Optimization of Culture Conditions for Production of Cellulase by *Stenotrophomonas maltophilia*

Carlos E. Molina-Guerrero,[a,c,*] Guadalupe de la Rosa,[b,c] Jaquelina González-Castañeda,[d] Yazmín Sánchez,[d] Hiram Castillo-Michel,[e] Idania Valdez-Vazquez,[f] Edgar Balcazar,[g] and Iván Salmerón [a]

Stenotrophomonas maltophilia is a microorganism that exhibits a wide range of applications. In this study, a Box-Behnken design was used to determine the optimum parameters for maximizing the cellulase production by *S. maltophilia* isolated from soil. The factors that were evaluated were the pH (3 to 10), carbon source (0.5 wt.% to 1.5 wt.% carboxymethylcellulose), and nitrogen source (0.5 wt.% to 1.5 wt.% yeast extract). The results showed that a maximum cellulase activity of 0.082 U/mL was achieved at a pH of 6.3, a 0.72% carbon source concentration, and a 1.5% nitrogen source concentration. *Stenotrophomonas maltophilia* was grown on sterilized wheat straw (WS) to evaluate the production of reducing sugars as a potential application in a biorefinery scheme. After 72 h of incubation at 30 °C, the final concentration of reducing sugars was 150 mg/L ± 2 mg/L and the corresponding yield was 10.16%. The hydrolyzed WS was mapped by synchrotron micro Fourier transform infrared spectroscopy, where a significant reduction in the characteristic signals for hemicellulose and cellulose was observed.

Contact information: a: Universidad Autónoma de Chihuahua, Facultad de Ciencias Química, Circuito Universitario, Campus Universitario II, Chihuahua, Chihuahua, 31125, México; b: UC Center for Environmental Implications of Nanotechnology (UC CEIN), The University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968, USA; c: Universidad de Guanajuato, Depto. de Ingenierías Química, Electrónica y Biomédica, Loma del Bosque 103, Col. Loma del Campestre, León, Guanajuato, 37150, México; d: Universidad de Guanajuato, Depto. de Ingeniería Ambiental, DICIVA, Carretera Irapuato-Silao Km 9.5, El Copal, Irapuato, Guanajuato, 36625, México; e: European Synchrotron Radiation Facility, ID21. BP220 – F-38000 Grenoble, France; f: Universidad Nacional Autónoma de México, Unidad Académica Juriquilla, Instituto de Ingeniería, Blvd. Juriquilla 3001, C.P. 76230, Querétaro, México; g: Universidad de Guadalajara, Departamento de Farmacología, Blvd. M. García Barragán # 1451, Guadalajara, Jalisco C.P. 44430, Mexico;
* Corresponding author: cemolina@uach.mx

INTRODUCTION

There are a large number of studies in the literature that focus on the development of biofuels with the purpose of finding renewable sources of energy that can diversify the energy supply and mitigate the negative impact of CO_2 emissions from the use of fossil fuels. One alternative to fossil fuels is the development of second generation biofuels, such as bioethanol, where hydrolytic enzymes are a key element for the conversion of lignocellulosic biomasses. Lignocellulolytic microorganisms can metabolize a wide range of biomass substrates and are widespread in the environment (Mosier *et al*. 2005). Recently, research has focused on finding efficient lignocellulolytic enzyme systems, with

the main goal of reaching a sustainable bioconversion. Until now, most cellulases have been isolated from fungal organisms, such as *Trichoderma* and *Aspergillus*, as well as anaerobic bacteria, such as *Clostridium* (Galperin 2008; Maki *et al.* 2009). However, cellulase enzymes that are produced by aerobic bacteria have received less attention. *Stenotrophomonas maltophilia* is a non-fermenting, gram-negative bacterium that is present in plants, soil, and water (Alonso *et al.* 2000; Ryan *et al.* 2009; Delafont *et al.* 2013). Because of the beneficial interactions with plants promoting plant growth, this gram-negative bacterium has become important for biotechnological applications in agriculture. Dantur *et al.* (2015) reported that *Stenotrophomonas* is not a phytopathogenic species, in contrast with *Xanthomonas* and *Xylella*, which have similar phylogenetic information. For example, *S. maltophilia* has been applied in the biological control of fungal plant diseases (Berg *et al.* 1996; Dunne *et al.* 2000). Additionally, there are a large number of studies in the literature on the industrial applications of *S. maltophilia* for the production of lipases, biosurfactants, keratinolytic enzymes, and proteases (Jeong *et al.* 2010; Ribitsch *et al.* 2012; Hemlata *et al.* 2015; Neethu *et al.* 2015). Furthermore, *S. maltophilia* is a microorganism that has the ability to produce extracellular cellulases (Huang *et al.* 2012). López-Mondéjar *et al.* (2016) published a study that shows that *S. maltophilia* possesses the genetic information required for the expression of cellulases and hemicellulases. Raj *et al.* (2013) studied *S. maltophilia* and evaluated the effects of different agro-industrial residues, alternative carbon and nitrogen sources, and effect of pH values on the xylanase production. Their results showed that the highest xylanase activity was achieved with wheat bran (26.4 IU/mL ± 0.6 IU/mL), yeast extract (YE) (25 IU/mL ± 0.6 IU/mL), and a pH of 8 (23.8 IU/mL ± 0.4 IU/mL). More recently, Dantur *et al.* (2015) isolated a strain of *S. maltophilia* that was capable of cellulase production using carboxymethylcellulose (CMC) as a carbon source and obtained a modest CMCase activity of 1 µU/mL after 5 d of incubation at 30 °C. However, they did not optimize the operative parameters for cellulase production.

In this study, the effects of the carbon and nitrogen sources, as well as the pH, on the cellulase activity in cultures of *S. maltophilia* were determined. A Box-Behnken design (BBD) was used to determine the optimum parameters for maximizing the cellulase production. Additionally, the transformation of wheat straw (WS) to reducing sugars by *S. maltophilia* was studied for possible application in a biorefinery scheme. The hydrolyzed WS was mapped with synchrotron micro Fourier transform infrared (FTIR) spectroscopy.

