This article may be downloaded for personal use only. Any other use requires prior permission of the author or publisher.

The following article appeared in *Journal of Scientific and Industrial Research, Vol. 69 No. 12, 2010, pp. 942-947;* and may be found at http://nopr.niscair.res.in/handle/123456789/10663

Molecular and biochemical characterization of extracellular tannin acyl hydrolase activity from a Mexican isolate of *Aspergillus niger*

Fabiola León-Galván^{1,2}, Irineo Torres-Pacheco³, Fernando Jiménez-Espinoza⁴, Sergio Romero-Gómez⁵, Lorenzo Guevara-Olvera⁴, Ana Paulina Barba de la Rosa¹, Mario M González-Chavira⁶ and Ramón Gerardo Guevara-González³*

¹División de Biología Molecular. Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José 2055. Col. Lomas 4 sección CP. 78216. San Luis Potosí, S.L.P. México

²Universidad de Guanajuato, División de Ciencias de la Vida, Campus Irapuato-Salamanca, Departamento de Ingenieria en Alimentos, Ex-Hacienda el Copal k.m 9, Carretera Irapuato-Silao. C.P 36500. Irapuato Gto. México

³CA Ingeniería de Biosistemas. División de Investigación y Posgrado, Facultad de Ingeniería, Universidad Autónoma de Querétaro. Centro Universitario Cerro de las Campanas, S/N, Col. Las Campanas. CP. 76010. Santiago de Querétaro, Qro, México

⁴Departamento de Ingeniería Bioquímica, Instituto Tecnológico de Celaya, Ave Tecnológico y A García-Cubas, S/N, Col FOVISSSTE CP 38010, Celaya, Gto, México

⁵Facultad de Química, Universidad Autónoma de Querétaro, Centro Universitario Cerro de las Campanas, S/N, Col Las Campanas, CP 76010, Santiago de Querétaro, Qro, México

⁶Unidad de Biotecnología, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Carr Celaya-San Miguel de Allende, Km6.CP.38010.Celaya, Gto, México

Received 28 June 2010; revised 07 September 2010; accepted 21 September 2010

Microbial tannase, a hydrolysable tannin-degrading enzyme, is extensively used in manufacture of instant tea, beer, wine, and gallic acid. *Aspergillus niger* strain, obtained from a Mexican tannery wastewaters rich in gallic acid [Quebracho Phenolics-rich Tannery Wastewaters, (QPTW)], displayed a good growth and tannase activity in a minimal medium added with 1% (w/v) QPTW (Kr= 0.451 mm.h⁻¹). Using PCR and RACE 3' and 5' methodologies, a complete cDNA of a tannase was cloned from this isolate. Nucleotide sequence of complete cDNA was of 4690 bp with a complete ORF of 1833 bp encoding 611 amino acids. Transcriptional induction was observed in mineral medium added with carbon sources as tannic acid alone (1 and 10 g/l), as well as mix of glucose (1 and 10 g/l) and tannic acid (1 g/l) in the media. However, neither glucose (1 and 10 g/l) and sucrose (1 and 10 g/l) nor (+)-catechin (1 and 10 g/l) as sole carbon sources displayed gene induction in *in vitro* assays. Thus, *A. niger*-GTO is a new strain with interesting characteristics for industrial tannase production purposes.

Keywords: Tannase, Aspergillus niger Guanajuato isolate, cDNA cloning

Introduction

Tannase (Tannin-acyl-hydrolase, E.C. 3.1.1.20) catalyzes hydrolysis of ester and depside bonds in gallotannins, as tannic acid, releasing glucose and gallic acid¹. Gallotannins display high antimicrobial activity due to complexation with proteins and enzymes, but few fungal species develop resistance against gallotannin by production of tannase². Family *Aspergilli* is widely used for production of food ingredients, pharmaceuticals and industrial enzymes, as well as for production of heterologous proteins³⁻⁵. In their natural habitat, *A. niger* strains secrete large amounts of a wide variety of enzymes needed to release nutrients from biopolymers. This high secretory capacity is exploited industrially in both solid and

submerged fermentations^{6,7}. In addition, A. niger has a long tradition of safe use in production of enzymes and organic acids, and these products have obtained GRAS status (generally regarded as safe)⁸. Species of Aspergilli are best producers of tannase9,10, which is useful in manufacture of instant tea, acron wine, cofee-flavoured soft drinks, clarification of beer and fruit juices¹¹, food and beverage processing¹². To increase production of tannase, identification of cognate ORF is to be expressed in homologous or heterologous systems¹³. So far, only tannase gene cloned and sequenced from *Asperlli* species is from A. $oryzae^{14}$. Complete genome sequence of A. *niger* CBS 513.88, an early ancestor of current *A. niger* strains, has recently been reported⁵. A new strain of A. *niger*, isolated from Mexican tannery wastewaters, is highly-rich in gallic and protocatechuic acids¹⁵ and expected to display enzymatic activities as tannase.

