JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

INFLUENCE OF CARBON SOURCE ON EXTRACELLULAR PROTEASE PRODUCTION BY SOIL STREPTOMYCES SP. AGS-10

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doi: 10.15414/jmbfs.2019.9.2.236-241

ARTICLE INFO

Received 7. 7. 2018
Revised 4. 4. 2019
Accepted 9. 4. 2019
Published 1. 10. 2019

Regular article

ABSTRACT

The aim of this study was to screen and select streptomycetes exhibiting the production of extracellular proteases and evaluates the effect of carbon sources on protease expression. An acidic precipitation of non-degraded casein was performed to monitor the hydrolytic activity of protease-producing isolates on proteolysis medium (PM). Furthermore, we assessed the induction/repression status of protease production depending on the carbon sources. Clear halos surrounded the colonies were measured to calculate the Enzymatic Index (EI) values. One-hundred and sixty-eight streptomycete strains were screened in order to identify protease producers. Among all of these, the *Streptomyces* sp strain AGS-10 was selected based on their highest EI value. When sucrose, xylose, and arabinose were added to PM as carbon source, the EI were not modified. Conversely, the presence of lactose, glucose, and fructose repressed the proteolytic activity displayed by the AGS-10 colonies. A supernatant obtained from a culture media seeded with AGS-10 also displayed an extracellular proteolytic activity that was detected by an agar well diffusion assay. However, when lactose or glucose was added to the culture media the resulting proteolytic activity from supernatant was lost. The study shows a negative influence of some carbon sources for protease production.

Keywords: protease, streptomycetes, carbon repression, casein precipitation

INTRODUCTION

The use of enzymes for industrial catalytic purposes has significantly increased during the last decades, estimating that the global market for these enzymes reached $3.3 billion USD in 2010, $4.4 billion in 2015 and it is estimated that by 2022 it will reach $6.30 billion (**Binod *et al*., 2013; Markets and Markets, 2018**). Because of their wide applications range in the food, beverage, detergent, and biofuel industries, proteases constitute the most important market share of the industrial enzyme production, accounting for 60% of sales worldwide (**Kirk *et al*., 2002; Sandhya *et al*., 2005**).
The species from the genus *Streptomyces* are the most widely used in this industry and comprise a diverse group of Gram-positive bacteria belonging to the phylum Actinobacteria. Distinctively, they undergo a complex process of morphological differentiation in which branched mycelium develops into aerial mycelia, sporophores and spores (Anderson and Wellington, 2001). They produce a broad spectrum of highly valued bioactive compounds for commercial purposes. Some of them are of medical interest, especially those displaying antifungal, antiviral, antitumor, anti-hypertensive, immunosuppressant, and antibiotic activities (**Takizawa *et al*., 1993; Berdy, 2005; Procópio *et al*., 2012**). Similarly, hydrolytic enzymes are of great importance for various industrial purposes, as they are used in the manufacture of food, drugs and agricultural inputs, such as celluloses, chitinases, amylases, xylanases, proteases, lipases and esterases (**Sathya and Ushadevi, 2014; Vaijayanthi *et al*., 2016**).
Some *Streptomyces* species produce different proteolytic enzymes according to the substrate in which they live, as well as their inherent capacity to degrade the organic matter contained in soil. However, a critical benefit of many proteolytic enzymes produced by streptomycetes is their secretion into extracellular compartments. This feature is generally regarded as safe (GRAS) by the U.S. Food and Drug Administration (**Ghorbel *et al*., 2014**). Moreover, this represents an advantage for purification purposes, since mycelia may be easily separated from the enzyme solution by simple filtration or centrifugation before the process of purification of enzyme in order to achieve homogeneity.
Several protease-producing *Streptomyces* spp. from a wide spectrum of substrate have been previously described. In this regard, *S. thermovulgaris* produced more than one type of serine- and metallo- proteases when grown on rapemeal-derived media (**Yeoman and Edwards, 1994**). An alkaline protease used to remove blood stains from surgical instruments was produced by *S. gulbangersis* using wheat bran in a solid-state fermentation process (**Vishalakshi *et al*., 2009**). A partial characterization of the extracellular proteases produced by *S. clavuligerus* was carried out and the enzyme was produced through a batch fermentation process using a soy bean filtrate (**Moreira *et al*., 2001**). Recently, an alkaline protease produced by *S. griseorubens* was used to control root rot disease caused by *Rhizoctonia* in corn (**Al-Askar *et al*., 2015**). The aim of this study is to screen and select streptomycetes exhibiting proteolytic activity and evaluate the effects of carbon sources on protease expression. Additionally, this study will show the characterization and molecular identification of the AGS-10 protease-producing isolate.