EXPERIMENTAL

Screening and Isolation

Reforested soil from Guanajuato, Mexico, served the purpose to bio-screen the hydrolytic microorganisms. A 5-g soil sample was diluted in 100 mL of sterilized 10 mM phosphate buffer. Sequential dilutions were performed until a concentration of 10^{-4} was reached. One milliliter of the resulting solution was spread onto an agar plate with CMC as the sole carbon source, with the following composition (in distilled water): 0.165 g/L K_2HPO_4, 0.16 g/L $(NH_4)_2SO_4$, 0.1 g/L YE, 0.096 g/L NaCl, 0.05 g/L cysteine-HCl, 0.0096 g/L $CaCl_2$, and 0.0096 g/L $MgSO_4$ (pH = 7.0) (Kim and Wimpenny 1981). The plates were incubated at 30 °C for 5 d. To observe the hydrolysis zone, the plates were flooded with an aqueous solution of Congo red (1% w/v) for 15 min and washed with 1 M NaCl. The formation of a clear zone around the colonies registered as positive for cellulase

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production. The 5 isolated colonies that presented the highest diameter of clear zone were selected to evaluate their enzymatic activity. The bacterial colony that showed the highest enzyme activity was chosen for molecular identification and to evaluate and optimize the operative parameters.

Seed Culture Preparation

A seed culture was prepared to obtain a homogenous inoculum for the subsequent experiments. A mineral methanol salts medium (MMSM) was used in this study with the following composition: 1 g/L KNO_3, 0.20 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.23 g/L Na_2HPO_4, 0.07 g/L NaH_2PO_4, 1 mg/L $FeSO_4 \cdot 7H_2O$, 5 µg/L $CuSO_4 \cdot 5H_2O$, 10 µg/L H_3BO_3, 1 µg/L $MnSO_4 \cdot 5H_2O$, 70 µg/L $ZnSO_4 \cdot 7H_2O$, and 10 µg/L MoO_3. The initial pH was adjusted to 7.0. After heat sterilization of the MMSM, methanol was added as the sole carbon source at a concentration of 0.05 wt.%. Methanol was selected as the carbon source for the production of biomass that was later used for cellulase production in the subsequent experiments. Then, 100 mL of sterilized medium was placed in 250-mL flasks and incubated at 30 °C for 3 d under static conditions. Five-milliliter samples were used to inoculate the media flask for enzyme production with a bacterial suspension, the optical density was adjusted to 0.2 at 500 nm.

Molecular Identification

For identification purposes, the 16S ribosomal gene was used as a molecular marker. The extraction of DNA was performed using the Power Soil® DNA Isolation kit (Mo Bio Laboratories, Carlsbad, USA), according to the instructions given by the manufacturer. A region of the 16S rDNA gene was amplified using 28F and 519R primer pairs based on the method used by Turner *et al*. (1999) and using a Quick Load Taq2x Master Mix kit (New England BioLabs, Ipswich, USA) in a thermal cycler (C1000 Touch, Bio-Rad, Hercules, USA). The polymerase chain reaction products were analyzed in a 0.8% agarose gel in TAE 1X buffer and purified using a QIA quick Gel Extraction kit (QIAGEN, Hilden, Germany). Sanger sequencing was performed at the National Laboratory for Genomics and Biodiversity (LANGEBIO, Guanajuato, Mexico). The obtained sequences were analyzed with the BLAST tool (NCBI, Bethesda, USA) and MEGA6 software (Tamura *et al*. 2013) for phylogenetic analysis (GenBank accession number: AJ131114.1).

Enzyme Production Medium

The broth for enzyme production contained the same components as were in the medium described by Kim and Wimpenny (1981), but without agar, and 25 mL of the medium were placed into 100-mL conical flasks. The flasks were autoclaved at 121 °C for 15 min. After cooling, the flasks were inoculated with 5 mL of the seed culture that was prepared previously. The inoculated flasks were incubated at room temperature (30 °C ± 5 °C) for 5 d under static conditions. Afterwards, the supernatant was centrifuged at 492 × g for 2 min and then used as the crude enzyme extract.

CMCase and FPase Activity

In 1.5-mL conical tubes, 0.5 mL of a 0.1 M phosphate buffer solution (pH = 7) that contained 0.05 g of carboxymethylcellulose (CMC) (CMC-Sigma Aldrich, St. Louis, MO, USA) or filter paper (FP) (Whatman #1, Sigma Aldrich, St. Louis, MO, USA) was added. Later, a Thermomixer (Eppendorf, Hamburg, Germany) maintained the tubes at 50 °C.

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Subsequently, 0.5 mL of the enzymatic extract was added to the tubes, and the reaction occurred for 60 min at 700 rpm. The reaction was stopped by placing the samples in an ice bath. The reducing sugars were quantified according to Miller (1959).

Evaluation of the Effect of the pH, Carbon Source, and Nitrogen Source on the Enzyme Production

The effect of the pH on the enzyme production was evaluated using the medium described above by Kim and Wimpenny (1981), but without agar. The pH ranged from 3 to 10 in step intervals of 1 unit. The experiments were conducted at room temperature (30 °C ± 5 °C) with the following buffers: 50 mM citrate (pH = 2 to 5), 50 mM phosphate (pH = 6 to 7), and 50 mM glycine–NaOH (pH = 8 to 10). At the end of the incubation period, the cell-free culture filtrate was used as an enzyme source.

The capacity of *S. maltophilia* to produce CMCase in the presence of different carbon sources (maltose, lactose, fructose, xylan, and CMC) was evaluated. A broth was prepared as described by Kim and Wimpenny (1981) and supplemented with a 1% (w/v) concentration of each carbon source. The experiments were conducted at 30 °C ± 5 °C for 5 d. At the end of the incubation period, the cell-free culture filtrate was obtained and used as an enzyme source.

Five nitrogen sources (NS) were evaluated to determine their effects on the enzyme production. The assays were performed using 0.5% of the selected NS. The NS sources that were evaluated were potassium nitrate (KNO_3), YE, ammonium chloride (NH_4Cl), peptone, and ammonium sulphate (($NH_4)_2SO_4$). The experiments were conducted at 30 °C ± 5 °C for 5 d. At the end of the incubation period, the cell-free culture filtrate was obtained and used as an enzyme source.

Statistical Analysis

The effects of the nitrogen source, pH, and carbon source on the CMCase and FPase activity were evaluated using an analysis of variance (ANOVA) and by applying the Tukey-Kramer test with the statistical software JMP 10.0.2 (SAS Institute Inc., Cary, NC, USA) ($p < 0.05$).

