	-
3	1.2	-	0.5	-
4	1.2	-	2.5	-
5	-	0.1	-	0.5
6	-	0.1	-	2.5
7	-	1.2	-	0.5
8	-	1.2	-	2.5
9	0.1	-	-	0.5
10	0.1	-	-	2.5
11	1.2	-	-	0.5
12	1.2	-	-	2.5
13	-	0.1	0.5	-
14	-	0.1	2.5	-
15	-	1.2	0.5	-
16	-	1.2	2.5	-

All media were supplemented with a salt mineral solution (see Material and Methods)

Cultures were incubated at 28 ºC (orbital shaker) at 200 rev.min-1 during 6 days with periodical sampling where the whole content of a shake flask was collected centrifuged for 10 min at 2,096 g at 4 ºC, and the supernatant passed over a 0.45 µm filtration unit. All experiments were carried out in duplicate.

Batch fermentation in 2-L bioreactor

The batch fermentation was carried out in a 2-L bioreactor (Setric Genie Industriel – France) equipped with two four-bladed disc impellers, oxygen, and pH electrodes, under the following conditions: mineral salt medium volume 1.0 L, WB 2.5% (w/v), CSL 1.2% (w/v), 30 ºC, pH 6.8. The values for aeration rate and agitation speed tested were 30 and 90 L.h-1, and 300, 450 and 600 rev.min-1 respectively, combined according to Table 2. The actinobacterial strain was inoculated with 1.0 mL of a spore suspension (4□109 spores□mL-1). Fermentation was conducted during 6 days. At periodical intervals 15.0 mL of the culture broth were collected, centrifuged for 10 min at 2,096 g at 4 ºC, and the supernatant, passed over a 0.45 µm filtration unit, was used for enzymatic assay.

Table 2. Different conditions used in bioreactor to conduct the submerged fermentation by *Streptomyces malaysiensis* AMT-3 grown on CSL 1.2% (*w/v*) and WB 2.5% (*w/v*)

Fermentation Condition	Aeration Rate ($L.h^{-1}$)	Agitation Speed ($rev.min^{-1}$)
1	30	300
2	90	300
3	90	450
4	30	600
5	90	600

Enzymatic assays

The endoxylanase (EC 3.2.1.8) activity was measured in a reaction mixture containing 750 µL of a solution of 1.5% oat spelts xylan (SIGMA®) in 50 mM sodium citrate buffer (pH 5.3) plus 0.25 □L of the supernatant. This system was incubated for 6 min at 50 ºC. The resulting reducing sugars concentration was determined by the dinitrosalicylic acid (DNS) method [19]. One unit (U) of enzymatic activity corresponded to the formation of 1 □mol of xylose equivalents released per minute, under the assay conditions [20]. All enzymatic assays were carried out in triplicate.

Optimal temperature and pH, thermostability and influence of metal ions

The culture supernatant of 6-day fermentation [WB 2.5% (w/v) and CSL 1.2% (w/v)] was used to investigate the temperature effect on enzymatic activity. The assay for endoxylanase was performed in the

temperature range of 20–90ºC, at pH 5.3, as described above. For the optimum pH determination, 50 mM buffer solutions ranging from pH 2.0 to 10.0 were used at the optimal temperature previously determined. Glycine–HCl buffer was used for pH 2.0–3.0, sodium citrate buffer for pH 3.0–6.0, phosphate buffer for pH 6.0–8.0 and tris-HCl buffer for pH 8.0–9.0, glycine–NaOH buffer for pH 9.0–10.0. To study the endoxylanase thermal stability, enzyme preparations, in 50 mM phosphate buffer pH 6.0, were incubated at 50 and 60 ºC. The residual enzymatic activity was assayed at the optimal conditions (pH 6.0 and 60 ºC).