^{*}Author for correspondence

E-mail: ramon.guevara@uaq.mx

This study characterized tannase activity, cloning a complete cDNA of a tannase gene (TG) and carried out studies on transcriptional regulation of TG from *A. niger* strain.

Experimental Section

Strain and Culture Medium

Quebracho phenolics-rich tannery wastewater (QPTW, 3001) was collected from effluents of a tannery located in León, Guanajuato, México. Effluent was serially diluted in phosphate buffer (pH 7.0) and plated on solid minimal medium M9¹⁶ [Na₂HPO₄-7H₂O (12.8 g/l), KH₂PO₄ (3 g/l), NaCl (0.5 g/l), NH₄Cl (1 g/l), CaCl₂ (0.1 g/l), MgSO₄ (2 g/l)], supplemented with tannic acid (1 g/l; SIGMA, St. Louis, MO) as only carbon source. Petri dishes were incubated at 30°C during 7 days and colonies obtained were further submitted to identification.

Identification of A. niger-GTO

Identification of *A. niger* strains were carried out first by microbiological and microscopic methodologies¹⁷. For further identification of suspected *A. niger*, genomic DNA was extracted using protocol reported¹⁸, and identified by amplifying and sequencing ITS1-5.8S-ITS2 regions of rRNA genes¹⁹ and sequence comparison with Genbank on NCBI.

Evaluation of Fungal Radial Growth

Radial growth of *A. niger*-GTO and a strain of *A. oryzae* (CINVESTAV-Zacatenco, México), was determined using mineral medium M9 alone or supplemented either with tannic acid (1 g/l) or QPTW (1 g/l) as carbon source. A fungus mycelium disc (diam, 7 mm) was inoculated in center of Petri dishes with reported media^{20,21}. Petri dishes were incubated at 30°C until mycelium growth covered all plate surface. Each 24 h radial mycelial growth was measured using a vernier (Industrias Vermar, Jalisco, México). Data were used to calculate velocity of radial growth as²²

$$Kr(mm.h^{-1}) = (r_2 - r_1)(t_2 - t_1) - 1$$
 ...(1)

where r_1 and r_2 are mycelium radio at beginning (t_1) and end (t_2) of exponential growth of fungi. All experiments were carried out in triplicate and in three independent assays.

Determination of Tannase Activity

Tannase activity²³ of *A. niger*-GTO and *A. oryzae* was determined by using erlenmeyer flasks (250 ml) with

sterile liquid medium M9 (50 ml) plus tannic acid (1 and 10 g/l) at an initial pH of 5.5, inoculated with 2.5 ml of a spore suspension (2 x 10^7 spores/ml) at 30°C on a rotary shaker (220 rpm); cultures were monitored every 2 h over a 20 h period. Biomass was separated by centrifugation at 13000 rpm during 10 min. Cell-free culture broth was assayed for tannase activity measuring absorbance at 260 nm. In addition, tannase activity was determined in mixture M9 media enmmended with tannic acid (10 g/l) plus glucose (1 and 10 g/l) for possible catabolite repression detection in the system. A tannase unit is amount of enzyme that catalyzes production of 1 mol of gallic acid per minute per ml of cell-free culture broth. All assays were carried out bytriplicate.