Biodegradation of Wheat Straw by *S. maltophilia*

Wheat straw (WS) was used as a carbon source using the media reported by Kim and Wimpenny (1981). Winter WS (*Triticum aestivum* L.) was obtained from local farmers in La Barca Province, Jalisco, Mexico (20º 15' 30" to 20º 26' 45" North latitude and 102º 20' 40" to 102º 21' 20" West longitude). The WS was harvested using conventional hay-harvesting equipment. Straw composition was determined according to AOAC INTERNATIONAL methods (AOAC Official Methods 4.6.03 and 4.6.04) with 44.45 ± 2.35, 19.23 ± 4.20, 5.78 ± 0.57 and 10.34 ± 1.1 of cellulose, hemicellulose, lignin and ash respectively (% w/w dry weight basis). The media was supplemented with 2% (w/v) WS that had been previously sterilized. The experiment was conducted in triplicate using 250-mL Erlenmeyer flasks with an effective work volume of 100 mL, which were inoculated with 10% of the seed culture prepared previously and the absorbance was adjusted to 0.2 at a wave longitude of 500 nm. The pH of the medium was adjusted to 6, and the incubation temperature was 30 °C. The experiment was incubated for 72 h. The reducing sugars concentration was determined using the Miller method (Miller 1959).

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Synchrotron µ-FTIR Analysis

An analysis of the WS using synchrotron µ-FTIR spectroscopy was completed to map the surface of the hydrolyzed WS. The exploration of the substrate surface was conducted in the ID21 at the European Synchrotron Radiation Facility (Grenoble, France). The WS samples were placed in a 200-µL Eppendorf tube that was saturated with distilled water, and the samples were frozen using liquid nitrogen. The obtained frozen samples were cut using a cryogenic microtome (RM2265, Leica Biosystems Inc., Buffalo Grove, USA) adapted with a diamond knife, and samples with 5-µm thicknesses were obtained. The samples were lyophilized and analyzed by synchrotron µ-FTIR spectroscopy. The beamline is equipped with a Thermo Nicolet Continuum (Thermo Scientific, Madison, WI, USA) microscope coupled to a Thermo Nicolet Nexus FTIR spectrometer (Thermo Scientific, Madison, WI, USA) with a 32x objective, a motorized sample stage, and a liquid nitrogen-cooled 50 µm HgCdTe detector.

Optimization of the Cellulase Production

Response surface methodology (RSM) using the BBD was used to determine the mathematical correlation between the three independent variables on the production of the CMCase; additionally, the BBD was used to determine the mathematical correlation between the three independent variables with the cell density (Box and Behnken 1960). The three independent variables for maximizing the enzyme activity were % CMC (X_3), pH (X_2), and % nitrogen source (N, X_1). The CMCase activity was the response variable (Table 1). The low, middle, and high concentration levels of each variable were designated as -1, 0, and 1, respectively.

Table 1. Values of the Factors Analyzed in the BBD

Factor	Actual Values of Natural Levels		
	-1	0	1
% N (w/v) (X_1)	0.5	1	1.5
pH (X_2)	5	6	7
% CMC (w/v) (X_3)	0.5	1	1.5

For the statistical calculations, the independent variable levels were coded as:

$$x_i = (X_i - X_0) / \delta X_i \tag{1}$$

where X_i is the real value of the variable, X_0 is the midpoint of the range of X_i, δX_i is the step change in X_i, and x_i is the coded value for X_i, where i is 1, 2, and 3.

The behavior of the system was explained with the following quadratic model equation,

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{\substack{ij=1 \\ i<j}}^{3} \beta_{ij} x_i x_j \tag{2}$$

where Y is the predicted response variable, β_0 is the intercept, β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficients, respectively, and x_i and x_j are the coded forms of the independent variables.

The analysis of the response surfaces, ANOVA, and optimization of the conditions were performed using the JMP 10.0.2 software. The significant effects on the dependent variables were determined with a t-test ($p < 0.05$).

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RESULTS AND DISCUSSION

Isolation and Molecular Identification of the Hydrolytic Strain

A bacterium that was capable of producing cellulases, identified as HCM-2, was isolated from the reforested soil from Guanajuato, Mexico. The HCM-2 colonies displayed a red color on the CMC agar plates and were identified as an aerobic, gram-negative bacterium. The HCM-2 was able to grow with several different carbon sources, such as betaine, CMC, dextrose, ethanol, methanol, L-ramose, maltose, mannose, ribose, and xylose. Figure 1 shows that the 16S rDNA sequence presented similarities with different *Stenotrophomonas* species according to the BLAST analysis. The phylogenetic analyses indicated that the identity of the HCM-2 strain was *S. maltophilia* with a 100% similarity.
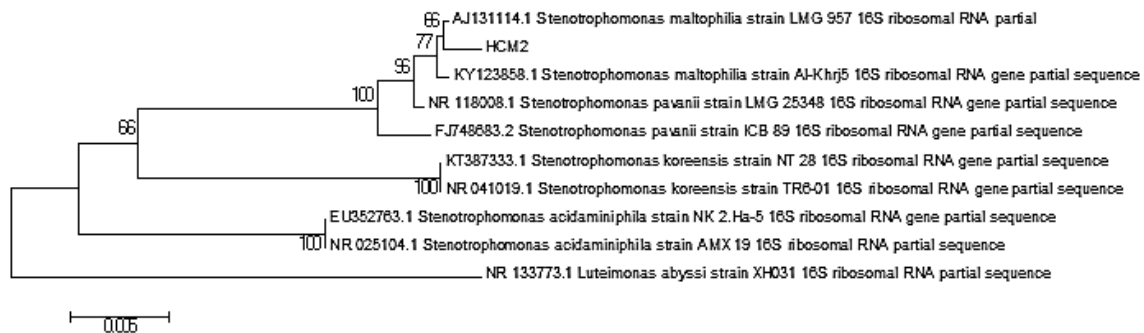

Fig. 1. Phylogenetic tree of the 16S rDNA sequence of HCM-2 isolated from a reforested soil. The values of the bootstrap that showed a probability value higher than 50% were included at the branching points.