MATERIAL AND METHODS

Culture media and growth conditions

ISP 2 (International Streptomyces Project) agar media was used to grow cells and to induce spore production, while ISP 9 agar medium was used during the processes of protein detection and biochemical characterization (Shirling and Gottlieb, 1966). Proteolysis Medium (PM) was prepared by adding 1% casein from bovine milk (Sigma-Aldrich® C7078) to the ISP 9 medium (HIMEDIA®). Strains were maintained at 29 °C for 14 days.

Screening streptomycetes displaying protease activity

A screening exercise was carried out in order to detect streptomycetes species producing extracellular proteases. One-hundred and sixty-eight strains preserved in the Germplasm Bank of Actinomycetes of the Center for Research and Assistance in Technology and Design of the State of Jalisco (CIATEJ), Southeast Unit, were used in the study. This collection preserves streptomycetes species from soil samples obtained from mesophyll mountain forests, wetland Ramsar´s

forests, caves and agricultural field's environments (**Caraveo *et al*., 2014; Evangelista-Martínez, 2014ab; Córdova-Dávalos *et al*., 2018**).
A 2-µL aliquot from a general inoculum (GI) of spores or cells, previously prepared (**Evangelista-Martínez, 2014b**), was placed in plates containing PM medium and incubated for 7 days at 29°C. Proteolytic colonies were detected by adding 5 ml of a 10% acetic acid solution to Petri plates for five minutes at room temperature. The remaining solution was poured-off from the plates and the clear zones surrounding the colonies were measured with Vernier caliper. Clear halos evidenced protease activity. The Enzymatic Index (EI) was assessed by calculating the hydrolyzed zone diameter/colony diameter ratio (**Florencio *et al*., 2012**). The strain with the highest EI value was selected for further studies.

Effect of sources of carbon on protease expression

The effect of different types of sugars on protease expression was evaluated by respectively adding 1% glucose, fructose, lactose, sucrose, xylose or arabinose to the PM medium containing 1% casein (**Caraveo *et al*., 2014**). As indicated above, a 2 µL-aliquot of a suspension containing the AGS-10 GI isolate was seeded and kept at 29°C for 14 days. EI values were determined using the ratios of the diameter of hydrolyzed zone and that of the colony.

Effect of casein concentration on proteolytic activity

The effect of casein concentration on proteolytic activity as indicated by the enzymatic index was studied for the selected AGS-10 streptomycete strain. PM plates were supplemented by three levels of casein namely 0.25, 0.5, and 1%. EI was calculated using the ratios of the diameter of hydrolyzed zone and that of the colony.

Proteolytic activity on a cell-free supernatant obtained from the *Streptomyces* sp AGS-10

Proteolytic extracts were obtained from AGS-10 strain culture grown on 1000 mL of ISP9 medium supplemented with 1% casein and 50 ppm antifoam A (Sigma-Aldrich®). The culture was kept at 29°C and 150 rpm for 96 h. Supernatant obtained by spinning down cells at 4000 g for 10 minutes was filtered twice with Whatman paper filter No. 1, and passed through a 0.45 µm sterile filter (Millex®GV, Merck-Millipore). The cell-free extract was dialyzed three times by using 50 mM sodium acetate buffer, pH 7.6 and 20% glycerol. It was subsequently stored at -20°C until further use. Protein concentration was determined using the Bradford assay.
The supernatant proteolytic activity was evaluated through the agar well diffusion assay. A 50 µL supernatant aliquot containing 15-100 µg protein was placed on the respective wells of a 6 mm-diameter multi-well plate containing 0.5% of casein dissolved in 1.2% agar. After a 24-h incubation at 37°C, 10 mL of 10% acetic acid were added. The diameter of the clear zones surrounding the wells was measured by using a caliper. For assay validation purposes, 60 µg of a commercial protease preparation obtained from *Streptomyces griseus* (Sigma-Aldrich® P5147) dissolved in 100 mM Tris-HCl buffer pH 7.2 was used.