cDNA Cloning of a Tannase Gene (TG) from A. niger-GTO

Based on sequence of TG from A. $oryzae^{14}$, several primers were synthesized and pairwise evaluated in order to PCR amplify a segment of ORF of a TG from A. niger-GTO. Primer sequences were as follows: forward primers, Tan 1TF (5'-ATGCGCCAACACTCGCGCAG-3') and Tan 2TF (5'-CCTCCATCATCGGCCAGTCC-3'); and reverse primers Tan 1TR (5'-CGGAGCCATTCTGGGCGGGC-3'), Tan 2TR (5'-GCTGGTCCAGAGGGGGGGGGGGGG3') and Tan 3TR (5'-TTAGAAAACGGGCATCTTGA-3'). Sterile liquid M9 medium supplemented with tannic acid (1 g/l) was inoculated with 2 x 107 spores/ml and incubated during 48 h; then mycelium was collected by centrifugation and total RNA extraction was carried out using RNeasy kit (QIAGEN). Once obtained high quality total RNA, cDNA synthesis was carried out using SMART[™] RACE cDNA amplification kit (ClonTech) according to manufacturer conditions. Amplicons obtained with different combinations of forward and reverse primers were sequenced and compared to Genbank, in order to determine homology to tannase from A. oryzae. Amplicons with high homology to TG of A. oryzae were obtained with primers Tan 1TF and Tan 2 TR and were used as substrate for 3'and 5'RACE methodology, using either forward (5'-GCTCTGCCAGTGGCCTTCCCG-3') or reverse (5'- GTGCCATTGGAAGGAAGGGC-3') primers synthesized based on nucleotide sequence of amplicon obtained from A. niger-GTO with homology to A. oryzae TG. Sequencing of TG was obtained by dideoxynucleotide chain termination method using an automatic sequencer (Applied Biosystems, Foster City, California).



Fig. 1—Tannase activity and tannic acid degradation in: A)A. oryzae and B) Aspergillus niger-GTO

Southern, Northern and Dot Blot Hybridizations

Both Southern and Northern blot analysis were carried out by standard protocols14. Dot blot analysis was carried out as reported²⁴. In all hybridizations, a fragment of 1833 bp encoding complete ORF of TG of A. niger-GTO was used as probe. For dot blot, probes were generated incorporating dUTP-11-fluorescein by a routine protocol (Gene Images CDP-Star Random prime labeling module; Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA). Probe detection was made by conjugate alkaline antifluorescein-phosphatase and CDP-Star detection reactive (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). For Southern and Northern blot, probe was labeled using ³²P-dCTP by random priming. Inducer molecules glucose, catechin, tannic acid and sucrose were purchased to SIGMA-ALDRICH (St. Louis, MO, USA). Exposed X-ray film was scanned, and hybridization signals were quantified with a digital image system (1D Image Analysis Software Version 3.0.2, Kodak digital system, Rochester, New York).

Sequence Analysis

Sequences were compared based on data conducted with blastx algorithm²⁵ of National Center of Biotechnology Information (NCBI) and MegAlign tool of LASERGENE (DNASTAR software; Madison, WI, USA). Sequence of cDNA of TG from *A. niger*-GTO was deposited in Genbank database with accession number DQ185610.

Results and Discussion

Isolation of A. niger-GTO

During isolation of microorganisms from QPTW studies, two types of fungal colonies were detected. Majority of these colonies (98%), based on morphological and microscopic studies, were identified as *A. niger*. Rest (2%) of colonies were identified as *Talaromyces* sp. Molecular identification of *A. niger* was confirmed using ITS1-5.8S-ITS2 sequencing of this isolate. *A. niger* strain was named as *A. niger*-GTO.

Evaluation of Radial Growth of Fungi

Radial growth (Kr) on mineral medium (M9) plus QPTW of *A. niger*-GTO and *A. oryzae*, respectively, was found as: tannic acid, 0.506, 0.536 mm/h; and QPTW, 0.517, 0.451 mm/h. These results and reports on tannase activities in *A. niger* strains¹² suggested that *A. niger*-GTO strain could be a good source for tannase activity. In addition, Kr levels obtained for both *A. niger*-GTO and *A. oryzae* using either tannic acid or QPTW as carbon sources were similar, and that *A. oryzae* is reported¹⁴ an efficient tannase producer.

Determination of Tannase Activity

Maximal tannase activity was detected at 8 h of culture, for A. niger-GTO (0.84 AU) and A. oryzae (0.68 AU) with a subsequent decline in enzyme activity, being more pronounced for A. oryzae (Fig. 1). Tannase activity increased to 1.82 AU when included glucose (10 g/l) plus tannic acid (10 g/l), indicating that catabolite repression was not present in the system at least in concentrations evaluated in this work (Fig. 2A). Decline in tannase activity of A. niger-GTO could be due to an enzyme inactivation as reports²⁶⁻²⁸. These results were similar to those reported for A. niger HA37, to degrade phenolics in wastewaters from olive mill²⁸. Mineral medium (M9) is more complex than AT medium reported²⁸, could be likely that this aspect influenced differences in time and quantity of tannase activity showed by A. niger-GTO (this work) and A. niger HA37. In addition, it was shown a concomitant diminish in tannic acid level, once tannase activity increased from 2 h incubation for both A. niger-GTO and A. oryzae evaluated (Fig. 1).