Effect of the Carbon Source on the Enzyme Activity

To evaluate the effect of the carbon source on *S. maltophilia* cellulase production, maltose, lactose, fructose, xylan, and CMC were tested. Figure 2a shows that the highest enzymatic activity was achieved when the strain was incubated with CMC. The ANOVA showed that treatment with CMC was significantly different compared with the other treatments, and it also presented the highest enzyme activity (0.093 U/mL $\pm$ 0.0025 U/mL). The decreasing order for the CMCase activity when the carbon source was evaluated was as follows: CMC > lactose > xylan > maltose > fructose. The ANOVA also showed that the treatments with fructose, maltose, and xylan were statistically equal when compared with one another. The treatment with lactose was significantly different compared with the other treatments, but the enzymatic activity response was 80% lower compared with that achieved with CMC. These results allowed for the selection of CMC as a suitable carbon source for conducting the subsequent optimization experiments. A similar CMCase activity was obtained by Das *et al*. (2010) for *Bacillus* sp. (0.037 IU/mL), and it was concluded that the best carbon source for cellulase production was CMC. Additionally, Ariffin *et al*. (2008) and Shabeb *et al*. (2010) presented similar results and also concluded that CMC, FP, and cellobiose were the best carbon sources for inducing cellulase production by bacteria, such as *Cellulomonas* sp. and *Clostridium* sp.

Effect of the Nitrogen Source on the Enzyme Activity

Nitrogen is the main building block of proteins (and enzymes) and is one of the main constituents of protoplasm (Sethi *et al*. 2013). Five different nitrogen sources, KNO_3,

YE, NH_4Cl, peptone, and $(NH_4)_2SO_4$, were tested to determine the effect on the enzyme activity. Figures 2b and 2c demonstrate that all of the nitrogen sources used in this research promoted CMCase and FPase production.
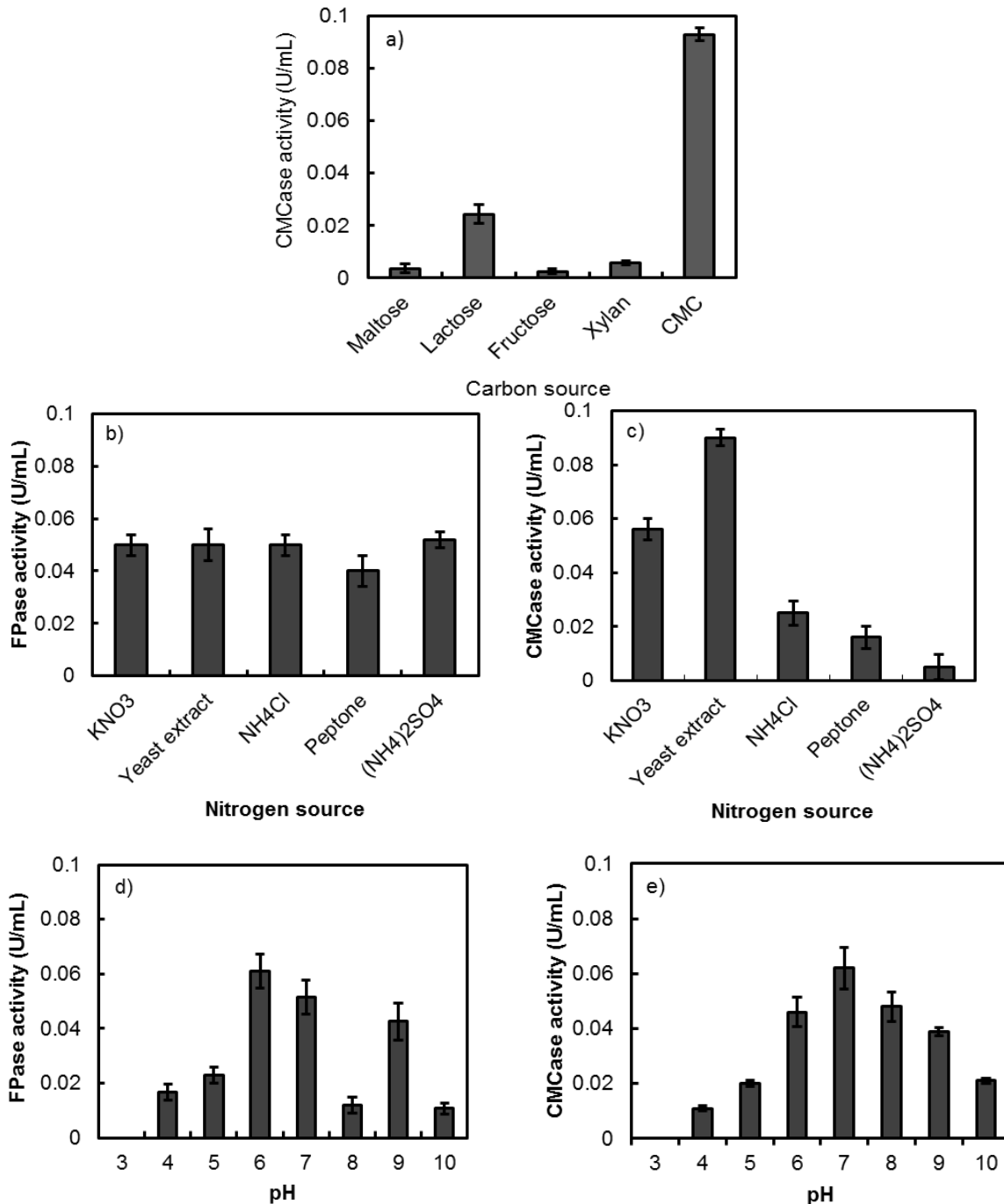

Fig. 2. Characterization of the enzymatic extract of *S. maltophilia* at 50 °C after 5 d of incubation: (a) effect of the carbon source on the CMCase activity; (b) effect of the nitrogen source on the FPase activity; (c) effect of the nitrogen source on the CMCase activity; (d) effect of the pH on the FPase activity; and (e) effect of the pH on the CMCase activity

The CMCase activity was evaluated, and the results obtained were as follows: YE (0.09 U/mL ± 0.003 U/mL) > KNO_3 (0.056 U/mL ± 0.004 U/mL) > NH_4Cl (0.025 U/mL ± 0.004 U/mL) > peptone (0.016 U/mL ± 0.004 U/mL) > $(NH_4)_2SO_4$ (0.005 U/mL ± 0.001 U/mL). The FPase activity was evaluated, and the results obtained were as follows: YE (0.05 U/mL ± 0.006 U/mL) = $(NH_4)_2SO_4$ = KNO_3 = NH_4Cl > peptone (0.04 U/mL ± 0.006 U/mL). The treatments that applied NH_4Cl, peptone, and $(NH_4)_2SO_4$ as a nitrogen source were statistically equal and presented the lowest CMCase and FPase activities. Similarly, Raj *et al*. (2013) found that YE induced a higher xylanolytic activity (25 IU/mL ± 0.6 IU/mL) with *S. maltophilia* compared with other nitrogen sources, such as NH_4Cl, peptone, and $(NH_4)_2SO_4$. Additionally, Sethi *et al*. (2013) evaluated the cellulase activity with *Pseudomonas fluorescens*, *Bacillus subtilis*, *Escherichia coli*, and *Serratia marcescens* using YE as the nitrogen source and found similar results. The results of these authors and this study indicated that YE is the nitrogen source that efficiently induces the production of enzymes, such as cellulases and xylanases. Yeast extract was the best nitrogen source because it was complemented by a mixture of amino acids, peptides, water-soluble vitamins, and carbohydrates.