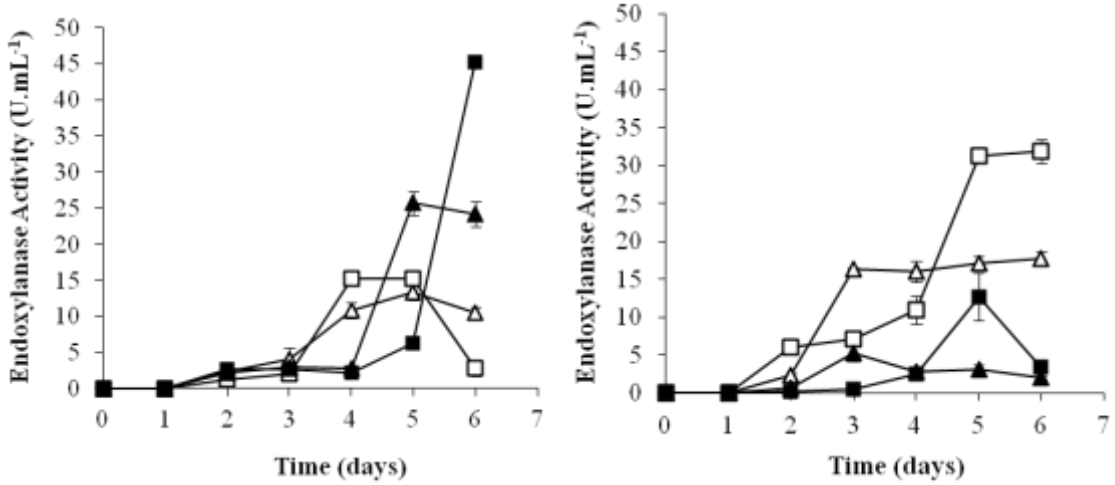
The influence of several metal ions on endoxylanase activity was evaluated performing the enzymatic assay at pH 6.0 and 60 ºC after addition of each ion (magnesium, calcium, manganese, zinc, copper, potassium, iron, cobalt and sodium in the chloride form) at 10 mM final concentration. The experiment was carried out in duplicate.

Zymograms

The extract obtained on the best growth condition in bioreactor was analysed by electrophoresis on denaturing 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel, with 0.2% (w/v) copolymerized oat spelts xylan (Sigma) as substrate. Electrophoresis was performed at constant voltage (100 V) at 4 ºC for 3 h. Samples containing 150 mU of activity were loaded. After electrophoresis, gel was incubated with Triton X-100 sodium acetate 1% buffer for 30 min in ice-bath, for SDS removal, and then incubated with 50 mM glycine-HCl buffer pH 1.5 for 10 min at 65 ºC. For the endoxylanase activity detection, the gel strip was submerged in 0.1% (w/v) Congo Red solution for 10 min, and then washed with 1M NaCl until visualisation of enzyme bands. Molecular masses were calculated from mobility of standards ranging from 67 to 669 kDa (HMW electrophoresis calibration kit – Pharmacia Biotech), and the corresponding gel was stained using the Comassie blue staining method [10,21,22].

RESULTS AND DISCUSSION

The time course for endoxylanase production by S. malaysiensis AMT-3 is presented in Figures 1 and 2. The highest endoxylanase production (45.8 U.mL-1) was observed within 6-days fermentation in the medium presenting WB 2.5% (w/v) and CSL 1.2% (w/v) as substrates (Figure 1A). The activity observed in these conditions was also 1.9-fold higher than the one observed in culture medium when using these same substrates, but with WB concentration at its minimum level (0.5% w/v), and after 5 days fermentation.



