CIÊNCIA e NATURA

UFSM

Ci. e Nat., Santa Maria v.42, e9, p. 1-22, 2020

DOI: 10.5902/2179460X42741

ISSN 2179-460X

Química

Submissão 05/03/20 Aprovação 28/04/20 Publicação 11/05/20

Optimization of production and partial characterization of xylanase from a newly isolated *Bacillus amyloliquefaciens*

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ABSTRACT

This paper reports the process of production optimization and partial characterization of xylanase from a newly isolated Bacillus amyloliquefacies VR002, isolated from local soil. The microorganism exhibited maximum xylanase production when 1.0% (v/v) of inoculum size was added to culture medium with initial pH 6, 1.0% (w/v) birchwood xylan, at 35 °C after 48h of incubation. Xylanase production in different carbon sources apart from birchwood xylan and xylose did not show high production levels. Optimum pH for xylanase activity was 6.0. The enzyme was alkali-stable and retained 100% of residual activity over the pH range from 6.0 to 10.0 for 24 h at 25°C. Optimum temperature for enzyme activity was 55°C. Xylanase was 100% stable at 4°C and 25°C even after 24h of incubation, a desirable characteristic for enzyme storage. Moreover, best crude extract volume and time reaction were found to be 10 µL and 5 min, respectively. After optimization of production and activity parameters, an increase of nearly 60-fold in xylanase activity (44.12 ± 4.36 U/mL) was achieved. Characteristics of B. amyloliquefaciens VR002 xylanase are particularly desirable for biotechnological applications.

Keywords: Xylanase; Bacillus amyloliquefaciens; Optimization; Characterization

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1 INTRODUCTION

Hemicelluloses are the second most abundant polysaccharides in nature, after cellulose. Xylan which is the main hemicellulosic polysaccharide found in plant cell wall is a β-1,4 linked polymer of xylose, often with side chains of o-acetyl, α-L-arabinofuranosyl, D-α-glucuronic and phenolic acid residues (BERNIER *et al.*, 1983; COUGHLAN; HAZLEWOOD, 1993). 20-35% of the total dry mass of hardwood and annual plants constitutes of xylan (HALTRICH *et al.*, 1996). The main xylanolytic enzymes responsible for converting this polysaccharide into a more readily fermentable form are xylanases and β-xylosidase (GHOSH *et al.*, 1993).

Endo-1,4-β-xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) catalyse hydrolysis of xylan to produce a blend of xylose and xylobiose (BERNIER *et al.*, 1983). Xylanases have been widely studied due to its industrial exploitation such as baking and pulp and paper industry, clarification of juices, xylooligosaccharides production and conversion of discarded and agricultural residues into useable products. (BHALLA; BISCHOFF; SANI, 2015; BUTT *et al.*, 2008; GOWDHAMAN; PONNUSAMI, 2015; GUPTA; KAR, 2009; KUMAR *et al.*, 2017; LI *et al.*, 2020; WALIA *et al.*, 2017). Cellulases and xylanases, together with pectinases compose 20% of world enzyme market (POLIZELI *et al.*, 2005).

Many microorganisms including bacteria, yeast, actinomycetes and filamentous fungi are capable of producing xylanase (BHARDWAJ; KUMAR; VERMA, 2019; DEEKER; ROBERTS, 1975). Furthermore, they have been also found in marine algae, protozoa, snails, crustaceans, insects, and seeds of terrestrial plants (SUNNA; ANTRANIKIAN, 2008). Due to advantages like availability, structural stability and easy genetic manipulation, microbial xylanase production is preferred over plant and animal sources. Bacteria from the genus *Bacillus* are widely used for production of extracellular hemicelullases due to their rapid growth rate and capacity of secret important extracellular enzymes and proteins in the medium (ADHYARU *et al.*, 2017; AMMONEH *et al.*, 2014; COUGHLAN; HAZLEWOOD, 1993; SRINIVASAN; RELE, 1999).

An efficient fermentation is of pivotal importance for production of any industrial enzyme. Each organism or strain has its own special conditions for maximum enzyme production. Factors such as pH, temperature, incubation time, substrate concentration, among others must be studied in order to reach the production of highest amount of xylanase. Moreover, enzyme characterization such as optimum temperature, pH and stability is also essential to recognize potential biotechnological applications. In the present study, our aim was to optimize production and partially characterize xylanase from a newly isolated *Bacillus amyloliquefaciens.*

2 MATERIAL AND METHODS

2.1 Microorganism identification

Genomic DNA was isolated using QIAmp DNA Mini Kit (Qiagen). The amplification of 16S rRNA was carried out by PCR technique with primers for Eubacteria fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (WEIBURG *et al*., 1991). Amplification conditions were: 4 min of denaturation at 94 °C followed by 25 cycles at 94 °C for 1 min, primer annealing at 55°C for 30 s and extension at 60°C for 4 min. The amplified products were purified using 70% ethanol and 100% isopropanol. The nucleotide sequence was determined by using an ABI Prism 3130 Genetic Analyser automatic sequence (Applied Biosystems, CA, USA) from Núcleo de Análise Genômica – UENF. 16S rRNA and a Big Dye Terminator Kit according to the manufacturer instructions. The partial sequence of 16S rDNA were assembled using by package software Phred/Phrap/Consed (EWING, *et al*., 1998; GORDON *et al*., 1998) and subjected to BLASTn analysis against GenBank database of NCBI (National Center for Biotechnology Information, Available at: http://www.ncbi.nlm.nih.gov).

The microorganism was isolated from the garden of Escola de Engenharia Metalúrgica de Volta Redonda (EEIMVR). Stock cultures were maintained on nutrient agar and stored at 4 °C.

2.2 Cultivation of bacterial cells

Cultivation of bacterial cells was performed according to Amore *et al.*, with modifications (2015). Fifty mililiters of nutrient broth (g/L: NaCl 5.0, meat extract 1.0, yeast extract 2.0, meat peptone 5.0) was sterilized in 250 mL Erlenmeyer flasks at 121 °C for 15 min. After sterilization, the media was inoculated with a loopful of strains of *B. amyloliquefaciens* VR002 incubated at 35 °C for 18 h under 150 rpm. Vegetative cells were used as a source of inoculum throughout the experiment.