Cloning a Complete cDNA of Tannase from A. niger-GTO

In order to provide a TG source for further studies on expression in homologous or heterologous systems, a complete tannase cDNA was isolated and sequenced from TG (Fig. 3). cDNA isolated corresponded to a 4673 bp sequence, with two putative polyadenilation signal and a



Fig. 2—Tannase activity in A. niger-GTO in: A) presence (□) and absence (□) of glucose 10g/l (panel A); B) Northern blot analysis for tannase gene expression [Tannic acid (10 g/l, lane 1), glucose (10 g/l, lane 2), and glucose plus tannic acid (10 g/ l both, lane 3)]; and C) Total RNA loaded in each lane as control

Table 1—Homologies among tannase C	ORF of	Aspergillus	niger
(GTO) and others asp	pergilli*	<	

	% Identity	% Positives
A. niger CBS513.88(No.accesion)	86	90
A. oryzae(No.accesion)	75	84
A. terreus NIH2624(No.accesion)	76	84
* Each value is the % between A. nig	er-GTO and r	espective fungi

in the first column

poly A tail. Complete ORF was of 1833 bp. The 5' untranslated (UTR) region consisted of 1822 bp and a 3' UTR of 1015 bp; both regions are substantially larger than the ones reported for a TG of A. oryzae¹⁴. UTR's length in tannase cDNA of A. niger-GTO could be related to a highly regulated mechanism in comparison to A. oryzae. mRNA untranslated regions in fungi are involved in many post-transcriptional regulatory pathways that control mRNA localization, stability and translation efficiency²⁹⁻³⁰. Homology analysis of tannase ORF from A. niger-GTO and other Aspergilli, displayed an 86% identity with A. niger CBS513.88 (Table 1). Comparing sequence of a PCR amplified-genomic region using 5' and 3' primers flanking tannase ORF region of TG, displayed that there was no intron in TG. These results were similar to those reported for TG of A. $Oryzae^{14}$.