Effect of the pH on the Enzyme Activity

Figures 2d and 2e display data on the effect of the pH on the enzyme activity. The pH range that was evaluated was 3 to 10. The results showed that a pH of 3 inhibited the enzyme activity. The highest CMCase activity obtained was observed at a pH of 7, with a value of 0.062 U/mL ± 0.003 U/mL. The treatments at pH values of 6, 8, and 9 did not present statistically significant differences. The treatments at pH values of 4, 5, and 10 were statistically equal. This result served to define the pH range for the following optimization experiments. Previous papers have reported that the highest enzyme activity occurs at a pH of approximately 7 for *E. coli*, *S. maltophilia*, *B. subtilis*, *Serratia marcescens*, *Pseudomonas fluorescence*, and *Cellulomonas* sp. ASN2, which was in accordance with the results of this study (Shabeb *et al*. 2010; Raj *et al*. 2013; Sethi *et al*. 2013). Nevertheless, these studies used an approximation method to find the highest enzyme activity value, and did not optimize the activity.

Effects of the Carbon Source, Nitrogen Source, and pH on the Enzyme Activity

The statistical treatment combinations of the test factors and the measured response values, expressed as the CMCase activity that corresponded to each combination, are shown in Table 2. An empirical equation was obtained from the RSM that related the CMCase activity and tested factors, which is shown in their encoded form with Eq. 3,

$$Y = -0.036 + 0.076X_1 - 0.120X_2 - 0.040X_3 - 0.010X_1X_2 - 0.021X_1X_3 + 0.003X_2X_3 + 0.020X_1^2 - 0.010X_2^2 + 0.023X_3^2 \quad (3)$$

where Y is the CMCase activity and X_1, X_2, and X_3 are the % N, pH, and % CMC, respectively.

The coefficient of determination (R^2) in the second-order equation was 0.89, which indicated that 89% of the data variation could be explained by the model. The adjusted R^2 value (84.82%) and predicted value (79.88%) revealed that the second-order model was an adequate mathematical equation for demonstrating the relationship between the variables and response.

Table 2. BBD Matrix with the Experimental and Predicted Values of the CMCase Activity

Experiment	Factor			CMCase Activity (U/mL)	
	X_1	X_2	X_3	Experimental	Predicted
1	-1	-1	0	0.040	0.039
2	1	-1	0	0.078	0.074
3	-1	1	0	0.029	0.029
4	1	1	0	0.046	0.051
5	-1	0	-1	0.032	0.032
6	1	0	-1	0.084	0.065
7	-1	0	1	0.032	0.032
8	1	0	1	0.063	0.054
9	0	-1	-1	0.045	0.046
10	0	1	-1	0.032	0.032
11	0	-1	1	0.039	0.040
12	0	1	1	0.033	0.033
13	0	0	0	0.041	0.050
14	0	0	0	0.057	0.050
15	0	0	0	0.057	0.050
16	0	0	0	0.062	0.050
17	0	0	0	0.050	0.050

BBD Matrix with the Experimental and Predicted Values of the Enzyme Activity

The response surfaces were plotted to analyze the effects of the interactions between the variables and to determine the optimum enzyme activity. The results indicated that the maximum enzyme activity (U/mL) given by the model was 0.084 U/mL. This value was achieved with an X_1 of 1.5%, X_2 of 6.3, and X_3 of 0.72%. The effects of the % N and pH on the CMCase activity when the other factor (% CMC) was at its center point are shown in Fig. 3a. A high N level allowed a higher CMCase activity when interacting with a lower pH level. Samira *et al*. (2011) reported enzyme activity for *S. maltophilia* isolated from Persian Gulf of 0.084 U/mL and 0.072 U/mL for CMCase and FPase activity respectively. These values are similar to that obtained in this research. Shankar and Isaiarasu (2011) reported that the highest enzyme activity occurred at a pH of 6 when they tested the crude enzyme from *Bacillus pumilus* EWBCM1. Additionally, Shankar and Isaiarasu (2011) showed a similar enzyme activity (0.1 U/mL) when 1% CMC or 1% cellulose (0.05 U/mL) was tested. Irfan *et al*. (2016) and Sethi *et al.* (2013) showed that a pH of 7 produced the highest enzyme activities for microorganisms such as *E. coli*, *Bacillus*, *Serratia*, *Pseudomonas*, and *Cellulomonas* sp. ASN2. *Stenotrophomonas maltophilia* and *Pseudomonas* are species that occur ubiquitously in the environment and can be found together in diverse niches, including the rhizosphere of plants (Ryan *et al.* 2009). The diverse class of γ-proteobacteria includes bacteria such as *E. coli*, *Pseudomonas*, *Cellulomonas*, and *S. maltophilia* (Ryan *et al.* 2009). The effects of % N

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and % CMC on the CMCase activity when the other factor (pH) was at the center point are shown in Fig. 3b. A pronounced effect on the CMCase activity was observed at a high N level and intermediate CMC level. In accordance with these results, Sethi *et al.* (2013), Shankar and Isaiarasu (2011), and Irfan *et al.* (2016) found that the use of YE enzymes increased the cellulase activity of different bacteria, such as *E. coli*, *Bacillus*, *Serratia*, *Pseudomonas*, and *Cellulomonas* sp. ASN2. These bacteria were comparable with *S. maltophilia* because they share phylogenetic information, which is because they belong to the γ-proteobacteria class.
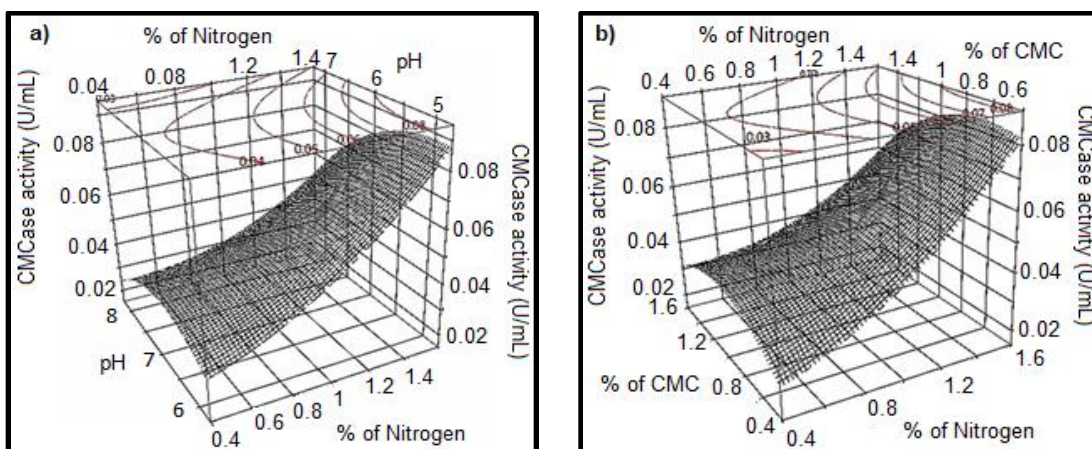