Characterization of the AGS-10 isolate

The morphological and biochemical characterization of the isolate was carried out (**Shirling and Gottlieb, 1966**). The effects on substrate mycelium, spore mass, and pigment production were evaluated under the following conditions: carbon source assimilation, different agar culture media, growth at several pH values (4-9), and different sodium chloride concentrations (2.5-15%). All tests were performed in 24-wells cell culture plates.
Antibiotic susceptibility assays were carried out by using Gram positive II multidiscs (Bio-Rad®). To perform this test, 100 µL of GI were homogenously dispersed in ISP 2 agar media. Subsequently, a multidisc was placed on the plate. Either resistance or susceptibility was evaluated after a 14-day incubation at 29°C, according to the manufacturer's instructions.

Molecular Identification

The identity of the selected strain was determined based on partial length 16S rRNA gene sequence analysis. The genomic DNA used as template for PCR was prepared according to the procedure described by **Evangelista-Martínez (2014b)**. The complete 16S rRNA gene fragment was prepared by PCR amplification using Platinum Taq DNA polymerase High Fidelity (Invitrogen®) and oligonucleotides 63F (**5'-CAGGCCTAACACATGCAAGTC-3'**) and 1389R (**5'-ACGGGCGGTGTGTACAAG-3'**) (**Osborn *et al*., 2002**). The PCR reaction was carried out in a total volume of 50 µl that contained 1X PCR buffer, 2 mM MgSO4, 0.2 mM of each dNTP, 2 ng of chromosomal DNA, 0.4 µM each primer and 2 units of Taq DNA polymerase. PCR conditions were: an initial denaturation step at 94°C for 5 min followed by 35 amplification cycles of 94°C for 1 minute, 55°C for 45 seconds, and 68°C for 1 minute. The amplified fragment was purified using the PureLink PCR Purification Kit (Invitrogen®) and was verified directly by nucleotide sequence determination of both strands. Sequencing was provided by LANGEBIO (National Laboratory of Genomics for Biodiversity, CINVESTAV Irapuato, Mexico). Sequences were assembled and trimmed using CLC Main Workbench 5.6 (Qiagen®). A 1236 bp DNA fragment was analyzed for homology using the BLASTN program and 16S rDNA gene sequences of typed strains of various genera were retrieved from the nonredundant GeneBank database (http:// blast.ncbi.nlm.nih.gov/) (Alschul et al., 1997). These sequences, in conjunction with additional streptomycete strains were used as reference in which unidentified and unpublished sequences were not included. Phylogenetic analysis was carried out at Phylogeny.fr web page: http://www.phylogeny.fr/phylogeny.cgi (**Dereeper *et al*., 2008; Dereeper *et al*., 2010**). Selected sequences were aligned with CLUSTAL W (v 2.1) configured for highest accuracy. After alignment, positions with gaps were removed from the alignment. The phylogenetic tree was reconstructed using the neighbor joining method implemented in the BioNJ program. The Jukes-Cantor substitution model was selected for the analysis. The confidence of the grouping was verified by bootstrap analysis (500 replications). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v 198.3). *Bacillus subtilis* subsp. *subtilis* was used as outgroup. Partial sequence of 16S rDNA gene of *Streptomyces* sp. isolate AGS-10 was deposited in GenBank database under accession number MF135615.

Statistical Analysis

All experiments were done in triplicate and data were analyzed using two-way analysis of variance (ANOVA) at P=0.05 with Statgraphics® Centurion XVI software version 10.

RESULTS AND DISCUSSION

Selection and characterization of protease-producing Streptomyces

An initial screening to detect proteases was essential in order to select previously unidentified producer strains (**Kasana *et al*., 2011**). One-hundred and sixty-eight streptomycetes isolates were initially screened in order to detect their proteolytic activity. Sixty-three of them exhibited clear 7-21 mm-diameter hydrolytic halos surrounding the colonies. These helped to discriminate precipitated casein from its non-hydrolyzed formed (**Figure 1**). The highest EI rate values (2.63 ± SD 0.06, 2.42 ± SD 0.2, 2.15 ± SD 0.06) were observed for three isolates (named AGS-10, AGS-17 and AGS-21, respectively). Because the AGS-10 isolate showed the highest EI value and superior cultural characteristics and profuse spore production, it was selected for further experiments.
The incorporation of skimmed milk, gelatin, egg-yolk or casein in solid agar media formulations have been used to perform microbial protease screening. Some of these methods have used developing agents intended to elicit the hydrolytic activity, e.g. trichloroacetic (TCA) or tannic acids (**Medina and Baresi, 2007; Saran *et al*., 2007; Suganthi *et al*., 2013**). In the present study, precipitation of casein with acetic acid correlate with the white zone of non-hydrolysed protein. In contrast, translucent zone around the colonies correlated with the hydrolytic activity of extracellular proteases.