	THE OTHER DESIGNATION AND ADDRESS AND ADDRESS ADDR	9.9
1.00		1.00
74	TAKTE CAUTOR TTUE THET TUGE CAUAGAOC ARAGE TATTE ACCURCACE TO TRETTE TATE TO BE TATE TATE TO BE TATE TATE TATE TATE TATE TATE TATE T	1.60
142	GETTIGAAL TIGALATTE TEAL FEET GOOTEGAL TO FETELELKGE TTEESGET CTGATEAL CATTIECGA	219
220	COCCE DE OFFICE AAC OF AC ET GA GECE DE CENERT TIT TA A ANGA NE TAAT DA TE DE GETTE NE DE TA GETTE DE CAAGTE	2.92
293	TOCTOTSCATOSTCSADSACATOSTSCAASSTACTOCTACAAASSTAGTATTTTCTSSTSCCACOSSAASSAT	365
366	ACCAMATCHTGDCAGTCTCGGAGTGATCTCTTCTGGAAADGTTACTTTGGGCTGTTTAAAAATATTGGCCAATGG	438
425	TAKOD TO TACA TO SO DONO TO TO COULT GA AT AUT ORIGAN ATT COMPANY TO A CADING ADDOLTANC ACTUAT TO A	\$11
\$12	TOPPE AT ACUTATION AT THE CACT THEFT OF A TIGHT OF CITERATION OF A TIGHT CAUGHTER CAUGHTER TO A SAC CARACTER	504
8.98	ACADEDICAN ACT CANDIDIC TO A THE THE CONTRACT TO A THE TOAC AND CONTRACT ADDRESS AND THE ANT	657
0.00	CATALOC TO TOTA OTTAL CATALOC AND	7.55
9.91		0.05
104	ADT INCOMENDATION CONTRACTOR AND A DESIGNATION OF TOWARD CALL TO CALL	803
804	CLTAINE CASE TALTUT THAT CITIAND FT TALIANS TO CTICARDA AND CONTRACT CONT	876
1072	ADDIDE GADE AT GGAD TO TO TO TO TO FOOTAT GADGAL TO TO TALE TO ARA TO TO ARAFINE TO ARAFE COOL TO	240
950	AGENT CACAMETERIA CAGEDETE TTUTAL CITURE DOCTTANE AFER TACTOR OF TEAD SAAGOC TUGALT THE	1022
1023	CACTRAC CARCEAUTORIC TTOPOCACEAUTOSCIC ADDAGE COARAGETTORIC CTTOPOCAACTOC	1095
1096	TTECT TO OGAD CAGAD AGAT CATTIC OCCT CAG TTE TAGAC AA TA TO OCTO STATAT STAT OG AACCTAGT T	1168
1169	GGACCITICTGT CGGC TGCC AAGGT DCC CGACTOCITI AAGCGGAGATAT GC TGACT CCACCC CGGA TAAGGT TAT	1241
1242	TACCED DE REALET ET TROCE DE AGRECOL TRAT TAGAT REMART TAGAT REALET RECATE DE LA TROCATE DE CECTER TAT	1014
1318	T OFTO TO ORGETT TO ADDIT OCOUTT TO GOODAAAT TO TO DO ADDA TO TAAD CAGOAAT DO CAGACAAT TAGACOT	1387
1200	TOPICAL FLOAD FTC TO DO TO THE TO CARGE TO AN A TATISTICT TO AT DO GATE USAGO FCT TO CARGO FCT F	1460
1461	A TC A A AC TO OCCUPATION TO A GAD CC ACCTORING CACITOR TO T AC TO A TO T CACCT A A A CC CA T CAT CT OCA.	1 53.5
10.94	TOUT OF DE TOUGOOOD OF AN EDDOUTOR CONCEPTING AND AN OTHER AT THE TOUT OF AN ANTIMAL	1006
i ciri		1674
1007	A server of the server and the server of the	1072
1000	C PERIOR TOP REPORT OF THE REPORT OF THE REPORT OF THE PERIOR PERIOR PERIOR	1.002
1763	TGATGATACGTTTTTACATRCTGTATCCTTTCAACACTAATTCATEGeteteseasteett.agtgtt.cgsAACATg	1828
1876	CITER & R. D. D. R. P. PILL V. T. T. J. M. R. T. T. L. R. D. LOL V. R. V. T. D. SCI. T. J.	1899
1899	CIGN IN CAR OF ILAST MATCHICCHIC CRARGE ACTIVITIES APART PRATTICITUDE IST	1971
1972	CREWER NUMERICAL COLOR OF A CALL OF AN ALC	2064
2045	GTURCET AC ACCERT ALL CONTRACTOR AND A CONTRACT OF CONTRACT AND A CONTRACT OF CONTRACT AND A CONTRACT	2117
21.18	NAM OF TO YOUR RECORDER AND RECORDER OF THE RECORD OF THE	2190
7191	THE COST IN CONTRACTOR CONTRACTOR OF A DAMAGE CONTRACTOR OF A	2263
2264	AN 15YO M REPORTED FOR THE REPORT OF THE PROPERTY AND THE	2336
2227	ADDITION OF THE ADDITION OF THE OWNER ADDITION OF THE ADDITION	2489
34.10	TO REPORT NEW YORK AND A DESCRIPTION OF	2482
1403		0.055
C104		2000
13.28	TRANSFERRET DISCONTINUE CONTINUES FOR DELLA TRANSFERRET	2628
15.45	CLEASE PRINT PULLE AND THE AND	2791
C102	THE SECOND COMPLETE AND A CONDUCTION OF THE REAL PROPERTY OF AN ADDRESS OF A DEALER AND ADDRESS OF A DEALER ADDRESS OF ADDRESS OF ADDRESS OF A DEALER ADDRESS OF ADD	2779
2775	ALL NOT CLUTCH ADARTIES ALL ATCALL TO CLUB, STUDIAL TYPEST CREEKE ACCURATE ACCURATION OF A CONSTICUTE A	2097
2849	CONTROLS AND TALE OF THE CHORE AND THE CONTROLS AND THE CARLES AND THE	2920
2921	CALVEST A CONTENT AND A CONTENT OF THE ACCOUNT OF THE ACTIVATION O	2991
1996	YOF TO NOT AN CAPTOR AND CAPITAL AND CAPACITY CAPITAL AND CAPACITY CAPITAL AND CAPACITY CAPACITY CAPITAL AND CAPACITY CA	3066
3067	CREWENCE AND REAL AND THE MEAN AS A SECOND OF THE AND THE ADDRESS THAT AND	8139
1140	REALT A THE RELEASED ON CONTRACT AND A CONTRACT A SECOND AND A CONTRACT OF	3212
1213	OT ADD CATCHARTER AND THE ACCENT OF TRANSPORTED COMPACT METALS TO THE TACK	3295
1286	CONTRACTOR OF A REPORT	3 25 8
20.00	BYC REVEN TO ODDITION OF THE PARTY AND CONCERNENT AND THE WAY TO THE PARTY AND AND	2421
14.94		3554
15.05		3533
16 34		acto
1374	In the second states where the in the second state in the second states	3420
36.61	TIBLE TAGETTGATE gas gas generate opposite to pychoot to TITGATAD GAT CTG TATIC OF THE ATGTA	3723
3724	GTATE IT OGET TE GATAGT OG IG TAET TE TTOCACT IT IG IGTET TE ET TTAATIT IT TATAOG TE MERAGAADG	3796
3797	CTTTGACOCGCTTACTTAGTCAACKATATTACTTGACTGTGTATACCTCTCACTCCATTATAAAATGAACATG	3869
3870	TTTTATTDCATCTCAATACATOGGARATTOCTDCACAAPCCACTCTAGCATATGCCTCTCCGCTGTCGBCACA	3942
1943	AND A CATATOC TAAR ARCAOC ARACANT TO GAC ATTETT CAUT OR TARGER GAC ARAT ARTS TATO TO CONC.	4015
4016	CALLARY OF ACCOUNT AS CAMP OF AN CALLARY AN ANY TYPANY AND STORE TAX CALLARY AN TAX OF TAX COUNT AND ANY	4000
4089	A FOUT TO TICT IGTT OF TO AT AGO OF TO TOGGA OF TAT TTO AS A COATA TO TO TAT TO AT A STORATOR TO TOT TO TO	4161
0.62	TAXOUGHT SOCIOUS AT AN TAXON SOCIAL SOCIAL SOCIAL SOCIAL SOCIAL SOCIOUS AT TOTAGE THOUGHT	4224
12.94		4900
4500	And a first and the second state of the second	4000
40.04	In a proving the second s	4,080
1981	TABLE ICTOTTOL TATE ADD. ODDCAMAAAAOOAAEOAAAAAOCATATAL TTADOAOCABAAATABTOGGTAC	4423
4454	TTACCGATGCAAACATCAATGGTAAGAAAAAAAACTATTTCTGTCAGCCACCAAAGCAAACGCAAAAAAAA	4526
4527	CGATT DEAGACIGACIOCTACTCACAGTAATGAGACTGATIC GTCT CATGAAAACTGTTCT TCAACGAAACC	4599
4600	TARCAAAATACTGTCARGTCATTCAAGTATTTAAAAATCTAATTTTATTAAGATCACCITGCTTEKTCCATCG	4672
4673	ATATY GATATT IT TAARAAAAAAAAAAAAAAAAAAAAAAAAAAA	