Fig. 3. Response surface plots and contour plots of the: (a) combined effects of the % YE and pH on the enzyme activity; and (b) combined effects of the % YE and % CMC on the enzyme activity

Biodegradation of the WS by *S. maltophilia*

Wheat straw, which contains cellulose, hemicellulose, and lignin, served as the carbon source for *S. maltophilia*. The experiment was conducted at a pH of 6 and 30 °C ± 2 °C for 72 h. The reducing sugars were measured and the final concentration was 150 mg/L ± 2 mg/L and the corresponding yield was 35.5%. At the final point, a WS sample was lyophilized for posterior analysis. The WS was analyzed before and after biodegradation by μ-FTIR coupled with a synchrotron light source in ID21 at the European Synchrotron Radiation Facility. A synchrotron is a high-energy electron storage ring that has brightness values 100 to 1000 times higher than a conventional thermal globar source (Miller and Dumas 2006). Synchrotron μ-FTIR allows for *in situ* analysis of biomass samples at a molecular level, while combining spatial and chemical information from IR absorbance to produce a chemical map that can be linked to specific functional groups (Dokken and Davis 2007). The behavior of an enzyme obtained from a microorganism, such as *S. maltophilia*, on natural substrates at a fundamental level can be better understood with the use of a powerful tool like a synchrotron. This approach allowed for high-quality IR spectra to be obtained because of high-IR photon flux on small surface areas (Miller and Dumas 2006).

Figure 4 shows the spectra obtained by μ-FTIR. The μ-FTIR bands that were associated with cellulose were 1434 cm^{-1}, 1376 cm^{-1}, and 1338 cm^{-1}, which are associated with CH$_2$ in-plane bending vibration, C-O stretching, and C-H ring in-plane bending vibration, respectively (Pandey 1999; Wilson *et al.* 2000; Yu *et al.* 2007). The intensities of these bands were reduced after bacteria action. The FTIR band at 1066 cm^{-1} was also associated with cellulose and can be associated with β(1-3)-polysaccharide, which is a strong signal that characterizes a high cellulose composition (Adapa *et al.* 2011;

Siengchum *et al*. 2013). Its intensity did not change after biological treatment. The FTIR band at 1743 cm^{-1} corresponded to C=O groups and is a typical band that is associated with hemicellulose (Siengchum *et al*. 2013). This band was present with a strong intensity in the non-treated WS; however, after the *S. maltophilia* treatment, the weak signal indicated that hemicellulose was removed. The FTIR bands that were associated with lignin were 1603 cm^{-1} and 1510 cm^{-1}, which are associated with quadrant ring stretching (aromatic lignin) and semicircle ring stretching (aromatic lignin), respectively. These bands were present with a weak signal in the non-treated WS and are maintained after the biological treatment. The FTIR band at 1167 cm^{-1} was associated with the glycosidic linkages of the glucose ring in cellulose, which includes symmetric polysaccharides (Adapa *et al*. 2011). The FTIR bands at 2956 cm^{-1} and 2906 cm^{-1} were attributed to the stretching of C-H (Kirtania *et al*. 2014). Kristensen *et al*. (2008) attributed the bands at 2950 cm^{-1} and 2906 cm^{-1} to waxes. These signals were modified after the biological treatment. These results indicated that *S. maltophilia* was able to degrade the waxes in biomasses and use them as carbon sources. Additionally, the hydrophilic tendency of the raw WS fibers was reflected in the broad absorption bands at 3413 cm^{-1}, 3343 cm^{-1}, and 3314 cm^{-1}. These bands were related to –OH groups that are their main components, which can include absorbed water, aliphatic primary and secondary alcohols in cellulose, hemicellulose, lignin, extractives, and carboxylic acids in extractives (Ibrahim *et al*. 2011; Kirtania *et al*. 2014). Additionally, the FTIR band at 1664 cm^{-1} was attributed to water absorbed in the cellulose (Siengchum *et al*. 2013; Kirtania *et al*. 2014). These results demonstrated that *S. maltophilia* degrades a natural substrate, such as WS, by hydrolyzing both cellulose and hemicellulose.
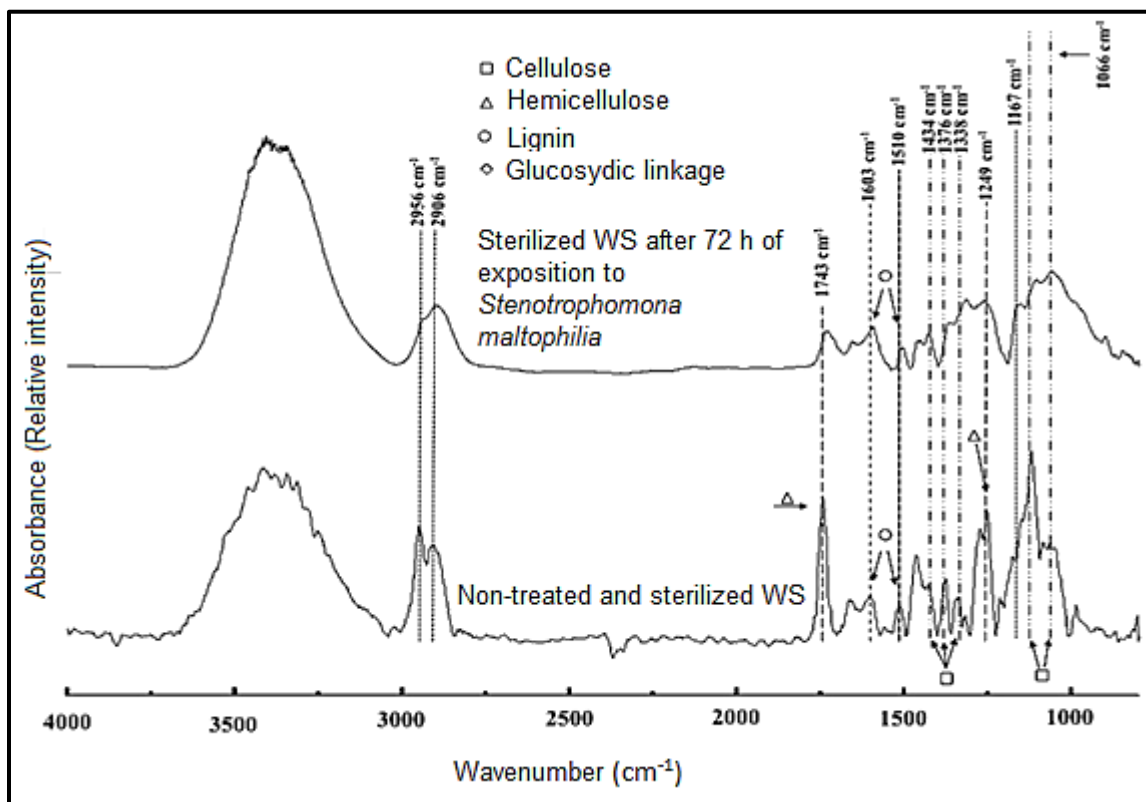