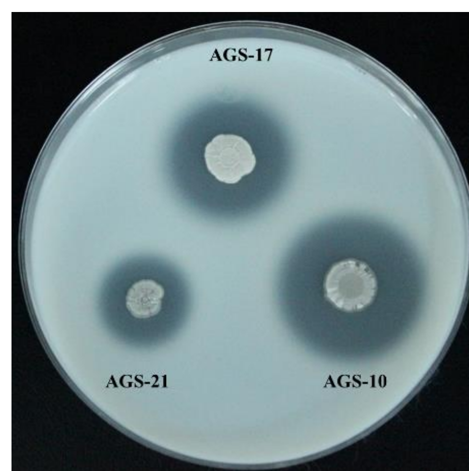

Figure 1 Screening of proteolytic streptomycetes. Isolates exhibit clear halos zone of different diameters around the colonies correlated with the hydrolysis of casein

Effect of carbon source on protease expression

It has been previously described that extracellular enzyme production is greatly influenced by ingredients on the media, especially carbon and nitrogen sources (**Caraveo *et al*., 2014**). The effect of glucose, fructose, lactose, sucrose, xylose, and arabinose on the extracellular protease production carried out by the AGS-10

isolate is shown in **Figure 2**. The obtained results revealed that sucrose, xylose and arabinose did not modify protease expression as protease activity was similar to that using casein alone (EI values = 2.8, 2.6, 2.8, and 2.9, respectively) (**Figure 2a**). However, it has been also observed that lactose, glucose or fructose addition produced a decreased proteolysis halo, and this may correlate with a catabolite repression effect as shown in **Figure 2b**. Moreover, EI values decreased from 4.1 to 2.9 after increasing the proteolytic substrate levels (**Figure 2c**; statistically significant differences, p = 0.05). This suggests that substrate concentration impacts on enzyme reaction rate or, if the product is in excess, protease expression is turned-off or repressed. Previous studies indicated that glucose was the optimal carbon source to induce extracellular protease production by *Streptomyces carpaticus* (**Haritha *et al*., 2012**). *Streptomyces* sp. DPUA1576, a suitable microorganism that produces proteases and fibrinolytic proteins, displayed a maximum protease production when grown on a medium consisting of soybean flour 1.26% and glucose 1.23% (**Silva *et al*., 2015**). Similarly, a high protease yield was detected when *S. albolongus* was grown in a medium containing 1% glucose, 2% beef extract, and 0.2% yeast extract as carbon and nitrogen source (**Akhtar *et al*., 2013**). Protease production by *S. griseus* increases when Triton X-100 and SDS were included in the basal medium (**El-Shahed *et al*., 2008**).

Proteolytic activity in supernatants obtained from culture media

The protease activity of supernatants obtained from cell cultures was evaluated on agar plates containing PM (**Figure 3**). Increasing supernatant amounts obtained from the AGS-10 strain were placed on well plates containing PM and the clear zones resulting from hydrolysis were measured. The hydrolytic activity was plainly visible and increased linearly with enzyme concentration (**Figure 3a**). As mentioned on the methods section, two supernatants used as control (40 µg) did not produce a clear hydrolysis zone because protease expression was decreased by the presence of glucose and lactose, as previously discussed (data not shown). A comparative assay performed by the well diffusion agar method confirmed that the proteolytic activity of the AGS-10 protease supernatants and a commercial protease obtained from *Streptomyces griseus* directly correlated with the clear area (**Figure 3b**). Additionally, these results confirmed that precipitation of casein micelles in acidic conditions may be useful to screen protease-producing microorganism in a straightforward manner. For instance, **Figure 4** showed a more conclusive hydrolytic halo (well-defined), resulting from the acidic precipitation assay when compared to an assay intended to detect extracellular protease activity using a Coomassie Blue staining (**Vermelho *et al*., 1996**).