Fig. 3—Complete cDNA sequence isolated from *A. niger*-GTO. [Open reading frame is marked shaded and in bold letter. Start (ATG) and termination codons (TAG) are underlined and written in italics. Putative polyadenilation signal at 3'unstranslated region are indicated underlined and written in bold]

Tannase Gene Copy Numbers and Gene Expression Studies

In order to analyze number of copies of TG throughout *A. niger*-GTO genome, a Southern blot assay was carried out using genomic DNA of this strain. It detected (Fig. 4) only one signal using restriction digestions that based on sequence, and will not cleave within TG sequence. TG is present in one copy in *A. niger*-GTO genome, which could facilitate future approaches to improve expression of TG. This result is similar to that reported in *A. oryzae* and *A. niger* CBS 513.88^{5,14}. On the other hand, mechanism of regulation of microbial TG induction is currently not deciphered although inducing effects of gallic acid fraction of tannic acid (or a derivative) have been suggested^{30,31}. Moreover, catabolite repression by several carbon sources could be part of the mentioned mechanism



Fig. 4—Tannase gene copy numbers in A. niger-GTO (Lane 1, genomic DNA from A. oryzae digested with Eco RI; lanes 2 and 3, genomic DNA from A. niger-GTO digested with Hind III and Eco RI enzymes, respectively. Complete ORF of tannase gene from A. niger radiactive ATP ³²P-labeled was used as a probe.)