Fig. 4. µ-FTIR spectra of the sterilized WS and sterilized WS after 72 h of exposure to *S. maltophilia*

CONCLUSIONS

1. Optimization of culture conditions for production of cellulase by *Stenotrophomonas maltophilia* was performed to study the effects of the carbon source, nitrogen source, and pH on this process. The carbon source, nitrogen source, and pH were sufficient to support biochemical conversion, which permitted found an maximum CMCase activity.

2. *Stenotrophomonas maltophilia* was grown on sterilized WS to evaluate the production of reducing sugars. After 72 h of incubation at 30 °C, the final concentration of reducing sugars was 150 mg/L ± 2 mg/L and the corresponding yield was 10.2%, showing its potential application such as in saccharification process

3. According to the synchrotron FTIR data, the bacterium was able to transform the WS using waxes, cellulose, and hemicellulose as carbon sources. This tool was able to provide information about the functionality of *S. maltophilia* for transforming biomass into fermentable sugars.

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REFERENCES CITED

Adapa, P. K., Tabil, L. G., Schoenau, G. J., Canam, T., and Dumonceaux, T. J. (2011). "Quantitative analysis of lignocellulosic components of non-treated and steam exploded barley, canola, oat and wheat straw using Fourier transform infrared spectroscopy," *J. Agric. Sci. Technol.* 1(12), 177-188.

Alonso, A., Sanchez, P., and Martínez, J. L. (2000). "*Stenotrophomonas maltophilia* D457R contains a cluster of genes from gram-positive bacteria involved in antibiotic and heavy metal resistance," *Antimicrob. Agents Ch.* 44(7), 1778-1782. DOI: 10.1128/AAC.44.7.1778-1782.2000

AOAC Official Methods 4.6.03 and 4.6.04

Ariffin, H., Hassan, M. A., Shah, U. K. M., Abdullah, N., Ghazali, F. M., and Shirai, Y. (2008). "Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by *Bacillus pumilus* EB3," *J. Biosci. Bioeng.* 106(3), 231-236. DOI: 10.1263/jbb.106.231

Berg, G., Marten, P., and Ballin, G. (1996). "*Stenotrophomonas maltophilia* in the rhizosphere of oilseed rape — Occurrence, characterization and interaction with phytopathogenic fungi," *Microbiol. Res.* 151(1), 19-27. DOI: 10.1016/S0944-5013(96)80051-6

Box, G. E. P., and Behnken, D. W. (1960). "Some new three level designs for the study of quantitative variables," *Technometrics* 2(4), 455-475.

DOI: 10.1080/00401706.1960.10489912

Dantur, K. I., Enrique, R., Welin, B., and Castagnaro, A. P. (2015). "Isolation of cellulolytic bacteria from the intestine of *Diatraea saccharalis* larvae and evaluation of their capacity to degrade sugarcane biomass," *AMB Express* 5, 15. DOI: 10.1186/s13568-015-0101-z

Das, A., Bhattacharya, S., and Murali, L. (2010). "Production of cellulase from a thermophilic *Bacillus* sp. isolated from cow dung," *American-Eurasian J. Agric. Environ. Sci.* 8(6), 685-691.

Delafont, V., Brouke, A., Bouchon, D., Moulin, L., and Héchard, Y. (2013). "Microbiome of free-living amoebae isolated from drinking water," *Water Res.* 47(19), 6958-6965. DOI: 10.1016/j.watres.2013.07.047

Dokken, K. M., and Davis, L. C. (2007). "Infrared imaging of sunflower and maize root anatomy," *J. Agr. Food Chem.* 55(26), 10517-10530. DOI: 10.1021/jf072052e

Dunne, C., Moënne-Loccoz, Y., de Bruijn, F. J., and O'Gara, F. (2000). "Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81," *Microbiology+* 146, 2069-2078. DOI: 10.1099/00221287-146-8-2069

Galperin, M. Y. (2008). "The quest for biofuels fuels genome sequencing," *Environ. Microbiol.* 10(10), 2471-2475. DOI: 10.1111/j.1462-2920.2008.01754.x

Hemlata, B., Selvin, J., and Tukaram, K. (2015). "Optimization of iron chelating biosurfactant production by *Stenotrophomonas maltophilia* NBS-11," *Biocatal. Agric. Biotechnol.* 4(2), 135-143. DOI: 10.1016/j.bcab.2015.02.002

Huang, S., Sheng, P., and Zhang, H. (2012). "Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (*Coleoptera*: *Scarabaeidae*)," *Int. J. Mol. Sci.* 13(3), 2563-2577. DOI: 10.3390/ijms13032563

Ibrahim, M. M., El-Zawawy, W. K., Abdel-Fattah, Y. R., Soliman, N. A., and Agblevor, F. A. (2011). "Comparison of alkaline pulping with steam explosion for glucose production from rice straw," *Carbohyd. Polym.* 83(2), 720-726. DOI: 10.1016/j.carbpol.2010.08.046