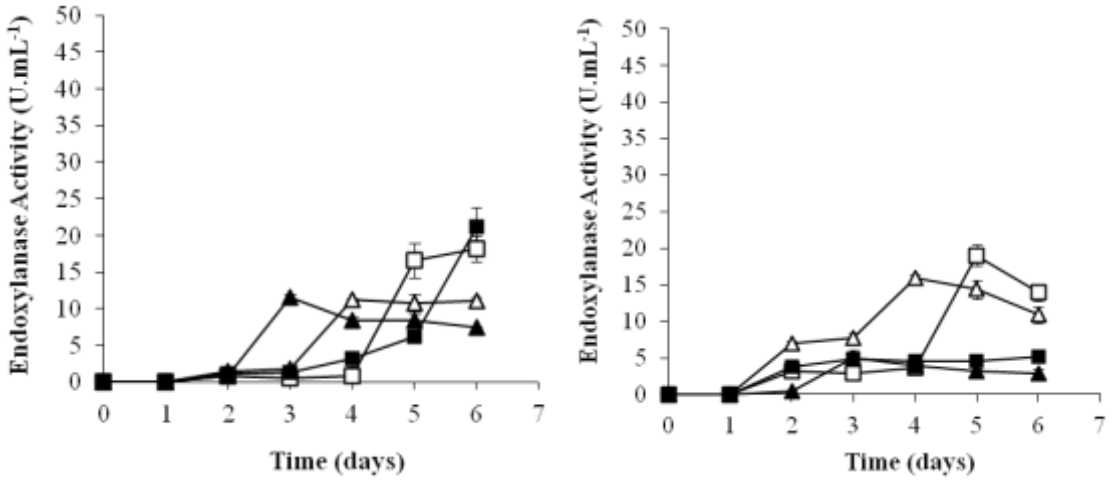
(a) (b)

Figure 1. Fermentations time-course for endoxylanase production by *S. malaysiensis* AMT-3 at 28ºC on culture media containing (A) WB and CSL; (B) WB and YE in different concentrations as described above: line 1 [WB 0.5% (*w/v*) and CSL or YE 0.1% (*w/v*)], (-△-); line 2 [WB 2.5% (*w/v*) and CSL or YE 0.1% (*w/v*)] (-□-); line 3 [WB 0.5% (*w/v*) and CSL or YE 1.2% (*w/v*)] (-▲-); line 4 [WB 2.5% (*w/v*) and CSL or YE 1.2% (*w/v*)] (-■-).

Another promising result was obtained when WB was tested with YE as nitrogen source in its lower concentration, 0.1% (w/v), when a maximum endoxylanase activity (31.85 U.mL-1) was observed within 6-days fermentation (Figure 1B). A very close endoxylanase activity (31.30 U.mL-1) was observed after 5-days fermentation. However, the high prices of this nitrogen source (approximately US$ 1,100.00/ton) preclude its use for the production of an industrial enzyme. Indeed, costs of endoxylanase production by Streptomyces malaysiensis AMT-3 can be greatly decreased using WB (approximately US$ 140.00/ton) and CSL

(approximately US$ 200.00/ton), in comparison to some fermentation media containing commercial substrates such as xylan as inducer substrate and YE as nitrogen supplement [23].

When BSG was used as the main carbon source results were around 20 U.mL-1, observed after 5–6 days fermentation (Figure 2A), using BSG 2.5% (w/v) combined with CSL 1.2% or 0.1% (w/v). The use of YE 0.1% (w/v) combined with BSG 2.5% gave results very near of those obtained using CSL instead. As already cited, its high prices preclude its used in the conditions of our experiments. Our results compare favourably to previous endoxylanase production reports, regarding high enzyme titres and potential production costs presented in this study. Based on earlier studies [10,11], carbon and nitrogen sources concentration were identified as the major factors affecting endoxylanase production by S. malaysiensis AMT-3, considering 6 days-fermentation. In comparison to a previous study [10], the endoxylanase production by S. malaysiensis AMT-3 increased 59.5% and 32.5% using WB and BSG, respectively, in the presence of CSL as nitrogen source, after 6-days fermentation. This stimulating effect was probably caused by certain nutrients present in the rich composition of CSL but absent in the formulation media formally used, as water soluble vitamins, minerals, amino acids, trace metals, some sugars and proteins [24,25]. Considering that yeast extract is an expensive source of nitrogen and other nutrients, CSL could be a cost-effective alternative for the production, on a large scale, of innumerous substances, including enzymes [25]. The best result obtained with WB, was probably caused by the high amount of arabinoxylans (70%) present in WB, in comparison to BSG (17-28% of arabinoxylan) [15,26].