2.3 Inoculation

Inoculation process was performed according to Amore *et al.*, with modifications (2015). Fifty millilitres of culture medium (g/L: KH_2PO_4 4.0, Na_2HPO_4 4.0, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.001, $FeSO_4.7H_2O$ 0.004, yeast extract 7.0) initially with different substrate concentrations was sterilized in 250 mL Erlenmeyer flasks at 121 °C for 15 min. After sterilization, the medium was initially inoculated with 2.0% of culture cells of *B. amyloliquefaciens* VR002 grew for 18h at 35 °C under 150 rpm. The flasks were initially maintained at 35 °C under 150 rpm for 4 days.

2.4 Sample Preparation

After incubation, xylanase was harvested by centrifuging medium samples at 10600 x G for 15 min at 4 °C to remove the bacterial cells and unwanted particles. The clear cell free supernatant (crude extract) was used for xylanase assay.

2.5 Analytical procedures

Xylanase assay was performed according to Bailey *et al*., with modifications (1992). Xylanase activity was determined by measuring the amount of reducing sugars released after hydrolysis of xylan. The reaction mixture containing 0.25 mL of enzyme extract and 0.50 mL of 1.0% (w/v) birchwood xylan (Sigma) was initially incubated at 45 °C for 30 min. After incubation, the reaction was then terminated by the addition of 0.75 mL of 3,5-dinitrosalicylic acid (DNS) and heated for 5 min in boiling water bath (MILLER, 1959). After cooling, 3.5 mL of water was added to solution and absorbance of reducing sugars released was measured by spectrophotometer at 540

nm using calibration curves of D-xylose. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol of reducing sugars equivalent to D-xylose per min. Bacterial growth was monitored by measuring optical density at 660 nm in a spectrophotometer.

2.6 Optimization of xylanase production

The optimization for xylanase production was carried out for the following parameters:

1. *Incubation period*: samples were harvested in intervals of 24h to determine xylanase production;
2. *Substrate Concentration*: culture medium constituted of 0.5, 1.0 and 1.5% (w/v) birchwood xylan was studied;
3. *Growth Temperature*: the microorganism was grown at 30-45 °C with regular interval of 5 °C;
4. *pH of the media*: the pH of the culture medium was studied from 5 to 9 with regular interval of 1.0;
5. *Inoculum Size*: culture medium was inoculated at a level of 1.0, 2.0, 5.0, and 10.0% (v/v);
6. *Carbon Sources*: Xylose, galactose, fructose, maltose, lactose, sucrose, and glucose were tested as carbon source at 1.0% (w/v) concentration.

2.7 Characterization of crude enzyme

The effect of temperature on xylanase activity was determined by incubating the reaction mixture at temperatures between 40 and 70 °C with regular interval of 5 °C. Thermo stability was determined by incubating the enzyme at 4, 25, 55, 65, 75, 85, and 95°C. Xylanase residual activity was calculated after different times. Enzyme activity was assayed as described above.

The effect of pH was determined by measuring its activity at various pH value using different buffers such as sodium citrate (pH 4.0-6.0) and Tris-hydrochloride (HCl) (pH 7.0-9.0) each at 100mM. pH stability was determined by mixing equal aliquots of crude enzyme and buffers described above, followed by incubation at room

temperature (25 °C). Xylanase residual activity was measured after different times. Enzyme activity was assayed as described above.

The effect of reaction time on xylanase activity was determined by measuring its activity after 5, 10, 15, 20, 30, 40, 50, and 60 min. Reactional mixture was prepared in 100 mM sodium citrate buffer (pH 6.0) and incubated at 55 °C. Enzyme activity was assayed as described above.

The crude extract volume was tested in order to examine its effect on xylanase activity. Volumes of 10, 25, 50, 75, 150, 250 µL were incubated for 5 min, at 55 °C, in 0.50 mL 1.0% (w/v) birchwood xylan prepared in 100 mM sodium citrate buffer (pH 6.0). In order for all samples have the same volume (0.75 mL) it was used different quantities of water for completing the final volume. Enzyme activity was assayed by DNS method as described above.

2.8 Statistical analysis

The experimental design was randomized with three replicates.

3 RESULTS AND DISCUSSION

3.1 Microorganism identification

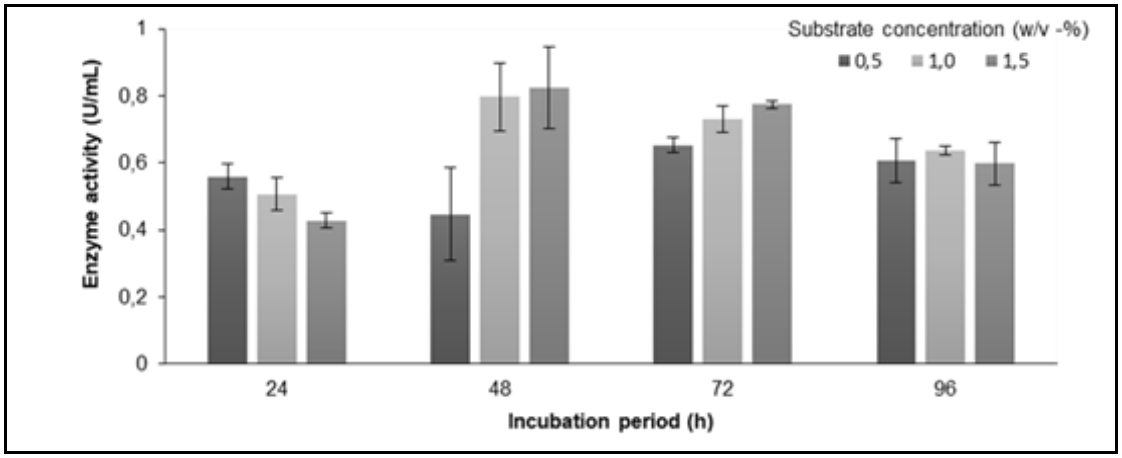
Analysis of 16S rRNA has shown 100% homology with *B. amyloliquefaciens* strains. Thus, the isolated microorganism was identified as *B. amayloliquefaciens* and designated as *B. amyloliquefaciens* VR002.