Irfan, M., Asghar, U., Nadeem, M., Nelofer, R., and Syed, Q. (2016). "Optimization of process parameters for xylanase production by *Bacillus* sp. in submerged fermentation," *J. Radiat. Res. Appl. Sci.* 9(2), 139-147. DOI: 10.1016/j.jrras.2015.10.008

Jeong, J.-H., Lee, O.-M., Jeon, Y.-D., Kim, J.-D., Lee, N.-R., Lee, C.-Y., and Son, H.-J. (2010). "Production of keratinolytic enzyme by a newly isolated feather-degrading *Stenotrophomonas maltophilia* that produces plant growth-promoting activity," *Process Biochem.* 45(10), 1738-1745. DOI: 10.1016/j.procbio.2010.07.020

Kim, B. H., and Wimpenny, J. W. T. (1981). "Growth and cellulolytic activity of *Cellulomonas flavigena*," *Can. J. Microbiol.* 27(12), 1260-1266. DOI: 10.1139/m81-193

Kirtania, K., Tanner, J., Kabir, K. B., Rajendran, S., and Bhattacharya, S. (2014). "*In situ* synchrotron IR study relating temperature and heating rate to surface functional group changes in biomass," *Bioresour. Technol.* 151, 36-42. DOI: 10.1016/j.biortech.2013.10.034

Kristensen, J. B., Thygesen, L. G., Felby, C., Jørgensen, H., and Elder, T. (2008). "Cell-wall structural changes in wheat straw pretreated for bioethanol production," *Biotechnol. Biofuels* 1, 5. DOI: 10.1186/1754-6834-1-5

López-Mondéjar, R., Zühlke, D., Becher, D., Riedel, K., and Baldrian, P. (2016). "Cellulose and hemicellulose decomposition by forest soil bacteria proceeds by the action of structurally variable enzymatic systems," *Sci. Rep.-UK* 6. DOI: 10.1038/srep25279

Maki, M., Leung, K. T., and Qin, W. (2009). "The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass," *Int. J. Biol. Sci.* 5(5), 500-516.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31(3), 426-428. DOI: 10.1021/ac60147a030

Miller, L. M., and Dumas, P. (2006). "Chemical imaging of biological tissue with synchrotron infrared light," *BBA—Biomembranes* 1758(7), 846-857. DOI: 10.1016/j.bbamem.2006.04.010

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresour. Technol.* 96(6), 673-686. DOI: 10.1016/j.biortech.2004.06.025

Neethu, C. S., Rahiman, K. M. M., Rosmine, E., Saramma, A. V., and Hatha, A. A. M. (2015). "Utilization of agro-industrial wastes for the production of lipase from *Stenotrophomonas maltophilia* isolated from Arctic and optimization of physical parameters," *Biocatal. Agric. Biotechnol.* 4(4), 703-709. DOI: 10.1016/j.bcab.2015.09.002

Pandey, K. K. (1999). "A study of chemical structure of soft and hardwood and wood polymers by FTIR spectroscopy," *J. Appl. Polym. Sci.* 71(12), 1969-1975. DOI: 10.1002/(SICI)1097-4628(19990321)71:12<1969::AID-APP6>3.0.CO;2-D

Raj, A., Kumar, S., and Singh, S. K. (2013). "A highly thermostable xylanase from *Stenotrophomonas maltophilia*: Purification and partial characterization," *Enzyme Research* 2013. DOI: 10.1155/2013/429305

Ribitsch, D., Heumann, S., Karl, W., Gerlach, J., Leber, R., Birner-Gruenberger, R., Gruber, K., Eiteljoerg, I., Remler, P., Siegert, P., *et al.* (2012). "Extracellular serine proteases from *Stenotrophomonas maltophilia*: Screening, isolation and heterologous expression in *E. coli*.," *J. Biotechnol.* 157(1), 140-147. DOI: 10.1016/j.jbiotec.2011.09.025

Ryan, R. P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M. B., Berg, G., van der Lelie, D., and Dow, J. M. (2009). "The versatility and adaptation of bacteria from the genus *Stenotrophomonas*," *Nat. Rev. Microbiol.* 7, 514-525. DOI: 10.1038/nrmicro2163

Rzedowski, J., and Calderón, G. (2009). "Lista preliminar de árboles silvestres del estado de Guanajuato [Preliminary list of wild trees in the state of Guanajuato]," *Flora del Bajío y de Regiones Adyacentes*, 1-14.

Samira, M., Mohammad, R., and Gholamreza G. (2011). "Carboxymethyl-cellulase and Filter-paperase Activity of New Strains Isolated from Persian Gulf," Microbiology Journal 1(1), 8-16.

Sethi, S., Datta, A., Gupta, B. L., and Gupta, S. (2013). "Optimization of cellulase production from bacteria isolated from soil," *ISRN Biotechnology* 2013. DOI: 10.5402/2013/985685

Shabeb, M. S. A., Younis, M. A. M., Hezayen, F. F., and Nour-Eldein, M. A. (2010). "Production of cellulase in low-cost medium by *Bacillus subtilis* KO strain," *World Applied Sciences Journal* 8(1), 35-42.

Shankar, T., and Isaiarasu, L. (2011). "Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions," *Middle East J. Sci. Res.* 8(1), 40-45.

Siengchum, T., Isenberg, M., and Chuang, S. S. C. (2013). "Fast pyrolysis of coconut biomass – An FTIR study," *Fuel* 105, 559-565. DOI: 10.1016/j.fuel.2012.09.039

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0," *Mol. Biol. Evol.* 30 (12), 2725-2729

Turner, S., Pryer, K., Miao, V., and Palmer, J. (1999). "Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis," *J. Eukaryot. Microbiol.* 46(4), 327-338. DOI: 10.1111/j.1550-7408.1999.tb04612.x

Wilson, R. H., Smith, A. C., Kacuráková, M., Saunders, P. K., Wellner, N., and Waldron, K. W. (2000). "The mechanical properties and molecular dynamics of plant cell wall polysaccharides studied by Fourier-transform infrared spectroscopy," *Plant Physiol.* 124(1), 397-405.

Yu, P., Block, H., Niu, Z., and Doiron, K. (2007). "Rapid characterization of molecular chemistry, nutrient make-up and microlocation of internal seed tissue," *J. Synchrotron Radiat.* 14(Pt. 4), 382-390. DOI: 10.1107/S0909049507014264