(a) (b)

Figure 2. Fermentations time-course for endoxylanase production by *S. malaysiensis* AMT-3 at 28ºC on culture media containing (A) BSG and CSL; (B) BSG and YE in different concentrations as described above: line 1 [BSG 0.5% (*w/v*) and CSL or YE 0.1% (*w/v*)], (-△-); line 2 [BSG 2.5% (*w/v*) and CSL or YE 0.1% (*w/v*)] (-□-); line 3 [BSG 0.5% (*w/v*) and CSL or YE 1.2% (*w/v*)] (-▲-); line 4 [BSG 2.5% (*w/v*) and CSL or YE 1.2% (*w/v*)] (-■-).

A highest endoxylanase production (24.0 U.mL^{-1}) was observed in 1.0% (w/v) birchwood xylan after 5-days fermentation by *Streptomyces cuspidosporus* [27]. Inversely, the maximum endoxylanase activity (176 U.mL^{-1}) was reached when 15% (w/v) WB was used. However, the high viscosity of WB posed handling problems in submerged fermentation. The endoxylanase production from *Streptomyces* sp. Ab106 using sugar cane bagasse was investigated [28]. The highest endoxylanase activity (13.0 U.mL^{-1}) was detected after 5-days fermentation. The endoxylanase production by *S. malaysiensis* AMT-3 (45.4 U.mL^{-1}) in wheat bran 2.5% (w/v) after 6-days fermentation 3.5- and fold higher in comparison to those reported [28]. In other experiment, a maximal endoxylanase (31.2 U.mL^{-1}) production by *Streptomyces* sp. S38 was observed when oat spelts xylan was used as carbon source, after 100 hours [29]. Wang and coauthors [30] studied the endoxylanase production by *Streptomyces actuosus* A-151 in rice bran. The maximal endoxylanase production reached 10.3 U.mL^{-1} after 144 hours.

The endoxylanase production by *S. malaysiensis* AMT-3 was also higher than that reported for some bacteria and fungi. Ko and coauthors [31] observed a maximum endoxylanase production (15.4 U.mL^{-1}) by *Paenibacillus campinasensis* using birchwood xylan 0.5% (w/v) and casein 1.0% (w/v) whereas Nawel and coauthors [32] obtained values of 10.80 U.mL^{-1} after 35h growing *Jonesia denitrificans* BN13 in birchwood xylan 0.7% (w/v).

Concerning fungi, Meshram and coauthors [33] showed the highest endoxylanase production (34.45 $U.mL^{-1}$) when *Penicillium janthinellum* NCIM 1169 was grown using birchwood xylan for 48 hours in Mandels-Weber medium. A maximal endoxylanase production (18.44 $U.mL^{-1}$) by *Gracilibacillus* sp. TSCPVG was detected in the presence of birchwood xylan 0.75% (w/v) after 60 hours incubation [34]. Li and coauthors [35] studied the endoxylanase production by *Penicillium oxalicum* ZH-30 in wheat bran, with maximal values 13.11 $U.mL^{-1}$ detected after 144 hours.

After experiments in conical flasks, a scale-up assay was performed in a STR 2-L bioreactor, maintaining the best concentrations of wheat bran and yeast extract for endoxylanase production. In these conditions, effect of aeration rate and agitation speed on endoxylanase production was verified. The maximal enzyme production was 81.3 $U.mL^{-1}$, observed when 450 $rev.min^{-1}$ and 90 $L.h^{-1}$ were used, after 5 days-fermentation (Figure 3). Considering productivity, these conditions were the only in which endoxylanase production reached 30.5 $U.mL^{-1}$ after 2 days-fermentation, which represents a very good production in a short time. Comparing maximal endoxylanase production in conical flasks (45.8 $U.mL^{-1}$) with that obtained in STR 2-L bioreactor (81.3 $U.mL^{-1}$), it was observed an increase of 77.5%. As shown in the Figure 3, the use of high stirring speed (600 $rev.min^{-1}$) induced a decrease in endoxylanase production, possibly due to shear force, which caused a morphological stress in filamentous bacteria. However, the stimulating effect observed in 450 $rev.min^{-1}$ and 90 $L.h^{-1}$ was probably caused by the oxygen transfer constant (KLa).