3.2 Effect of incubation time and substrate concentration on xylanase production

Analysis of effects of substrate concentration and incubation period on xylanase production revealed (Fig.1) that maximum production by *B. amyloliquefaciens* occurred after 48 h at 1.0 and 1.5% (w/v) substrate concentration (0.72 ± 0.10 and 0.75 ± 0.12 U/mL, respectively). As similar results were observed between these concentrations, 1.0% (w/v) was chosen to proceed the experiments in order to reduce the use of substrate. Further incubation at both 1.0 and 1.5% (w/v) did

not affect the enzyme production. Regardless of the period, 0.5% (w/v) was not capable of supporting high production levels. Results reported by Bocchini *et al*. (2003) and Prakash *et al*. (2012) also showed 48h as optimum period to xylanase production. In contrast, *Bacillus* such as *B. tequilensis* ARMATI (KHUSRO *et al.*, 2016) and *B. pumilus* ASH (BATTAN *et al.*, 2007) were capable of reaching maximum production spending just 24 and 26h, respectively, while *Arthrobacter* sp. MTCC 6915 (MURUGAN *et al.*, 2011) demanded 96h to reach its peak enzyme production. According to Kulkarni *et al*. (1999) xylanases are commonly expressed in the end of exponential phase. Time-course profile of enzyme production depends on factors such as source of isolation, types, genetic makeup of strains, and their cultivation conditions (KHUSRO *et al.*, 2016). Regarding substrate concentration, similar observations were also recorded for fungi *Aspergillus foetidus* MTCC 4898 (SHAH; MADAMWAR, 2005) and *Clostridium absonum* CFR-702 (RANI; NAND, 2000). Substrate cost plays an important part in the economy of an enzyme production process (HALTRICH *et al.*, 1996).

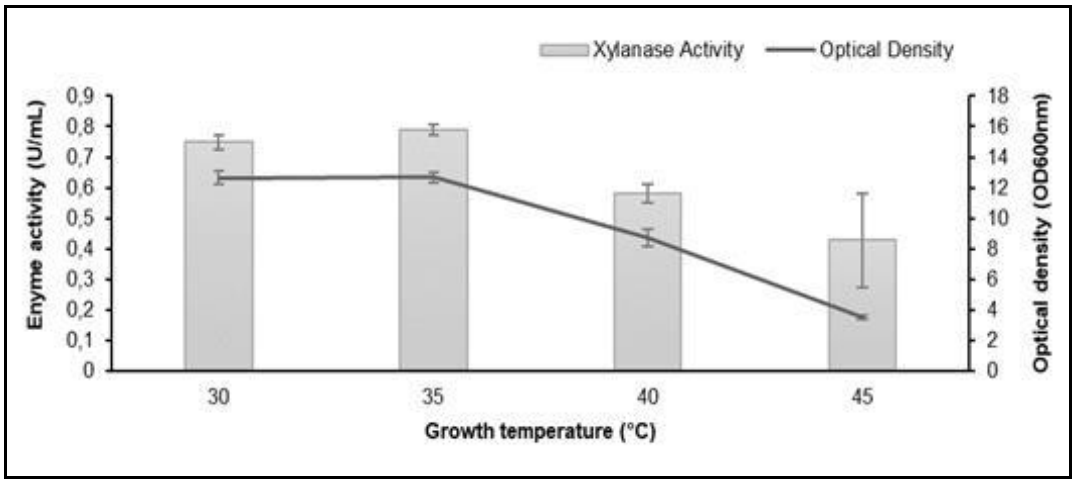
Figure 1 - Effect of substrate concentration and incubation period on xylanase production. Enzyme production was conducted at 35 °C, 150 rpm, 2.0% (v/v) inoculum size and initial pH 7.0. Enzyme activity was measured using standard assay. Bacterial growth was measured as described above. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.3 Effect of growth temperature on xylanase production and bacterial growth

Cultivation temperature affects not only the growth rate of an organism, but also has an evident effect on xylanase production levels (KAVYA; PADMAVATHI, 2009). Regarding growth temperature, xylanase production was maximum at 35 °C (0.78 ± 0.01 U/mL), which bacterial growth was maximum too (12.66 ± 0.33 OD_{600nm}) (Fig.2). By increasing growth temperature (40-45 °C) xylanase production was reduced by 53% (0.42 ± 0.15 U/mL) and bacterial growth decreased by 72% (3.55 ± 0.12 OD_{600nm}). Temperature may affect the secretion of extracellular enzymes possibly due to alterations of physical properties of the cell membrane (RAHMAN *et al.*, 2005). Moreover, lower growth and enzyme production at higher temperatures might have happened due to the lack of dissolved oxygen in the medium. Different *Bacillus* species also exhibited optimum temperature about 35-37°C (AKHAVAN SEPAHY; GHAZI; AKHAVAN SEPAHY, 2011; BATTAN *et al.*, 2007; NAGAR *et al.*, 2010; PRAKASH *et al.*, 2012). Nonetheless, this temperature range contrasts with results reported such as *Geobacillus* sp. (BHALLA; BISCHOFF; SANI, 2015) strain WSUCF1, showing maximum amount of xylanase at 60 °C, and *B. circulans* BL53, that obtained highest production at 25 °C (HECK; DE BARROS SOARES; ZÁCHIA AYUB, 2005). *Geobacillus* was isolated from a composting facility while *B. circulans* was isolated from aquatic ambient. The environment where the microorganism is isolated strongly affects its temperature profile of xylanase production.