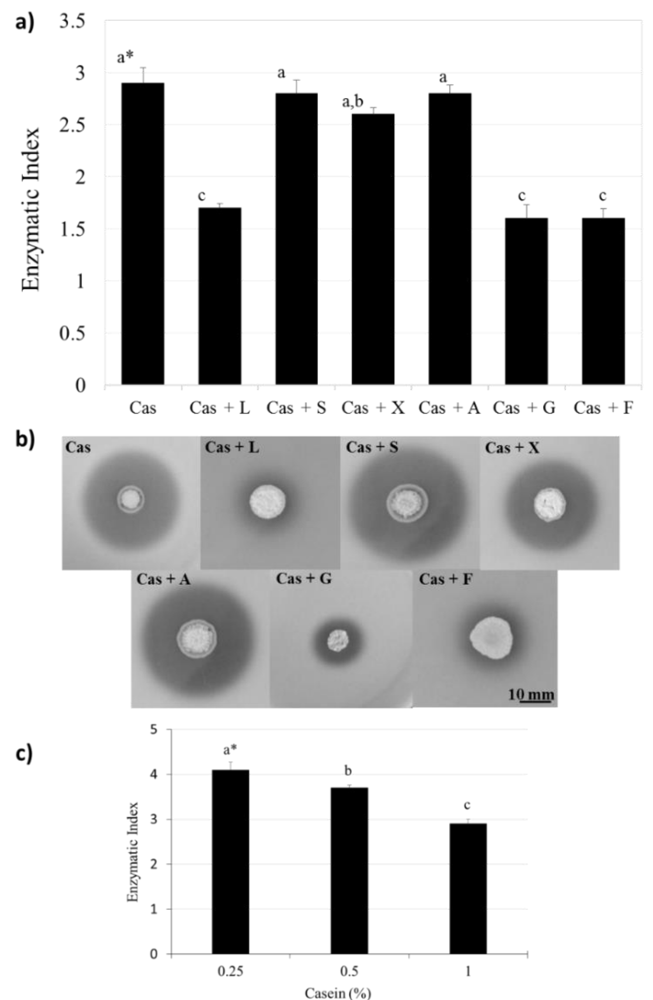

Figure 2 Effect of carbon source on protease expression from *Streptomyces* sp AGS-10. a) Enzymatic Index obtained from 7 days old cultures. b) Visualized effect of carbon source over the protease production. c) Enzymatic Index of *Streptomyces* sp AGS-10. Spores were inoculated on PM agar plates with 0.25, 0.5 and 1% of casein and incubated at 29°C, hydrolysis halos were measured after 7 days. * The bars that show the same letters are not significantly different (P = 0.05)

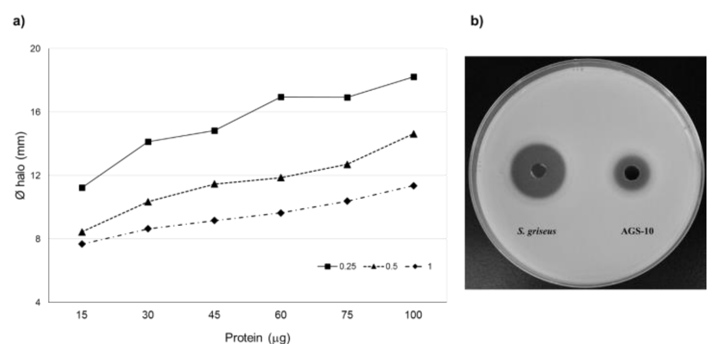

Figure 3 Proteolytic activity of *Streptomyces* sp AGS-10. a) Increased concentrations of the enzymatic extract supernatant were deposited into 6 mm wells on Petri plates with three casein concentrations and measured after 24 hours of incubation at 29°C. b) Proteolytic activities of enzymes from *Streptomyces griseus* and concentrated supernatant from *Streptomyces* sp AGS-10. Left halo, 10 µg of *S. griseus* protease (diameter: 22.0 mm ± 1.3 SD); right halo, 40 µg of the concentrated supernatant from AGS-10 (diameter: 16.2 ± 1.8 SD)