as reported²⁸. Thus, in order to evaluate TG induction in A. niger-GTO, several transcriptional studies of TG were carried out using several carbon sources (Fig. 2B and 2C, and Fig. 5). Tannic acid (conc., 10 g/l) induced transcriptional TG expression in A. niger-GTO (Fig. 2B and 2C, lane 1). Moreover, when besides the latter carbon source, glucose 1 g/l was added, gene expression displayed no significant increase (Fig. 2B and 2C, lane 3). Tannase activity in these latter conditions (Fig. 2A) displayed a threefold increase (1.83 AU/ml) in comparison to tannic acid (10 g/l) alone. Thus, increase displayed in tannase activity was not associated with a concomitant increase in induction in TG, suggesting a post-transcriptional mechanism involved. This result agrees with Aguilar et al²⁷ who found in A. niger Aa-20, that in submerged fermentation tannase activity increased from 0.57 to 1.03 IU/ml, when initial glucose concentration increased from 6.25 to 25 g/l, but a strong catabolite repression of tannase synthesis was observed in SmF when an initial glucose concentration of 50 g/l was used. Thus, although different media were used in present work, it is likely that glucose concentrations used in present work were down the threshold required for catabolite repression of TG. For further TG induction studies, other several putative inducers as sucrose and catechin were also evaluated



Fig. 5—Transcriptional studies of tannase gene expression of *A. niger*-GTO using different carbon sources. [Lane 1, tannic acid (1 g/l); lane 2, glucose (1 g/l); lane 3, glucose (10 g/l); lane 4, tannic acid + glucose (1 g/l both); lane 5, tannic acid (1g/l) + glucose (10 g/l); lane 6, sucrose (1 g/l), lane 7, tannic acid + sucrose (1 g/l both), lane 8, tannic acid (1g/l) + sucrose (10 g/l), lane 9, catechin 1g/l), lane 10, tannic acid + catechin (1g/l both), lane 11, tannic acid (1g/l) + catechin (10g/l), lane 12, tannase ORF used as a probe (positive control). Each lane was loaded with 10 mg of total RNA]

(Fig. 5). In two concentrations evaluated, neither glucose alone (Fig. 4), sucrose nor catechin were TG inducers (Fig. 5, lanes 2, 5 and 6 respectively). Catechin was a repressor for TG expression in *A. niger*-GTO (Fig. 5, lanes 6 and 7), the first report, in which it is demonstrated that TG repression using catechin.

Conclusions

Extracellular tannase from *A. niger*-GTO displayed molecular and biochemical features with potential applications in several industries. Also, authors claim that this is the first report on cDNA sequence for tannase gene in *A. niger*, which could be useful to future research in tannase activity improvement using molecular biology tools.

Acknowledgements

One of the authors (RGG-G) thanks to FOMIX-Qro 2008, SEP-CONACyT, PROMEP and FIFI-UAQ 2009, for partial support of this research. F León-Galván and F Jiménez-Espinoza also acknowledges to CONACyT for a grant provided.

References

- 1 Pinto G, Leite S, Tarzi S & Couri S. Selection of tannaseproducing *Aspergillus niger* strains, *Brazilian J Microb*, **32** (2001)24-26.
- 2 Banerjee D & Pati B R, Optimization of tannase production by Aureobasidium pullulans DBS66, J Microb Biotechnol, 17 (2007) 1049-1053.
- 3 Punt P J, van Biezen N, Conesa A, Alberts A, Mangnus J & van den Hondel C, Filamentous fungi as cell factories for heterologous protein production, *Trends Biotechnol*, **20** (2002) 200-206.
- 4 Archer D B & Turner G, Genomics of protein secretion and hyphal growth in Aspergillus, in The Mycota XIII, edited by A J P Brown (Springer, Berlín Heidelberg) 2006, 75-96.
- 5 Pel H J, de Winde J H, Archer D B, Dyer P S, Hofmann G, Schaap P J, Turner G, et al, Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88, *Nature Biotechnol*, **25** (2007) 221-231.
- 6 Berka R M, Dunn-Coleman N & Ward M, Industrial enzymes from *Aspergillus* species, in *Aspergillus Biology and Industrial Applications*, edited by J W Bennett & M A Klich (Butterworth-Heinemann, Stoneham, MA) 1992, 155-202.
- 7 Pandey A, Selvakumar P, Soccol C R & Nigam P, Solid state fermentation for the production of industrial enzymes, *Curr Sci*, 77 (1999) 149-162.
- 8 Schuster E, Dunn-Coleman N, Frisvad J C & van Dijck P W. On the safety of Aspergillus niger -a review, Appl Microb Biotechnol, 59 (2002) 426-435.
- 9 Bhat T K, Singh B & Sharma O P, Microbial degradation of tannins. A current perspective, *Biodegradation*, 9 (1998) 343-357.
- 10 De Vries R P & Visser J, Aspergillus enzymes involved in degradation of plant cell wall polysaccharides, Microb Mol Biol Rev, 65 (2001) 497-522.
- 11 Mohapatra P K D, Mondal K C & Pati B R, Production of tannase by the immobilized cells of *Bacillus licheniformis* KBR6 in Caalginate beads, *J Appl Microb*, **102** (2006) 1462-1467.
- 12 Aguilar C N, Rodríguez R, Gutiérrez-Sánchez G, Augur C, Favela-Torres E, Prado-Barragan L A, Ramírez-Coronel A & Contreras-Esquivel JC, Microbial tannases: advances and perspectives, *Appl Microb Biotechnol*, **76** (2007) 47-59.
- 13 Zhong X, Peng L, Zheng S, Sun Z, Ren Y, Dong M & Xu A, Secretion, purification and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*, *Protein Exp Purif*, 36 (2004) 165-169.
- 14 Hatamoto O, Watarai T, Kikuchi M, Mizusawa K & Sekine H, Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*, *Gene*, **175** (1996) 215-221.
- 15 Marín-Martínez R, Veloz-García R, Veloz-Rodríguez R, Guzmán-Maldonado S H, Loarca-Piña G, Cardador-Martínez A, Guevara-Olvera L *et al*, Antimutagenic and antioxidante activities of quebracho phenolics (*Schinopsis balansae*) recovered from tannery wastewaters, *Biores Technol*, **100** (2009) 434-439.
- 16 Sambrook J, Fritsch E F & Maniatis T, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press,) 1989,1531-1532.