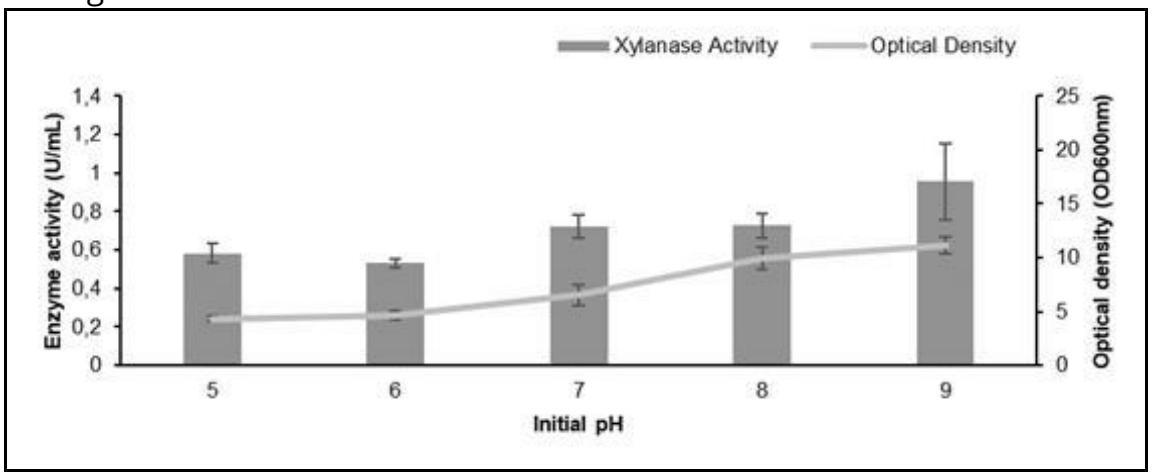
Figure 2 - Effect of growth temperature on xylanase production and bacterial growth. Enzyme production were conducted after 48h of incubation, 150 rpm, 2.0% (v/v) inoculum size, pH 7.0 and 1.0% (w/v) birchwood xylan. Enzyme activity was measured using standard assay. Bacterial growth was measured as described above. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.4 Effect of pH media on xylanase production and bacterial growth

Several bacteria are dependent on pH and produce xylanase under high pH (AKHAVAN SEPAHY; GHAZI; AKHAVAN SEPAHY, 2011; BATTAN *et al.*, 2007; IRFAN *et al.*, 2015; RANI; NAND, 2000). Many enzymatic processes and transport of various components across the cell membrane are strongly affected by the pH of the medium (MOON; PARULEKAR, 1991). Results revealed that *B. amyloliquefaciens* exhibited its peak production (0.95 ± 0.19 U/mL) at pH 9.0 (Fig.3). Slightly acid conditions resulted in low xylanase production – about 40% lower (0.58 ± 0.05 U/mL). By increasing pH, bacterial growth also increased and reached its peak at pH 9 (11.13 ± 0.75 OD_{600nm}). Results of pH and temperature indicated that *B. amyloliquefaciens* may have its profile of xylanase production strongly related to its growth rate. *B. pumilus* MK001 (KAPOOR; NAIR; KUHAD, 2008) and *Bacillus licheniformis* P11(C) (BAJAJ; MANHAS, 2012) also showed maximum xylanase production at pH 9.0. Some bacteria such as *B. pumilus* SV-205 (NAGAR *et al.*, 2012) and *B. holodurans* PPKS-2 (PRAKASH *et al.*, 2012) exhibited optimal xylanase production at higher pH levels (pH 10 and pH 11, respectively). On the other hand, the enzyme production by *B. pumilus* SV-85S (NAGAR *et al.*, 2010) and *B. circulans* AB 16 (DHILLON *et al.*, 2000) reached maximum levels at pH 6.0.

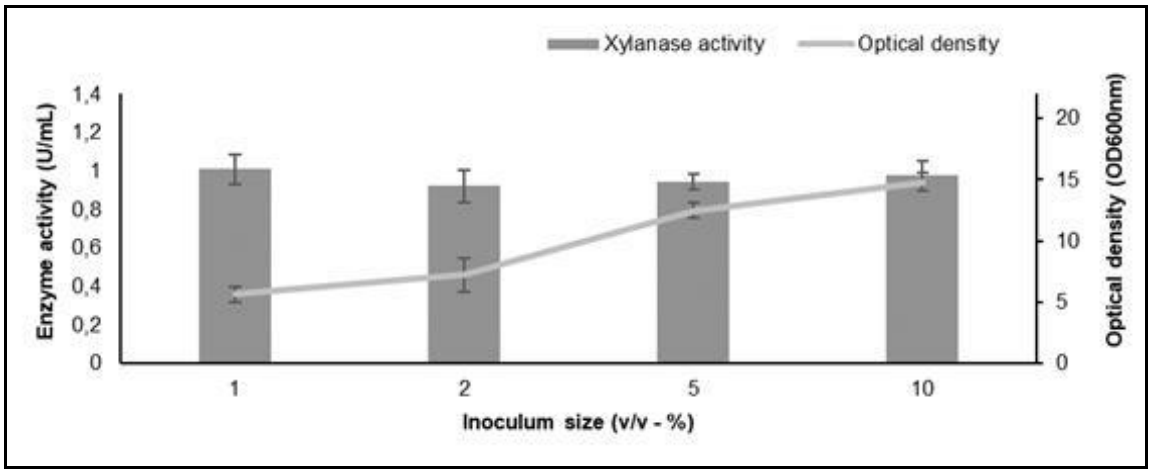
Figure 3 - Effect of initial pH on xylanase production and bacterial growth. Enzyme production was conducted at 35 °C during 48h of incubation, 150 rpm, 2.0% (v/v) inoculum size and 1.0% (w/v) birchwood xylan concentration. Enzyme activity was measured using standard assay. Bacterial growth was measured as described above. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.5 Effect of inoculum size on xylanase production and bacterial growth

Different inoculum sizes (1.0-10.0%) were tested for enhancing production of xylanase by *B. amyloliquefaciens*. Results (Fig.4) revelead that xylanase production was optimized (1.00 ± 0.07 U/mL) when 1% (v/v) of inoculum of *B. amyloliquefaciens* was added to the production medium. Further increase in inoculum size directly affected bacterial growth, however xylanase production was maintained. Researchers reported the use of 1.0-3.0% (v/v) inoculum for hyper production of xylanase (ADHYARU *et al.*, 2017; BATTAN *et al.*, 2007; IRFAN *et al.*, 2015; NAGAR *et al.*, 2012). According to Lincoln (1960) higher concentration of inoculum is not desirable in industrial fermentation. This may be due to the depletion of nutrients available from the fermentation medium, reducing enzyme synthesis (KHUSRO *et al.*, 2016).