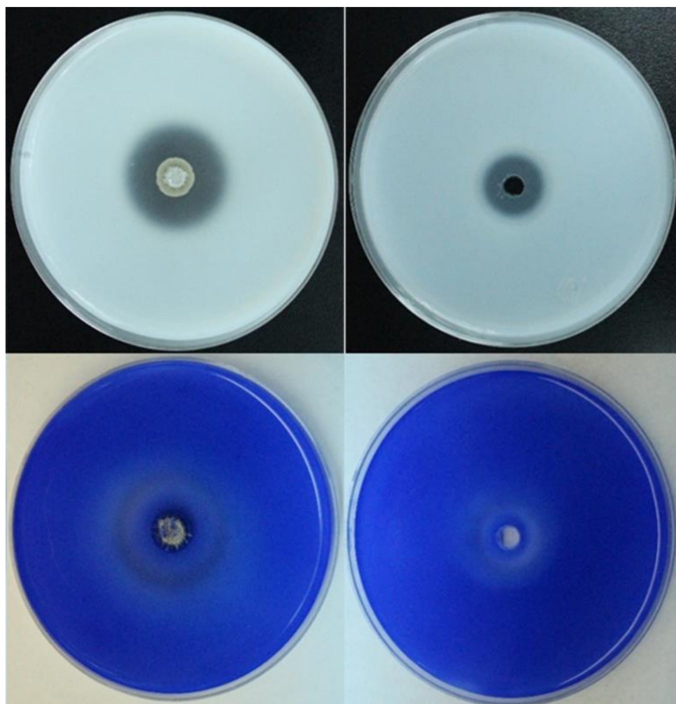

Figure 4 Comparative methods to detect extracellular protease activity from colonies (left column) and enzyme extract (right column) of *Streptomyces* sp AGS-10 on PM plates with 1% casein. Upper panel, plates flooded with acetic acid; botton panel, plates flooded with Coomassie blue stain.

Mokashe and Patil (2016) described an alternative method to quantify the enzymatic activity of purified proteases using a substrate-agarose plate diffusion method. Their bioassay avoids the use of a detection reagent (e.g. Coomassie blue dye or Trichloroacetic acid), and quantifies the enzyme units in a simple way. It could be interesting to evaluate the protease expression of *Streptomyces* sp AGS-10 under these conditions.

Phenotypic characterization and molecular identification of the AGS-10 isolate

The morphological features displayed by the selected isolate were observed for 21 days. The strain was characterized by an extensively branched mycelium that developed into an aerial mycelium with a flocculate appearance and a mass of mature spores possessing grey to black coloration. The details of this characterization are shown in **Table 1**. It shows a suppressive effect on sporulation mediated by some types of sugars. Raffinose, fructose and sucrose suppressed growth and spore production. The adaptability of AGS-10 isolate to different substrates was evidenced by its growth in the presence of different carbon and nitrogen sources. Vegetative growth and sporulation were noticeable when cells were cultured on the following media: International Streptomyces Project 2 and 3, nutrient agar, potato-dextrose agar, and tryptone-yeast extract agar. The observed physiological features showed that the isolate under study develops an extensive substrate mycelium producing a massive spore mass at pH values ranging from 6 to 9. Optimal growth and a moderate spore mass were observed up to 7.5% sodium chloride. The antibiotic susceptibility profile showed that the isolate was resistant to gentamicin and levofloxacin, as shown on (**Table 2**).

Table 1 Characterization of *Streptomyces* sp. AGS-10

Test		Carbon source	Growth*	Spore
Gram staining	+	None	++	++
Starch hydrolysis	+	D-glucose	+++	+++
Casein hydrolysis	+	Sucrose	+	-
Melanin production	-	D-xylose	+++	++
		D-mannose	+++	+++
Growth on ISP2		D-arabinose	+	-
Substrate mycelium	Yellow	D-raffinose	+	+
Aerial mycelium	White	D-cellobiose	++	+++
Spore mass	Black	D-fructose	+	+
Pigment production	None	L-rhamnose	+++	++
		Galactose	++	++
		Myo-inositol	++	-

* The classification of growth and spore production was: (+++), excellent; (++), moderate; (+), poor; (-), not detected