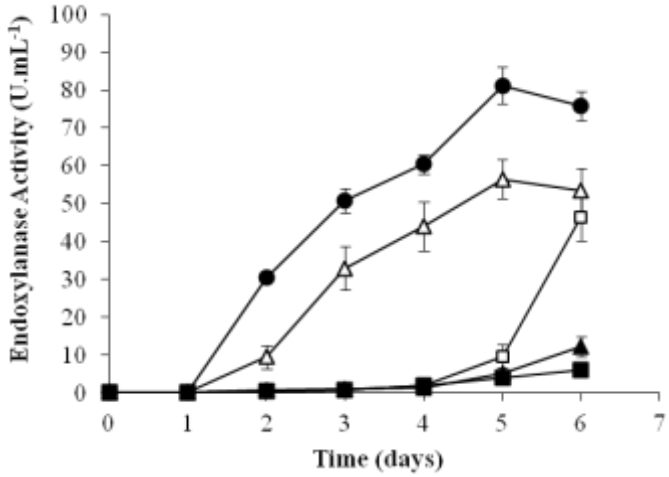
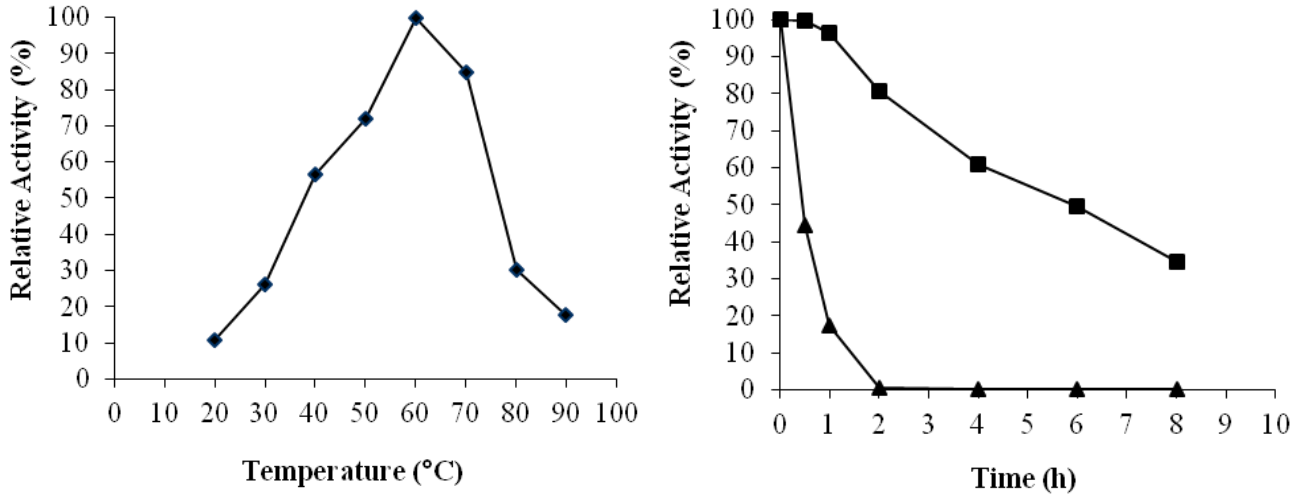


Figure 3. Fermentations time-course for endoxylanase production by *S. malaysiensis* AMT-3 at 28ºC in bioreactor STR 2l grown on CSL 1.2% (*w/v*) and WB 2.5% (*w/v*) using the following conditions: 300 $rev.min^{-1}$ and 30 $L.h^{-1}$(-△-); 300 $rev.min^{-1}$ and 90 $L.h^{-1}$ (-▲-); 450 $rev.min^{-1}$ and 90 $L.h^{-1}$ (-●-); 600 $rev.min^{-1}$ and 30 $L.h^{-1}$ (-□-); 600 $rev.min^{-1}$ and 90 $L.h^{-1}$ (-■-).