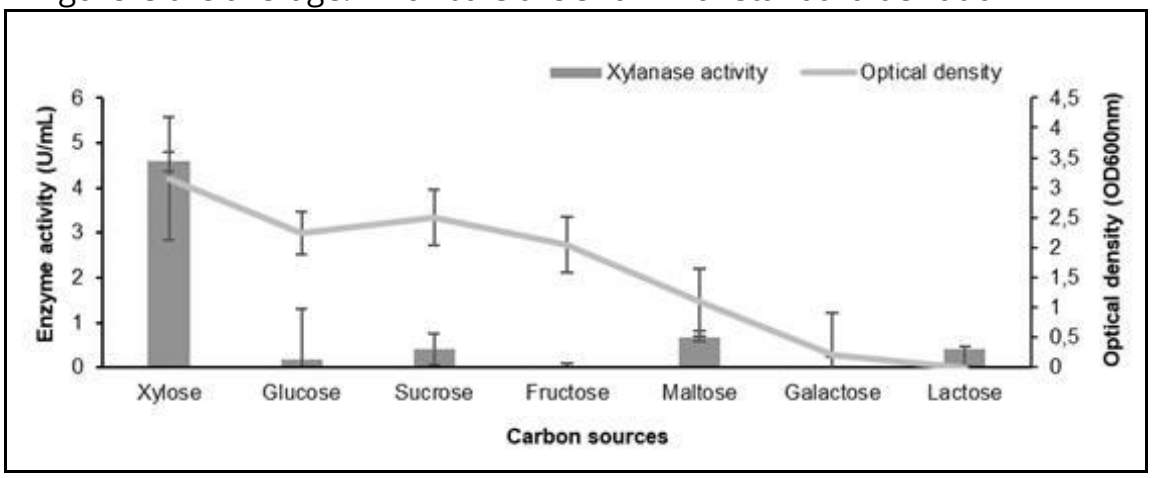
Figure 4 - Effect of inoculum size on xylanase production and bacterial growth. Enzyme production was conducted at 35 °C during 48h of incubation, 150 rpm, pH 9.0 and 1.0% (w/v) birchwood xylan concentration. Enzyme activity was measured using standard assay. Bacterial growth was measured as described above. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.6 Effect of different carbon sources on xylanase production and bacterial growth

Carbon source is one of the essential constituents of the microbial fermentation medium which affects the overall cellular growth and metabolism (NAGAR *et al.*, 2012) (10). The present study about effect of different carbohydrates on xylanase production revealed that except for xylose (4.58 ± 1.10 U/mL), all tested sugars repressed enzyme production (Fig.5). As xylan is unable to directly enter the cell, it may not be a direct inducer to xylanase synthesis. However, hydrolysis products like xylobiose, xylotriose, and so on, generated by constitutive xylanase action may act as inducers (KAPOOR; NAIR; KUHAD, 2008). Only bacterial growth was observed when the medium was supplemented with Glucose, Sucrose, Fructose and Maltose (Fig.5). The availability of rapidly metabolizable carbon sources causes catabolite repression which affects the synthesis of many enzymes (LENGELER, 2013). On the other hand, *B. amyloliquefaciens* was unable to grow and produce xylanase using Lactose and Galactose as carbon source. Studies reported by Nagar *et al*. (2012) and Kapoor *et al*. (2008) demonstrated that birchwood xylan and oat spelt xylan supported high production. In contrast, maltose was the best substrate for xylanase production by *B. circulans* D1 as reported by Bocchini *et al*. (2008).

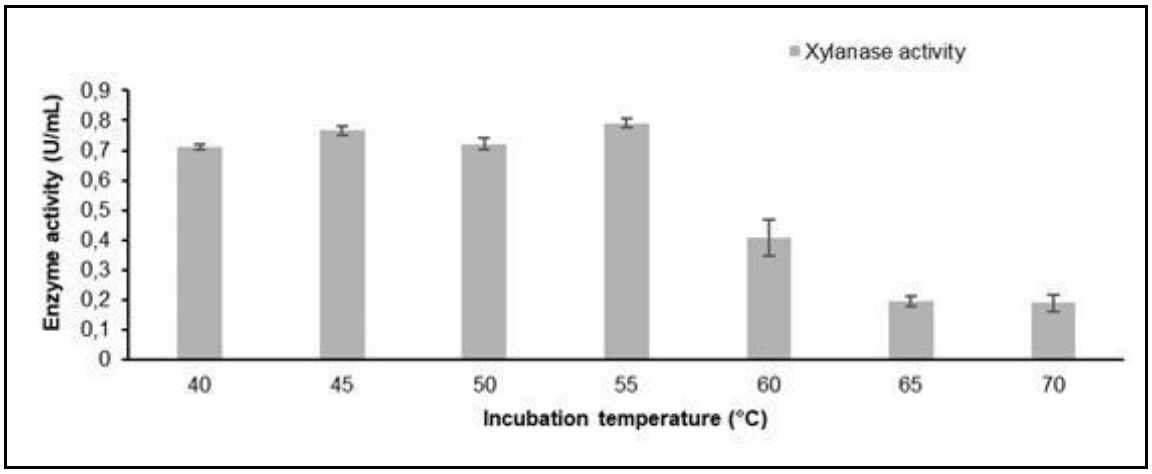
Figure 5 - Effect of different carbon sources on xylanase production and bacterial growth. Enzyme production was conducted at 35 °C during 48h of incubation, 150 rpm, pH 9.0 and 1.0% (v/v) inoculum size. Enzyme activity was measured using standard assay, except for incubation time of 5 min. Bacterial growth was measured as described above. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.7 Effect of temperature on xylanase activity