Table 2 Biochemical and physiological properties of *Streptomyces* sp AGS-10

Culture media	Growth*	Spore	Antibiotic†	Sensitivity
ISP9	++	++	Ampicillin 10 mg	R
ISP2	+++	+++	Cefalotin 30 mg	R
MH	++	+	Cefotaxime 30 mg	R
NA	+++	+++	Levofloxacin 5 mg	S
PDA	+++	+++	Cefuroxime 30 mg	R
TSI	+	-	Dicloxacillin 1 mg	R
LB	++	+	Erythromycine 15 mg	R
TYE	++	++	Gentamicin 10 mg	S
LIA	+++	++	Cefepime 30 mg	R
SDA	+++	+	Penicillin 10 U	R
SCA	+	+	Tetracycline 30 mg	R
ISP3	+++	+++	Tp-Sm§ 25 mg	R
pH				
6 to 9	+++	+++		
NaCl (%)				
0 – 2.5	+++	+++		
5.0 – 7.5	+++	++		

* The classification of growth and spore production was: (+++), excellent; (++), moderate; (+), poor; (-), not detected.
† Results regarding antimicrobial susceptibility were: (R), resistant; (I), intermediate; (S), susceptible.
§ Tp-Sm= Trimethoprim-sulfamethoxazole

Phylogeny of *Streptomyces* sp. AGS-10

A fragment of the 16S rRNA gene sequence of *Streptomyces* sp AGS-10 was analysed and it exhibited high similarity (100%) to other streptomycete species in the GenBank database (NCBI), especially *S. mutabilis*. A phylogenetic tree was constructed based on the neighbor-joining method and it showed that this strain is allocated to a defined branch along with *S. mutabilis*, *S. albogriseolus*, *S. goshikiensis*, *S. radiopugnans*, and *S. finlayi* (**Figure 5**). This cluster includes species previously studied as biocontrol agents for fungal plant pathogens (**Faheem *et al*., 2015; Luo *et al*., 2015**), for nematicidal production (**Zeng *et al*., 2013**), and for resistance to ^{60}Co gamma (**Mao *et al*., 2007**).

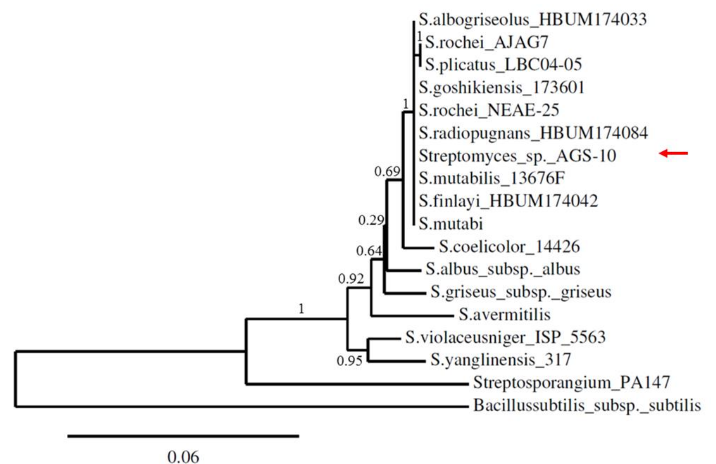

Figure 5 Phylogenetic tree based on 16S rDNA sequences showing the relationship between *Streptomyces* sp. AGS-10 and related species of the genus. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 500 resampled data sets.

CONCLUSION

Novel proteases displaying different catalytic activities are highly required by some industrial sectors, particularly those producing detergents, leather-derived products, medical devices, processed foods, feeds, and chemicals. In this regard, some *Streptomyces* species should be considered as protease producers because of their ability to secrete many extracellular enzymes, generally regarded as safe (GRAS).
This study revealed a direct correlation between protease production and carbon sources. An induced or repressed effect on protease production was observed based on hydrolysis halos used to select those proteolytic strains. The detection of hydrolysis halos by using our established procedure is based on the measurement of a clear area. This is a straightforward and efficient way to generate semi-quantitative data. This simple procedure was based on casein's physicochemical property to unfold when exposed to acid conditions, the rearrangement of the long polymer chains and its precipitation. This procedure may be adapted in order to perform a primary or a secondary screening of a large number of strains.

Acknowledgments: This study was supported in-part by grants CAMP-2008-C01-96874 and AGS-2011-C02-181930. To Dr. Alvaro Hernández-Flores and Babatunde O. Musa for English corrections.

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