The temperature profile of endoxylanase activity from *Streptomyces malaysiensis* is presented on Figure 4A. The maximal activity was observed at 60 ºC. The profiles obtained for thermostability at 50 and 60 ºC of endoxylanase activity produced by *Streptomyces malaysiensis* are represented on Figure 4B. Concerning 50 ºC, 99.7% of its original activity was retained after 30 min, 96.4% after one hour, 80.5% after two hours and 34.4% after eight hours of pre-incubation at pH 6.0. However, at 60 ºC, only 44.4% of its original activity was retained after 30 min and it was almost totally inactivated after 2-hours pre-incubation.



(a) (b)

Figure 4. Effect of temperature (A) and thermostability (B) at 50º (■), or 60º (▲) on endoxylanase activity produced by *S. malaysiensis* AMT-3 grown on CSL 1.2% (*w/v*) and WB 2.5% (*w/v*). Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 51.9 U·mL^{-1}).

According to temperature profile, the performance of *S. malaysiensis* AMT-3 is similar to those reported for different *Streptomyces* strains [36,37]. However, Li and coauthors [38] studying the effect of temperature on endoxylanase activity from *Streptomyces rameus* L2001 observed a maximal relative activity at 70ºC. Several other bacterial and also fungal endoxylanase activity have presented maximal activity at 35ºC (*Bacillus tequilensis*) [3], 50 ºC (*Jonesia denitrificans*) [32], 52 ºC (*Bacillus subtilis*) [39], 60 ºC (*Bacillus subtilis*) [40], 65 ºC (*Clostridium thermocellum*) [9], or even 70 ºC (*Chaetomium thermophilum*) [41]. So, the optimum of temperature of 60 ºC, and also the fact that almost 90% of this activity is still retained at 70 ºC, seems to be a good characteristic for endoxylanase activity from *S. malaysiensis* AMT-3, in comparison to endoxylanases from other microorganisms, indicating a real possibility to industrial application. Wang and coauthors [30] observed that after 30 min of pre-incubation at 60 ºC, endoxylanase activity of *Streptomyces actuosus* A-151 retained more than 80% of its original activity, whereas Osiro andf coauthors [9] observed a 40% decrease of endoxylanase activity from *Clostridium thermocellum* after 48 hours of its original activity at 60 ºC. Li and coauthors [35] observed a slight decreased (around 14%) of endoxylanase (from *Penicillium oxalicum* ZH-30) activity after 60 min at 50 ºC. We observed only around 4% of decrease at the same time and temperature for endoxylanase from *S. malaysiensis* AMT-3. Xylanases from various microorganisms, such as *Bacillus firmus*, *Geobacillus thermolevorans*, *Actinomadura* sp. Cpt20, *Streptomyces* sp. S27, and *Saccharopolyspora pathunthaniensis* S582, showing optimum activity at higher (65 to 90 ºC) temperatures are reported in literature [42,43].

When testing a pH range between 2.0 and 10.0 to study the effect of pH on endoxylanase activity the optimum pH was found to be 6.0, but significant levels of activity (above 80% of maximal activity) were still detected between pH 5.0 and 7.0 (Figure 5). The behaviour of pH profile from enzymatic crude extract of *S. malaysiensis* AMT-3 is similar to that described for other endoxylanases from several *Streptomyces* [29,36,37,44] and fungi [41,45,46] strains. Nawel and coauthors [32] detected the best condition of pH for endoxylanase activity between pH 6.0 and 9.0 from *Jonesia denitrificans*, with relative activity around 100%. Our results indicate an enzymatic activity profile which could be applied in industrial processes that needs a neutral or slightly acid pH values. The pH optimum in this range is a very common characteristic in actinobacterial species.