Results about effect of temperature on xylanase activity from *B. amyloliquefaciens* showed that optimum temperature was found to be 55 °C (0.78 ± 0,01 U/mL) as depicted in Fig.6. Further increase in temperature reduced gradually xylanase activity - at 70 °C was observed only 30% (0.18 ± 0.02 U/mL) of maximum activity found at 55 °C. High temperatures interfere the interactions between amino acids residues of enzymes resulting in denaturation and loss of catalytic activity. Most of the bacterial xylanases showed optimum temperature at 50-65°C (ADHYARU *et al.*, 2017; AKHAVAN SEPAHY; GHAZI; AKHAVAN SEPAHY, 2011; BAJAJ; MANHAS, 2012; MURUGAN *et al.*, 2011; NAGAR *et al.*, 2010; NASCIMENTO *et al.*, 2002; RATANAKHANOKCHAI; KYU; TANTICHAROEN, 1999). Nonetheless, Lama *et al*. (2004) and Dhillon *et al*. (2000) *et al*. reported xylanases that exhibited maximum activity at 80 °C.

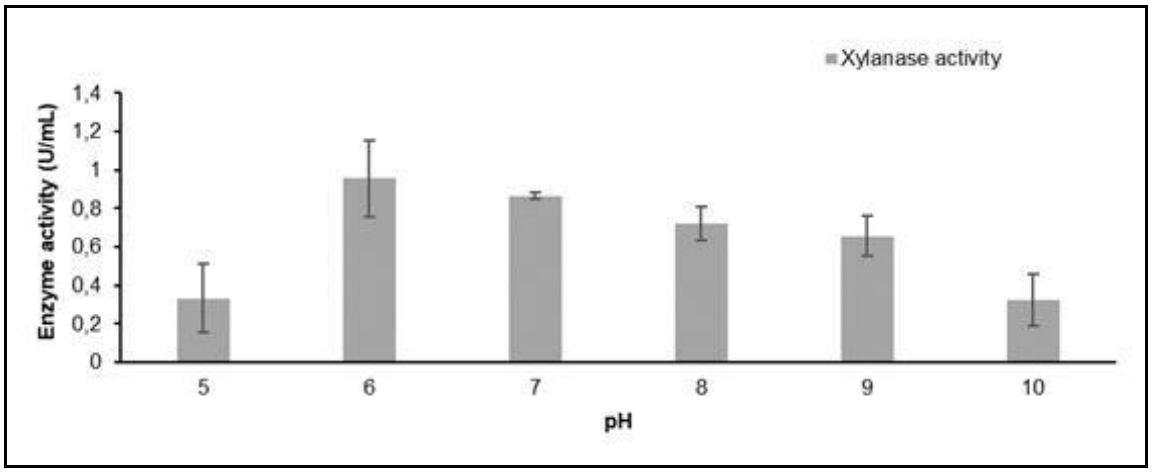
Figure 6 - Effect of temperature on xylanase activity. Enzyme activity was measured using standard assay, except temperature was as indicated in figure. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.8 Effect of pH on xylanase activity

Enzyme activity is significantly influenced by pH. Substrate binding and catalysis are often dependent upon charge distribution on both substrate and in particular enzymes (SHAH; MADAMWAR, 2005). Results concerning the effect of pH on xylanase activity are shown on Fig. 7. Although optimum pH was found to be 6.0 (0.95 ± 0.19 U/mL), near levels of activity was also observed in pH 7.0 (0.86 ± 0.01 U/mL). Different researchers reported optimum pH between 6.0 and 6.5 not only for *Bacillus* sp. (KHASIN; ALCHANATI; SHOHAM, 1993; NAGAR *et al.*, 2010, 2012) but also for another bacterial genus such as *Geobacillus*, *Streptomyces*, and *Clostridium* (BÉRENGER *et al.*, 2010; BHALLA; BISCHOFF; SANI, 2015; NASCIMENTO *et al.*, 2002). In contrast, xylanase for *B. halodurans* PPKS-2 exhibited optimum activity at pH 11.0 according to Prakash *et al*. (2012).

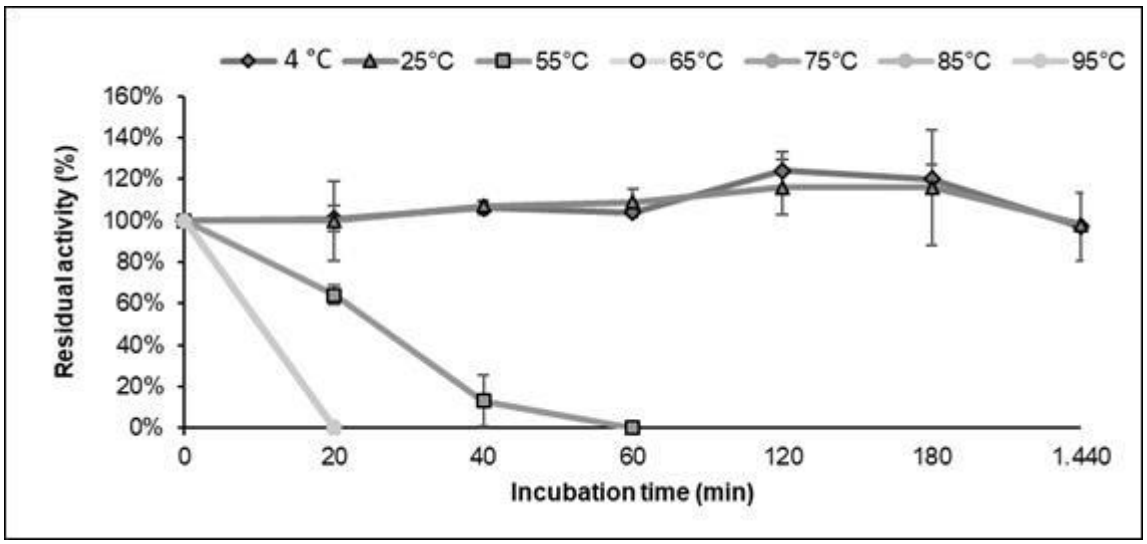
Figure 7 - Effect of pH on xylanase activity using different buffers: pH 5.0-6.0, sodium citrate; pH 7.0-10.0, Tris-hydrochloride (HCl) each at 100 mM. Enzyme activity was measured using standard assay, except that pH was as indicated in the figure. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.9 Xylanase thermostability