- 17 Samson R A & Pitt J I, Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification (Amsterdam: Harwood Academic Pubs.) 2000, 225-231.
- 18 Dellaporta S L, Word J & Hicks J B, A plant DNA minipreparation: version II, *Plant Mol Biol Rep*, 1 (1983) 19-21.
- 19 Henry T, Iwen P C & Hinrichs S H, Identification of Aspergillus species using internal transcribed spacer regions 1 and 2, *J Clin Microb*, **38** (2000) 1510-1515.
- 20 Müller-Riebau F, Berger B & Yegen O, Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in Turkey, *JAgric Food Chem*, **43** (1995) 2262-2266.
- 21 Veloz-García R, Marín-Martínez R, Veloz-Rodríguez R, Rodríguez-Guerra R, Torres-Pacheco I, González-Chavira M M, Anaya-López J L *et al*, Antimicrobial activities of cascalote (Caesalpinia cacalaco) phenolics-containing extract against fungus *Collectotrichum lindemuthianum*, *Ind Crops Prod*, **31** (2010) 134-138.
- 22 Trinci A P J, Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media, *JGen Microb*, **67** (1971) 325-344.
- 23 Ajay-Kumar R, Gunasekaran P & Lakshmanan M, Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent, *J Basic Microb*, **39** (1999) 161-168.
- 24 Barrera-Pacheco A, Joaquín-Ramos A de J, Torres-Pacheco I, González-Chavira M M, Pérez-Pérez C, Guevara-Olvera L & Guevara-González RG, Analysis of transcriptional expression induced in *Capsicum chinense* BG-3821 under conditions of biotic and abiotic stress, *Agrociencia*, **42** (2008) 95-106.
- 25 Altschult S F, Gish W, Miller W, Myers E W & Lipman D J, Basic local alignment search tool, *J Mol Biol*, **215** (1990) 403-410.
- 26 Suseela, R G & Nandy S C, Decomposition of tannic acid and gallic acid by *Penicillium chrysogenum*, *Leath Sci*, **32** (1985) 278-280.
- 27 Aguilar C N, Augur C, Favela-Torres E & Viniegra-Gonzalez G, Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid, *J Ind Microb Biotechnol*, **26** (2001) 296-302.
- 28 Aissam H, Errachidi F, Penninckx MJ, Merzouki M & Benlemlih, M, Production of tannase by *Aspergillus niger* HA37 growing on tannic acid and olive mill waste water, *World J Microb Biotechnol*, **21** (2005) 609-614.
- 29 Jöchl C, Rederstorff M, Hertel J, Stadler P F, Hofacker I L, Schretti M, Haas H & Hüttenhofer A, Small ncRNA transcriptome analysis from *Aspergillus fumigatus* suggests a novel mechanism for regulation of protein synthesis, *Nuc Acids Res*, **36** (2008) 2677-2689.
- 30 Tuller T, Ruppin E & Kupiec M, Properties of untranslated regions of the S. Cerevisiae genome, BMC Genomics, 10 (2009) 391-402.
- 31 Mohapatra P K D, Maity C, Rao R S, Pati B R & Mondal K C, Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology, *Food Res Intl*, **42** (2009) 430-435.