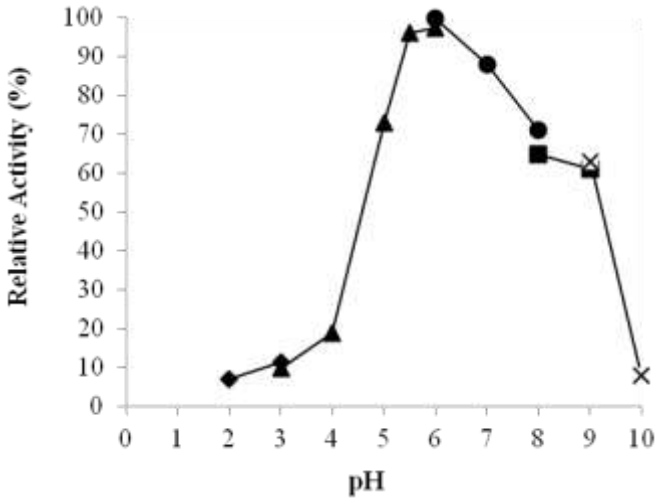


Figure 5. Effect of pH on activity (60ºC) of the endoxylanase produced by *S. malaysiensis* AMT-3 grown on CSL 1.2% (*w/v*) and WB 2.5% (*w/v*). The ionic strength for all buffers was 50mM: (-♦-), glycine–HCl; (-▲-), sodium citrate; (-●-), phosphate; (-■-), tris-HCl; (-**x**-), glycine–NaOH. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 58.1 U·mL^{-1}).

We have also investigated the effects of metal ions and EDTA on endoxylanase activity of the crude extract from *S. malaysiensis* AMT-3 (Table 3). Except for Ca^{2+} and Ba^{2+}, which almost did not interfere on enzyme activity, the presence of all other metal ions and EDTA (10mM) tested had a decreased effect on enzyme activity. The presence of EDTA, Mg^{2+}, Na^{+} and K^{+} have reduced activity to 50-65% of its original, However, a considerable decrease was observed in the presence of Cu^{2+}, Zn^{2+} and specially Mn^{2+}, which presence decreased activity to around 10-15% of its original.

Table 3. Effect of different ions on xylanolytic activity. Enzyme was produced by *S. malaysiensis* grown on CSL 1.2% (w/v) and WB 2.5% (w/v)

Ion [a]	**Relative activity** (%) [b]
Control (no addition)	100.0 ± 1.5
EDTA	58.0 ± 6.6
Mg^{2+}	54.4 ± 12.2
Zn^{2+}	14.6 ± 2.6
Cu^{2+}	13.2 ± 1.7
Na^{+}	56.3 ± 6.5
Ba^{2+}	94.5 ± 11.3
Mn^{2+}	9.5 ± 0.1
Ca^{2+}	98.9 ± 9.5
K^{+}	65.1 ± 2.4
Fe^{2+}	29.8 ± 4.6

[a] The final concentration in the reaction mixture was 10 mM.

[b] Relative activity is expressed as a percentage of Control (100% of enzyme activity = 58.1 U·mL^{-1}). The standard deviation is presented beside main values.

The study of ions effects (positive and negative) on enzymatic activity is very important to for the biochemical knowledge of the enzyme. Most endoxylanases described in literature were also found to be inhibited by Mn^{2+} [32,42,46,48,49]. This effect precludes the use of this preparation in industrial processes were these ions are present in relevant concentrations. It could be supposed that these ions exert their effect by interacting with some amino acid residues involved in the active site, which causes a change in conformation leading to enzyme inactivation. Nascimento and coauthors [10] have also observed a decrease on endoxylanase activity from *Streptomyces* sp. AMT-3 strain observed in the presence of Cu^{2+} (18.3%) and Mn^{2+} (17.7%), when xylan oat spelts was used as substrate for enzyme production. However, only a slight decrease on the endoxylanase activity was observed in the presence of Zn^{2+} (88.5%) in comparison to current results. Li and coauthors [44] detected a strong increase on endoxylanase activity from *Streptomyces rameus* L2001 in the presence of Co^{2+} (229%), Fe^{2+} (166%) and Mg^{2+} (109%). In our study none of metal ion tested had a positive effect on endoxylanase activity.