As stated by Scandurra *et al*. (1998) thermostability seems to be a property acquired by a protein through many small structural modifications obtained with the exchange of some amino acids and the modulation of the canonical forces (hydrogen bonds, ion-pairs and hydrophobic interactions) found in all proteins. Thermostability tests showed that crude xylanase was stable at 4 and 25 °C after 24h of incubation (Fig.8). According to (SHAH; MADAMWAR, 2005) storage of the enzymes at room and/or refrigerated temperature considerably keeping their activity is a desirable characteristic in industrial applications. Xylanase produced by *B. amyloliquefaciens* has this potential. The enzyme exhibited 64% of residual activity after 20 min and 20% after 40 min at 55 °C. Enzyme stability was largely reduced above 55 °C after 5 min. Nagar *et al*. (2010) demonstrated that xylanase by *B. pumilus* SV-85S held 40% and 30% of activity at 65 and 70 °C, respectively, after 30 min. Xylanase from *B. tequilensis* strain ARMATI (KHUSRO *et al.*, 2016) retained up to 51% of activity at 60 °C after 4h of incubation. Kumar *et al*. (2017) reported 65% of activity retention at 50 °C after incubation period of 180 min.

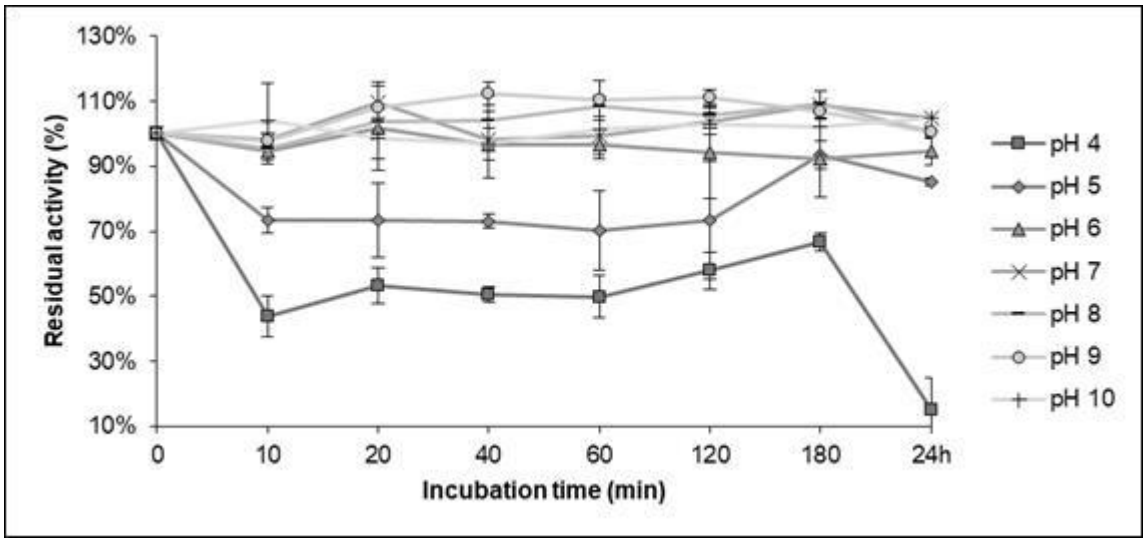
Figure 8 - Temperature stability of crude xylanase extract. The enzyme was incubated at 4 ( ), 25 ( ), 55 ( ), 65 ( ), 75 ( ), 85 ( ) and 95 ( ) °C. Residual activity was assayed after time intervals of 20, 40, 60, 120, 180 min and 1440 min (24h). The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.10 Xylanase pH stability

pH stability studies showed that xylanase was alkali-stable (Fig.9). The enzyme retained 100% of activity at pH range 6.0-10.0 even after 24h of incubation at room temperature. The presence of charged amino acid residues makes the enzymes to be stable at alkaline pH (KHUSRO *et al.*, 2016). *B. amyloliquefaciens* may be a good source for biotechnological applications because of its enzyme stability at alkaline pH. Nonetheless, at pH 5.0, xylanase retained 85% of residual activity, and at pH 4.0 only 15% of residual activity after 24h of incubation. Xylanase from *B. pumilus* SV-85S showed stability at pH 5.0 to 11.0 after 1h of incubation (NAGAR *et al.*, 2010). Ratanakhanokchai *et al*. (1999) reported that xylan-binding xylanase had stability at pH 5.0 to 9.0 after an incubation period of 24h. Xylanase from *B.subtilis* ASH was stable over the pH range 6.0-9.0 after 10 min of pre-incubation (SANGHI *et al.*, 2010).