Zymogram of culture supernatant of *S. malaysiensis* AMT-03 grown in lignocellulosic biomass (WB or BSG) have shown the presence of three multiple endoxylanase isoforms (Figure 6) with estimated molecular masses of 690.0, 180.0 and 142.0 kDa, respectively. The utilization of WB and BSG as carbon sources for enzyme production seems to have lead to a higher production of high molecular weight endoxylanases.

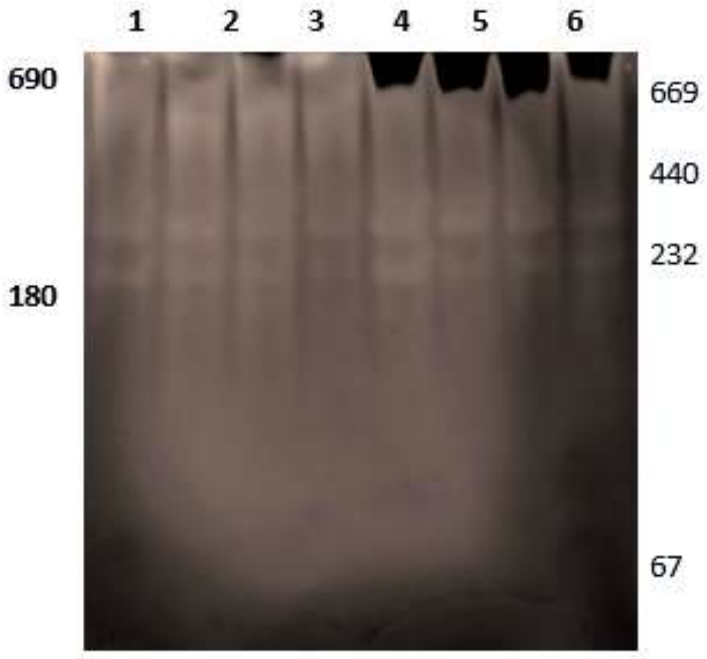


Figure 6. Zymogram analysis of endoxylanase activity in the supernatant of *Streptomyces malaysiensis* AMT-3 strain cultures grown on: lane 1 (WB + YE, medium 1, 4th day); lane 2 (WB + YE, medium 2, 5th day); lane 3 (BSG + YE, medium 2, 5th day); lane 4 (BSG + YE, medium 1, 4th day); lane 5 (WB + CSL, medium 4, 6th day); lane 6 (WB + CSL, medium 3, 5th day); lane 7 (BSG + CSL, medium 2, 6th day) and lane 8 (BSG + CSL, medium 4, 6th day). The amounts loaded in the gel contained 150 mU of endoxylanase activity. The gel containing the MW markers (kDa) was stained for proteins using the silver staining method and the molecular masses are shown on the right side of the figure.

In previous studies, *S. malaysiensis* AMT-3 was grown on different carbon sources and the expression profile of the endoxylanases was observed by zymography. As carbon sources xylan birchwood, xylan oat spelts, xylan larchwood, wheat bran, wheat germ, brewer's spent grain have been tested, and sodium nitrate was used as nitrogen source [10]. The crude extract was collected after 10 days-fermentation and three xylanolytic bands (690.0, 240.0 and 170.0 kDa) were detected in all substrates used. The small difference in the values obtained can be explained by the fermentation conditions that were different, especially the wheat bran concentration and the use of yeast extract as nitrogen source. Li and coauthors [38], for instance, detected an endoxylanase with 21.1 kDa from *Streptomyces rameus* L2001, a very low molecular mass when compared to *S. malaysiensis* AMT-3. According to literature, these high molecular weight bands could correspond to variable degrees of monomeric associations [10,49]. Also, the production of multiple endoxylanases with different specificities is common in nature, in spite of the heterogeneity and complexity of xylan, especially in actinobacterias [10,14,29].

CONCLUSION

As the use of agro-industrial by-products allowed high levels of this thermotolerant enzyme production it can be envisaged the production of a low cost biocatalyst with potential biotechnological application. These enzymes are of paramount importance for lignocellulosic biomass breakdown, assisting in the release of reducing sugars.

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