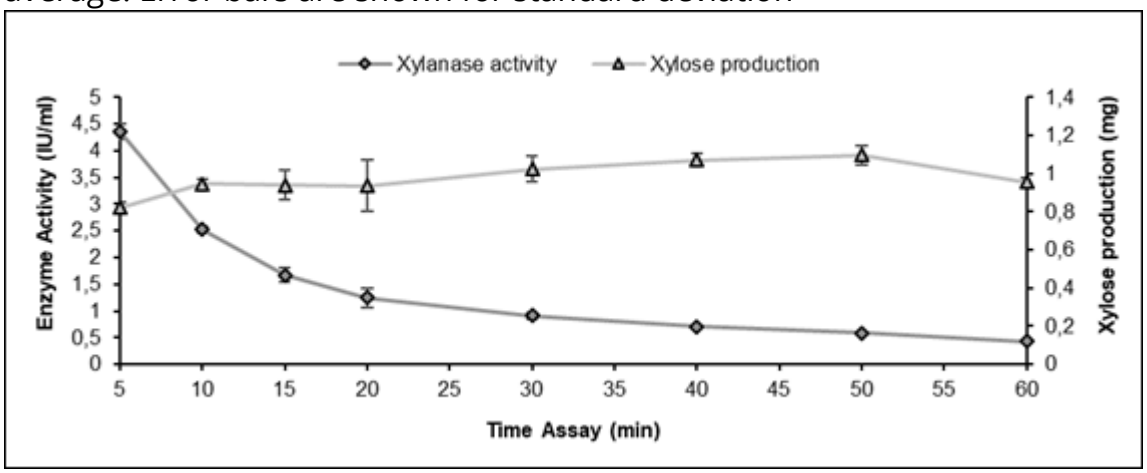
Figure. 9 - pH stability of crude xylanase extract. The enzyme was incubated at 25°C and diluted in different buffers: pH 4.0 ( ), 5.0 ( ), 6.0 ( ), sodium citrate; pH 7.0 (X), 8.0 (-), 9.0 ( ), and 10.0 (+), Tris-hydrochloride (HCl) each at 100 mM. Residual activity was assayed after time intervals of 20, 40, 60, 120, 180 min and 1440 min (24 h). The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.11 Effect of time reaction on xylanase activity

Another important factor of enzyme assay is time reaction. Fig.10 depicts the effect of different time assays on xylanase activity and xylose production. Highest activity (4.36 ± 0.14 U/mL) occurred after 5 min of incubation. Further increase in time assay resulted in depletion of activity – up to 9% after 60 min. Measurement of reducing sugars showed that xylose production slightly increased in function of time, but not enough to keep enzyme activity constant. Xylanase produced by fungi *Pleurotus eryngii* exhibited maximum activity after 15 min and then started to decrease (ALTAF *et al.*, 2016).

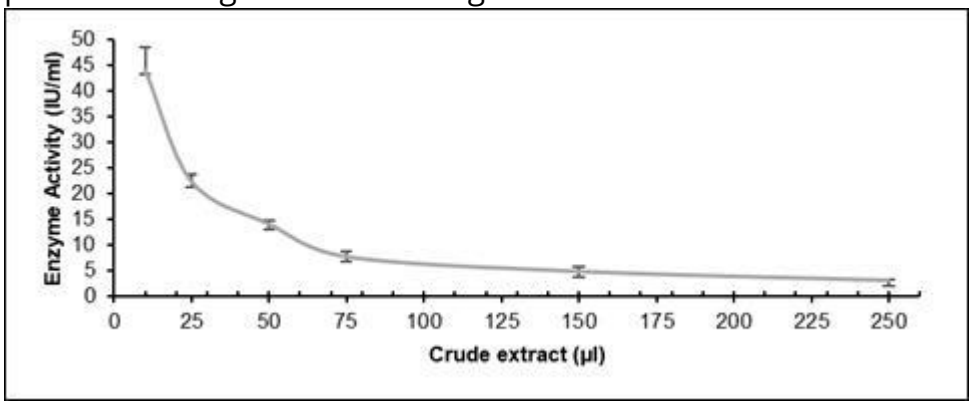
Figure 10 - Effect of reaction time on crude xylanase extract. 0.25 mL of xylanase was incubated at 55°C and diluted in sodium citrate buffer (pH 6.0). After different times, the reaction was stopped by adding DNS and absorbance was measured at 540 nm. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



3.12 Effect of crude extract volume on xylanase activity

The reaction velocity can be regulated by varying the amount of enzyme. Results concerning the effect of crude extract volume on xylanase activity are shown in Fig.11. Enzyme activity and crude extract volume were inversely proportional and highest activity (44.12 ± 4.36 U/mL) was obtained when lowest volume was used. According to Reymond *et al*. (2009) the concentration of enzyme involved in reaction should be as low as possible, only catalytic amounts are need. Occupation of the binding sites occurs more efficiently at lower concentrations. By increasing amount of enzyme, enzyme activity was reduced up to 7% (3.01 ± 0.18 U/mL) (Fig.11). On the other hand, Altaf *et al*. (2016) reported maximum fungal xylanase activity using 500 µL enzyme volume while minimum activity was observed when lowest amount of enzyme volume was used.

Figure11 - Effect of crude extract volume on xylanase activity. Different crude extract volumes diluted in sodium citrate buffer (pH 6.0) were incubated at 55°C. After 5 min of incubation the reaction was stopped by adding DNS reagent and absorbance was measured at 540 nm. The experiments were performed in triplicate and data presented in figure is the average. Error bars are shown for standard deviation



4 CONCLUSION

In this study, it was possible to determine the optimal conditions to reach maximum production and partially characterize xylanase from a newly isolated *Bacillus amyloliquefaciens* VR002. Maximum production was reached with the fermentation conditions of substrate concentration (1.0% - w/v), incubation temperature (35°C), initial pH (9.0), inoculum size (1.0% - v/v) and incubation period (48h). Maximum activity occurred after 5 min of incubation at 55°C, pH 6.0, using 10 µL of enzyme volume. After optimization of production and partial characterization, we could increase activity in 60-fold. Xylanase was alkali-stable and stable at room temperature for 24h, both desirable characteristics for industrial applications. Thus, *B. amyloliquefaciens* VR002 is a microorganism capable of producing xylanase with strong biotechnological potential.

ACKNOWLEDGMENT

Thanks to reviewers, collaborators and development agencies.

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